A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 1–14 (2024)
DOI: 10.3897/neobiota.94.126283

Research Article

A hitchhiker's guide to Europe: mapping human-mediated spread of the invasive Japanese beetle

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Abstract

Early detection of hitchhiking pests requires the identification of strategic introduction points via transport. We propose a framework for achieving this in Europe using the Japanese beetle (*Popillia japonica*) as a case study. Human-mediated spread has been responsible for its introduction into several continents over the last century, including a recent introduction in continental Europe, where it is now listed as a priority pest. Furthermore, recent interceptions far from the infested area confirm the risk of unintentional transport within continental Europe. Here, we analysed how three modes of transport - air, rail and road - connect the infested area to the rest of Europe. We ranked all European regions from most to least reachable from the infested area. We identified border regions and distant major cities that are readily reachable and observed differences between modes. We propose a composite reachability index combining the three transport modes, which provides a valuable tool for designing a continental surveillance strategy and prioritising highly reachable regions, as demonstrated by recent interceptions.

Key words: Biological invasion, hitchhiking pest, likelihood of introduction, pest risk assessment, *Popillia japonica*, surveillance, transport network

Introduction

The increasing global movement of goods and people provides countless opportunities for species to move around the world outside of their natural range, increasing the rate of biological invasion (Hulme 2009; Blackburn et al. 2011). Insects in particular can hitchhike on a variety of modes of transport, including planes, ships, trains and trucks (Saccaggi et al. 2016; Turner et al. 2021). Hitchhiking, which is part of unintentional human-mediated transport, facilitates the introduction of insects into new regions and, where conditions are suitable, their subsequent establishment (Early et al. 2016; Rosace et al. 2023). Predicting the risk of introduction of invasive insect pests through transport is therefore crucial for developing effective surveillance strategies (Essl et al. 2011).

Risk assessments of Invasive Alien Species (IAS) are becoming increasingly quantitative, particularly with the advent of environmental distribution models used to estimate suitability and hence establishment risk (Venette 2015). However, IAS introduction risk analyses are still often qualitative and expert-based, especially when considering human-mediated transport rather than active spread (accounted for by diffusion models) (Hulme 2009). Human-mediated introduction can occur by

Academic editor: Jianghua Sun
Received: 26 April 2024
Accepted: 1 July 2024
Published: 15 July 2024

Citation: Borner L, Martinetti D, Poggi S (2024) A hitchhiker's guide to Europe: mapping human-mediated spread of the invasive Japanese beetle. NeoBiota 94: 1–14. https://doi.org/10.3897/neobiota.94.126283

transport of traded goods and hitchhiking on cargo, on passengers or in vehicles. To be relevant to surveillance strategies for hitchhiking invasive insects, the analysis of risk of introduction should include directional (from infested regions to other regions) and weighted (volume measure) transport data, especially passenger travel (Hulme 2021). Most studies that use directional and weighted data focus on the trade of specific commodities that are pathways of entry for IAS (Piel et al. 2008; Yemshanov et al. 2012; Meurisse et al. 2019; Jamieson et al. 2022). Few studies include passenger movement data, and these studies usually focus on a specific region (Perry and Vice 2009; Szyniszewska et al. 2016) and rarely extend at a continental scale (Frem et al. 2020). As there is no framework for predicting the risk of hitchhiker IAS spreading across Europe through anthropogenic dispersal, we propose a method using directional and weighted transport data, including passenger movements, on a continental scale and at the finest possible spatial resolution. We use the Japanese beetle as a case study.

The Japanese beetle (*Popillia japonica*) is a prime example of a hitchhiker pest. Native to Japan, it was accidentally introduced into the United States of America at the beginning of the last century, causing a major invasion that still persists today (Frank 2016). From there, it was introduced to the Azores archipelago in the 1970s, and more recently to continental Europe. After its first detection in Italy in 2014, the beetle has spread to an area of more than 16,500 km^2 covering parts of northern Italy and southern Switzerland (Gotta et al. 2023). Due to its potential impact on the environment, food safety and economic balances, it has been listed as a priority pest by EU authorities (Commission Delegated Regulation (EU) 2019). Furthermore, several interceptions of the beetle far from the infested area have raised concerns about possible introductions to other parts of Europe. Although the source of the beetle's introduction into Europe has been phylogenetically reconstructed (Strangi et al. 2023), its potential spread in Europe and the risk of introduction through human movement have not been investigated.

In this paper, we present a novel approach to map the potential human-mediated spread of the beetle from the infested area to the rest of Europe. We considered three transport networks - air, rail and road - that are relevant to the beetle's pathways of entry from the infested area. We examined how reachability, i.e. the likelihood of introduction from the infested area, varied according to mode of transport. Finally, we combined transport modes to identify the most likely points of introduction and used interception sites to assess our reachability map.

Materials and methods

Data processing and analyses were performed using R version 4.2.1 (R Core Team 2021).

2022 European infested area

We first assessed the extent of the European infested area, taking into account both the municipalities where the presence of the beetle was confirmed and the neighbouring municipalities included in the buffer zone, according to 2022 official reports (Poggi et al. 2022a). This area will be considered as the origin for human-mediated spread of the Japanese beetle in Europe. The 2022 infested area covers approximately 16,500 km^2, spanning over five regions and more than 1900 municipalities

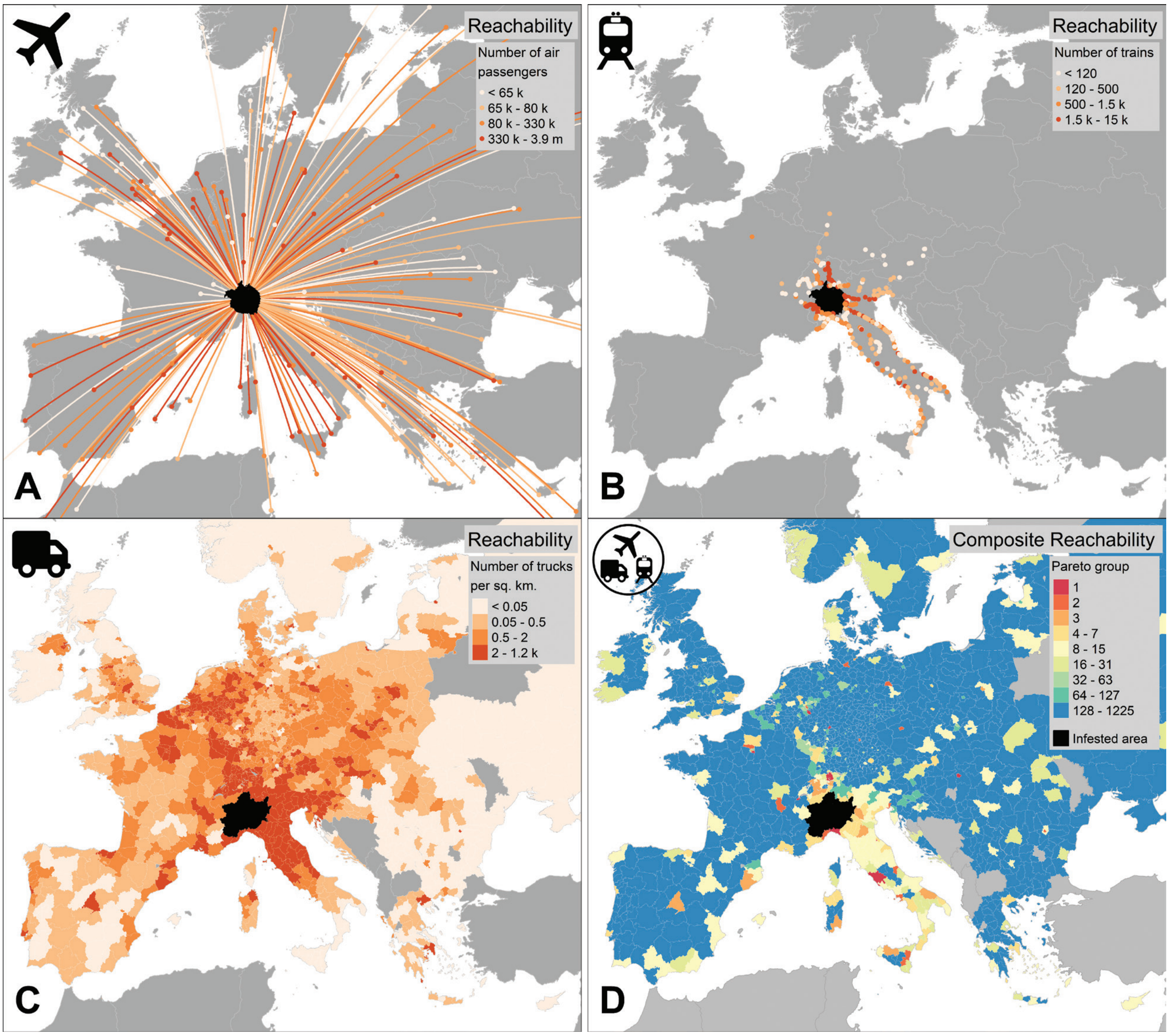

Figure 1. Reachability of Europe for *Popillia japonica* from the infested area (in black), by air, rail, road transport, and a combination of modes (composite index). Quantile-classified reachability maps showing: **A** the number of passengers arriving at airports **B** the number of trains arriving at stations, and **C** the number of trucks per square kilometre reaching NUTS 3 regions, departing from the infested area. Darker colours correspond to higher reachability **D** composite reachability map, i.e. risk of introduction for NUTS 3 regions ordered by Pareto fronts from most to least reachable. Warmer colours correspond to higher reachability.

in northern Italy and southern Switzerland (in black on Fig. 1). Six interceptions of *Popillia japonica*, i.e. captures of an isolated adult without establishment of a population, were made in Europe since 2018, far away from the infested area: one in the Netherlands (Amsterdam, EPPO 2019), one in Germany (Freiburg, EPPO 2022a), two in Italy (Udine, Bassi et al. (2022) & Cagliari, EPPO (2022b)) and two in Switzerland (Basel, NPPO of Switzerland (2021) & Zurich, EPPO (2023)).

Popillia japonica's pathways of entry and spread

Popillia japonica's pathways of entry and spread include national and international trade in commodities such as plant products, soil, fruits; and hitchhiking on cargo, on passengers (including in their baggage, Early et al. 2016) and in the vehicle itself (aircraft, train, car, truck, ship, EFSA Panel on Plant Health (PLH) et al.

2018; Poggi et al. 2022b). Here, we study hitchhiking by targeting three modes of transport that are relevant for the case study and for which comprehensive databases were readily available. Firstly, we assessed the air traffic via flights from the infested area to the rest of Europe. We focused on passenger air travel, since it has already been recognized as a major mode of introduction of IAS (e.g., 73% of interceptions at the US ports of entry between 1984 and 2000 occurred at international airports (Early et al. 2016)). Secondly, we considered terrestrial transport by assessing both road and rail traffic from the infested area to the rest of Europe (Hulme 2021). Private cars have been excluded due to lack of data, while maritime transport has been excluded from this analysis as there is no seaport in the area currently infested.

Data sources

Air transport

We used the Eurostat detailed air passenger transport by reporting country and routes (available at https://ec.europa.eu/eurostat/databrowser/explore/all/transp?lang=en&subtheme=avia&display=list&sort=category&extractionId=AVIA_PAR) and the World Bank - Global airports database (available at https://datacatalog.worldbank.org/search/dataset/0038117), which are complementary. From Eurostat, we extracted the number of air passengers between the main airports within the infested area of Italy and Switzerland, and their destinations in Europe (routes data, https://doi.org/10.2908/AVIA_PAR_IT and https://doi.org/10.2908/AVIA_PAR_CH). World Bank data provides the number of passengers on connecting flights between airports worldwide for 2019.

Rail transport

Data related to rail transport were retrieved from the EuroGlobalMap 2022 dataset (EGM 2022.2 © EuroGeographics, available at https://www.mapsforeurope.org/datasets/euro-global-map) and the Deutsche Bahn Transport Rest API V5 database. The EuroGlobalMap 2022 dataset includes locations of railway stations in Europe. Based on these locations, we have extracted data on train travel between railway stations in Europe, by querying the Deutsche Bahn Transport Rest API V5. Deutsche Bahn Transport Rest API is an open database that returns real-time data on most long-distance and regional traffic, as well as international trains, in Central Europe. This database has previously been used to display European train journeys, showing how far one can travel from any station in Europe in less than 8 hours (https://www.chronotrains.com/). Queries to Deutsche Bahn Transport Rest API V5 were made using the httr2 package 0.2.2 (Wickham 2022).

Road transport

Data related to road transport were retrieved from a recently published dataset on European road freight traffic (Speth et al. 2022). These data describe the flows of trucks (both in tonnes and number of trucks) between 1675 regions in Europe at NUTS 3 spatial resolution during 2019.

Reachability analysis

Our introduction risk assessment framework is based on three main steps. First, for each transport mode, we identify all source locations within the infested area (e.g. airports or railway stations). Then, we measure the intensity of connections to all possible destinations elsewhere in Europe. Finally, reachability by all modes of transport is combined using a Pareto optimality method to rank regions according to their risk of introduction. The following sections describe this framework in more detail.

Air transport

We selected the airports located in the infested area and all the European airports reachable from these airports from the Eurostat and World Bank databases. For each reachable European airport, we summed the total number of passengers on flights departing from airports within the infested area. For the World Bank database, these data were available for 2019, and for Eurostat, we extracted data during the beetle emergence period, from May to August, for years 2010 to 2019. Some major reachable airports were missing from the Eurostat database and were present in the World Bank database. We predicted Eurostat missing data using the World Bank data as there was a strong correlation in the total number of passengers at reachable airports shared between the two databases ($R=0.95$, $p<0.001$, Pearson correlation). On a subset of the data made of airports found in both World Bank and Eurostat databases, we fitted a Generalized Additive Model (GAM) with a Poisson distribution. We used World Bank number of passengers as the only explanatory variable to predict Eurostat number of passengers using the gam function of mgcv package 1.8–42 (Wood 2011) (k = 4, family = “poisson”). This model explained 84.6% of deviance found in the data with an adjusted R-squared of 0.89. We applied this model to predict the number of passengers where data was missing from the Eurostat database. The obtained value, accounting for the cumulated number of passengers arriving at any European airport from all airports located within the infested area, was used as a proxy for the risk of introduction by air.

Rail transport

We identified the spatial coordinates of all railway stations located within the European infested area from EuroGlobalMap 2022. We fed these coordinates to the “GET /stops/nearby” query to extract the railway stations identifier from the Deutsche Bahn Transport Rest API V5, hence locating the closest railway station within a 500-meter radius from given coordinates. We retrieved the trip identification number (tripID) for all trains departing from these stations during the adults’ emergence period, between 2022-05-01 and 2022-08-31, using the “GET /stops/:id/departures” query. For each tripID, we retrieved all railway stations where the train stopped on its trip using the “GET /trips/:id” query. The final database contains all tripID with corresponding information on the stations of departure and destination, as well as the train stops (station id, name and coordinates, as well as the time of arrival and departure).

We mapped the resulting railway stations, excluding those that were already within the infested area. We computed the cumulated number of trains reaching these stations by counting the number of unique trip ids at these stations. The obtained value, accounting for the total number of trains arriving at European railway stations from stations in the infested area during the chosen period, was used as a proxy for the risk of introduction by rail.

Road transport

Road transport sources were identified as the NUTS 3 regions (ID_origin_region in the database from Speth et al. 2022) which were either completely or partially covered by the infested area. For each destination region (ID_destination_region), we cumulated the number of trucks (Traffic_flow_trucks_2019) departing from the NUTS 3 located within the infested area. Finally, for each destination region, we weighted the total number of trucks arriving from the infested area by the area of the destination region (in km^2) in order to account for the variable NUTS 3 sizes and to avoid underestimating the importance of smaller regions. The obtained value, the total number of trucks per square kilometre reaching NUTS 3 regions from the infested area, was used as a proxy for the risk of introduction by road.

Combining air, rail and road transport - composite reachability

We combined reachability by air, rail and road transport using a Pareto front ranking method (Roocks 2016). This method is based on a well-known multi-objective optimization algorithm, where all feasible solutions of a given problem are characterized by a vector describing their score with respect to different objectives. A solution is said to be non-dominated (or Pareto optimal) if it cannot be improved in any of the objectives without degrading at least one of the other objectives. The set of all non-dominated solutions (that may include one or multiple feasible solutions) is called the Pareto front.

Our method iteratively searches for the Pareto front that maximises the risk of introduction for the three modes of transport combined (no priority is given to any of the transport modes, which are therefore considered to be equally risky). The set of feasible solutions consists of all non-infested NUTS 3 regions of Europe, each one characterized by a three-dimensional vector reporting its reachability index for the three modes of transport. For air and train transport modes, we aggregated the number of passengers reaching an airport and the number of trains reaching a station across all airports and railway stations located within each NUTS 3 region in order to assign a unique reachability value for these two modes of transport.

All NUTS 3 belonging to the first Pareto front that maximize the reachability are labelled as 1 and then removed from the dataset. A new Pareto front is then identified, whose solutions are labelled as 2 and removed afterwards. This process continues until all NUTS 3 have been labelled and assigned a composite reachability index value from 1 to 1225, with 1 being the most reachable and 1225 being the least reachable when air, rail and road transport from the infested area are combined. The Pareto front analysis was performed using the psel function of rPref package 1.4.0 (Roocks 2016).

Results

Among the 1675 European NUTS3 regions, twenty were considered infested in 2022 because they contained at least one infested municipality. Within this infested area, there are 6 airports and 540 railway stations. Outside of that area, a total of 160 airports (from 30 different countries), 422 railway stations (located in 5 different countries), and 1446 NUTS 3 regions (from 33 countries) can be reached by planes, trains, and trucks, respectively. Reachability from the infested area varies between modes of transport (Fig. 1A–C). With the exception of a few distant major European cities, the rail transport network mainly connects areas adjacent to the infested area (7 NUTS 3 in Germany, 6 in Austria and 4 in France), most of Italy (65 NUTS 3 out of 107) and Switzerland (13 NUTS 3 out of 26). The road freight network is both local, with Switzerland, northern and central Italy highly reachable from the infested area; and international, with many major European cities also highly reachable. Finally, the air transport network is mostly international, with direct access to all major cities in Europe. Our analysis shows that of all NUTS 3 regions directly reachable by at least one transport mode from the infested area, 10% and 7% are reachable by air and rail respectively. On the other hand, almost all of these regions (99.8%) are reachable by road freight.

Interestingly, the distribution of planes, trains and trucks that reach NUTS 3 regions in Europe is far from uniform, with very few NUTS 3 concentrating most of the traffic from the infested area. Indeed, the 1% of NUTS 3 most reachable by rail (14 NUTS 3) account for over 60% of all trains leaving the infested area. Similarly, the top 1% of NUTS 3 reachable by air account for 52% of all flights, and the same is true for road freight, with the top 1% of NUTS 3 reachable by trucks accounting for 46% of all trucks leaving the infested area.

The composite reachability index, which combines air, rail and road transport, ranks NUTS 3 regions into ordered groups, from most to least reachable (Fig. 1D). The five groups of most reachable regions contain 62 NUTS 3 (from 16 different countries), of which 13 are reachable by all three modes of transport. Furthermore, these 62 NUTS 3 account for 70%, 72%, and 47% of the total number of planes, trains and trucks leaving the infested area, respectively.

The distribution of composite reachability by number of NUTS 3 per country is shown in Fig. 2: Italy (IT), Germany (DE), United Kingdom (UK), France (FR), Switzerland (CH), and Spain (ES) contribute with both a high number of reachable NUTS 3 and their relative importance in terms of the composite reachability index. On the other hand, eastern countries like Hungary (HU), Romania (RO) and the Czech Republic (CZ) have fewer, but highly reachable NUTS 3. The large number of small NUTS 3 regions in Germany explains the over-representation of this country in Fig. 2.

Finally, reachability correlates negatively with distance from the infested area for train, trucks and the composite index (Kendall correlation of -0.25***, -0.44*** and -0.32***, respectively), which means that more distant destinations are less reachable than closer destinations (Fig. 3). On the contrary, reachability by flight correlates positively with distance (Kendall correlation of 0.11***). Interestingly, reachability is anisotropic, meaning that it does not distribute uniformly in all direction (see the circular bar plots within the four panels of Fig. 3).

Discussion

In this study, we have mapped the risk of introduction of the Japanese beetle in continental Europe by air, rail and road transport from the infested area as defined in 2022. We found that reachability of regions varies by mode, and detected topological features of transport networks, ranging from a local and national predominance (rail and road transport) to an almost exclusively international dimension (air transport)

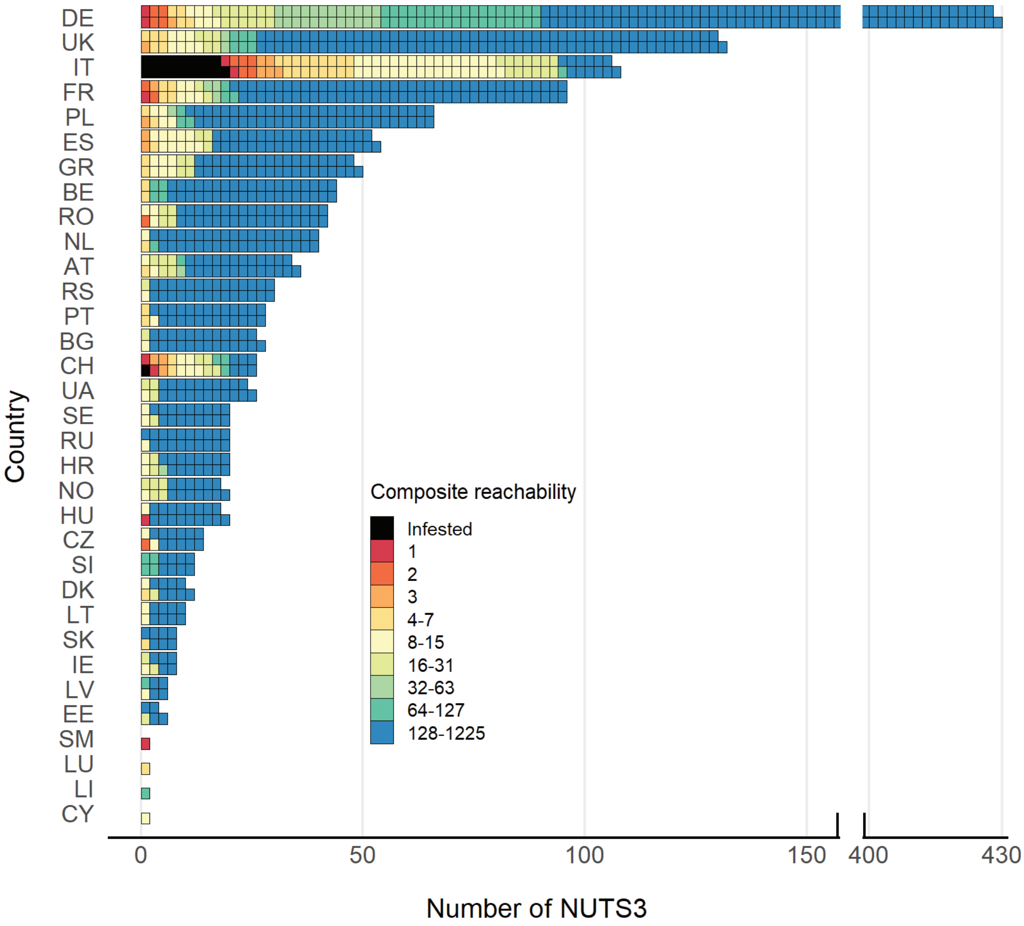

Figure 2. Distribution of reachable NUTS 3 regions per country when combining air, rail and road transport (composite reachability). Warmer colours correspond to higher reachability. Countries are shown using alpha-2 country ISO codes as described in the ISO 3166 international standard.

(Banks et al. 2015; Tatem 2017). The proposed composite reachability index, which combines the three transport modes, highlights a few scattered highly-reachable major cities across Europe, as well as a cluster of high reachability comprising many regions of Italy, Germany, Switzerland and France surrounding the infested area.

As this is the first analysis of the risk of Japanese beetle spread through human-mediated transport across continental Europe, our identification of likely introduction points cannot be compared with previous results. Nevertheless, the BeNeLux countries and northern Italy have also been identified as presenting a high risk of IAS introduction into Europe by a previous study that examined risk as a function of climate, soil, water, and anthropogenic factors (Schneider et al. 2021). Our results highlight transport network characteristics that have also been observed in previous studies, such as the international nature of air transport and the predominantly regional and national nature of rail and road freight transport (Hulme 2021). In addition, we have identified a disproportionate distribution of connections between the infested area and a small number of distinguished NUTS 3: indeed, for each of the three modes of transport, the 1% of the most reachable NUTS 3 accounts for more than 45% of the total outbound flow from the infested area. This demonstrates an assortative type of mixing (Newman 2003), a property of complex networks where nodes (here NUTS 3 regions) that are in some sense similar tend to be more connected. In our case, the infested area encompasses a major European hub (MXP-Milan

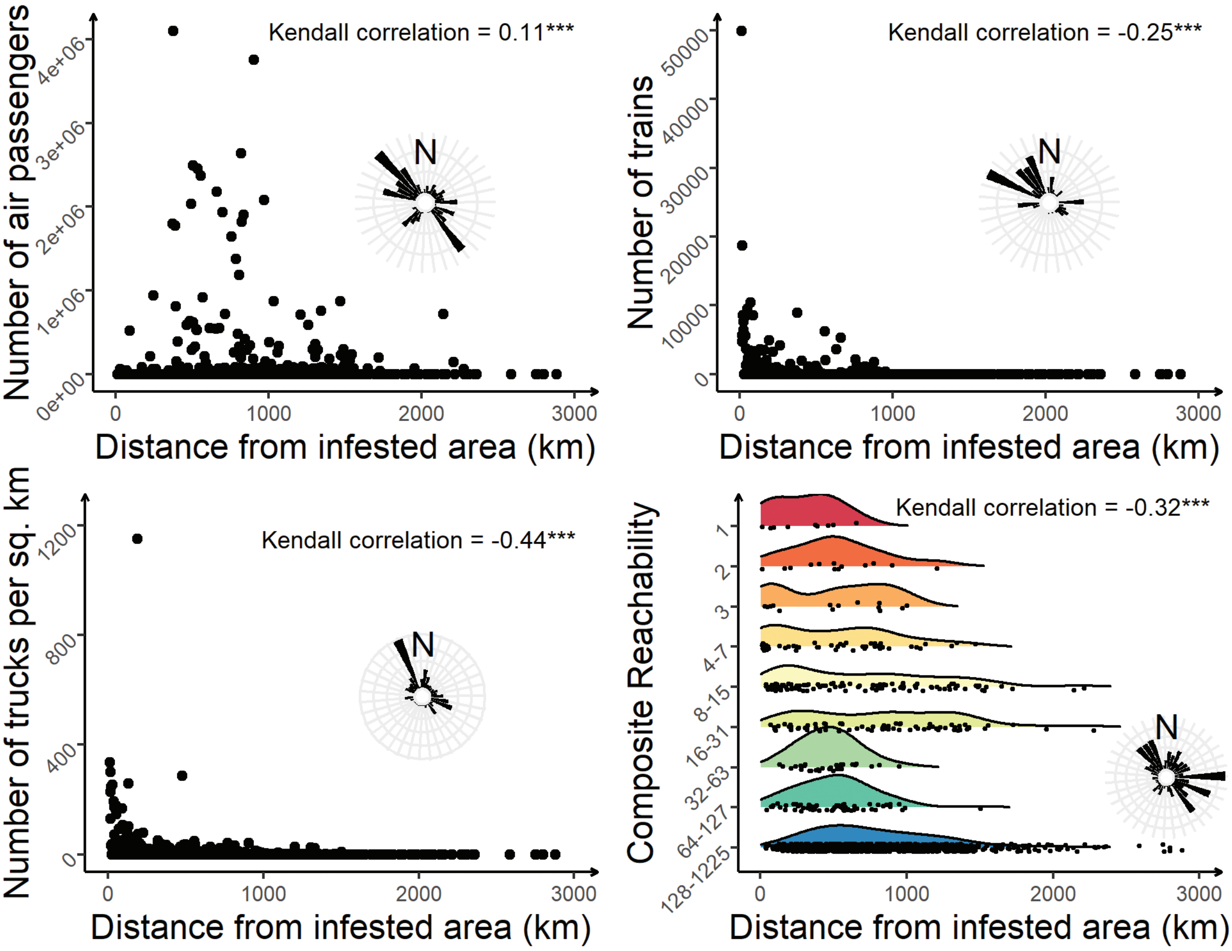

Figure 3. Distribution of air, rail, road and composite reachability of European regions for *Popillia japonica* as a function of distance from the infested area (in km), with the corresponding value of the Kendall correlation test. Within each panel, a circular bar graph shows the main directions in which the four reachability indices spread with respect to the infested area. Air, rail and road reachability are expressed as the number of passengers arriving at airports, the number of trains arriving at stations, and the number of trucks per square kilometre reaching NUTS 3 regions, departing from the infested area, respectively. Composite reachability of NUTS 3 regions is displayed from most reachable (group 1) to least reachable (groups 128-1225).

Malpensa Airport), which is naturally well connected to other hubs on the continent. On the other hand, Figs 1, 2 also show that the infested area is simultaneously connected to scattered, highly reachable hubs and is capillary-linked to surrounding, less reachable regions. This feature is typical of core-periphery graphs, where a subset of nodes in the network (the core) is connected to a few nodes of very high degree, as well as to many peripheric nodes (Pittel et al. 1996; Malliaros et al. 2019).

The highly-reachable hubs identified by combining air, rail and road transport, have already been shown to have particular potential for the spread of IAS (Banks et al. 2015). Interestingly, all Japanese beetle interceptions that have occurred in Europe over the last 5 years have been reported in regions that our analysis identified as highly reachable (Fig. 4), providing preliminary evidence of the robustness of our approach. Furthermore, an outbreak was detected in July 2023 in one of the nine regions we identified as being the most reachable from the infested area (Zurich region, Switzerland). Of all the interceptions known to date, only those in Zurich and Amsterdam could have originated from outside the continental European infested area, as they occurred at or near airports with direct connections to

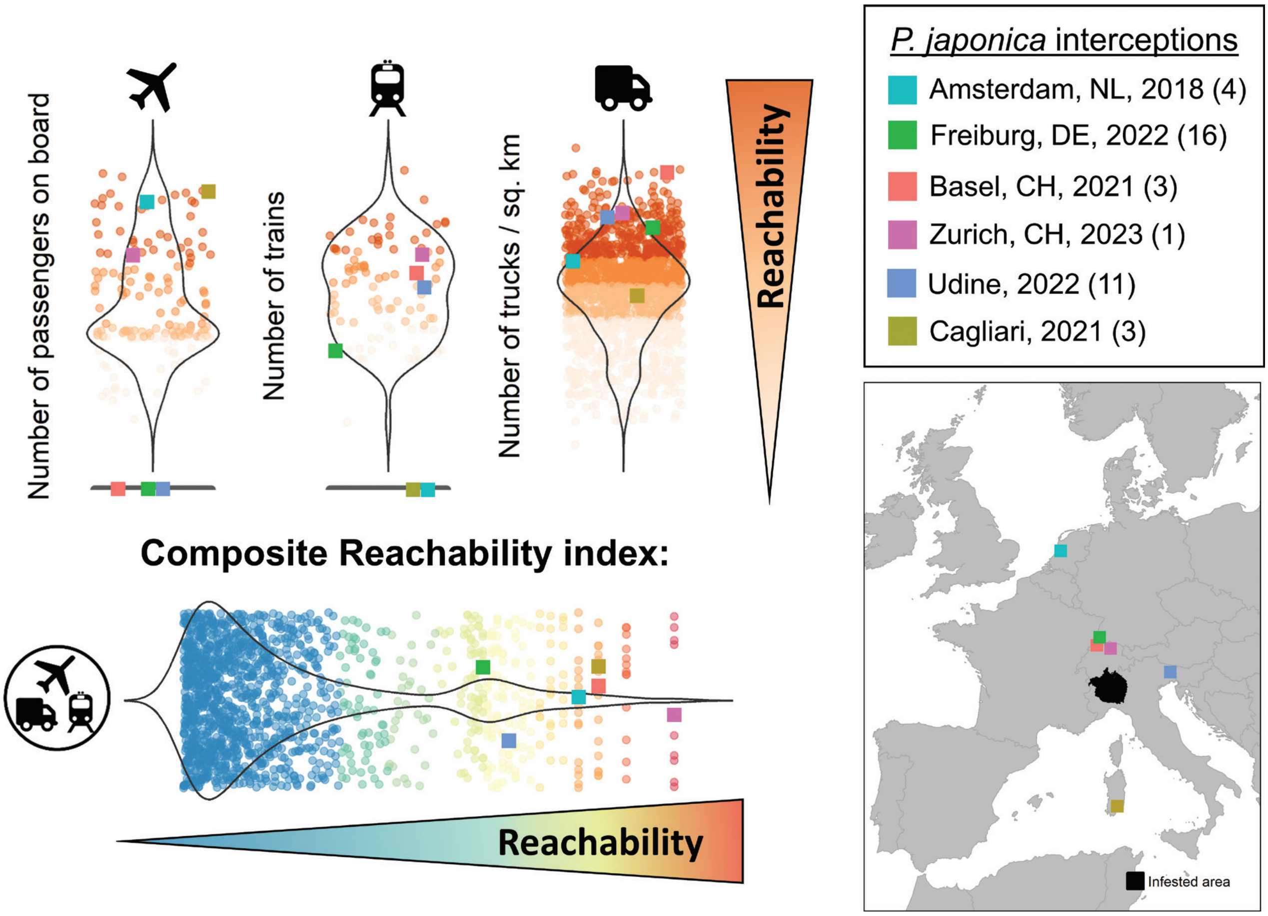

Figure 4. Interceptions of *P. japonica* made in Europe since 2018 and their position in relation to the distribution of reachability indices (air, rail, road, and composite). The number in brackets to the right of the interception site name indicates the group number assigned to the NUTS 3 region by the Pareto ranking method, from most reachable (1) to least reachable (16).

North America and/or Japan. Molecular methods could be used to further assess the most likely origin of these introductions (Strangi et al. 2023).

Although our results appear to be relevant based on interceptions and the published literature on transport networks, this analysis could be improved by considering hitchhiking on air freight, rail freight and private cars (domestic travel). Call detail record (CDR) could be a useful source for domestic travel, which could play an important role in facilitating the spread of the Japanese beetle, especially around the infested area (Tatem 2017).

The proposed framework provides a rapid response tool for decision-makers and phytosanitary services to anticipate the likelihood of hitchhiking pest introduction on a continental scale. Informing risk-based surveillance strategies with likelihood of introduction can significantly reduce surveillance efforts and promote early detection of invasive species (Parnell et al. 2014). As data become available, further improvements may be achieved, for example by targeting commodity movements specifically identified as pest carriers (Fenn-Moltu et al. 2023), or by including other transport modes. Highly reachable regions could also be surveyed for the presence of susceptible host plants or favourable environmental conditions (Tatem et al. 2006; Borner et al. 2023). Our framework highlights the need for local surveillance combined with a transboundary strategy, involving official authorities and stakeholders, and adapted to the scale and means of spread of the pest under surveillance (Radici et al. 2023).

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

This research was supported by the IPM-*Popillia* project, funded by the European Union Horizon 2020 research and innovation programme under grant agreement No 861852.

Author contributions

Conceptualization: SP, DM, LB. Formal analysis: DM, LB. Funding acquisition: SP. Methodology: SP, DM, LB. Project administration: SP. Supervision: SP, DM. Visualization: DM, LB. Writing - original draft: LB, DM. Writing - review and editing: SP, LB, DM.

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Data availability

The datasets generated during the current study are available in the French Research Government repository, https://doi.org/10.57745/3WUVWJ.

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Supplementary material 1

Reachability of European regions

Authors: Leyli Borner, Davide Martinetti, Sylvain Poggi

Data type: csv

Explanation note: Reachability of European regions by air, rail and road and combining the three modes of transport.

Link: https://doi.org/10.3897/neobiota.94.126283.suppl1

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 15–30 (2024)
DOI: 10.3897/neobiota.94.117313

Research Article

Invasive fish species in Romanian freshwater. A review of over 100 years of occurrence reports

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Abstract

Effective management of invasive alien species requires location-specific strategies involving the regular update of distribution maps to identify spatial patterns, trends, and pathways of entry and the spread and hotspots of those invasions. However, a comprehensive overview of invasive alien fish species in Romania is lacking. To fill this gap, we compiled a database with occurrences of alien fish species in Romania from diverse sources, including published literature, our own field data, online databases, social media, and online questionnaires. Occurrence data covers the 1910–2022 period. From a total of 52 alien fish species reported as present in Romania's waterways, we assigned an invasive status to 11 species, of which *Pseudorasbora parva*, *Lepomis gibbosus*, *Carassius gibelio*, and *Ameiurus* spp. are widespread. Based on the currently available occurrence records, we evaluated the presence and distribution of invasive alien fish species at the watershed level, concluding that invasive alien fish species are present in all Romanian watersheds. We identified several hotspots consistent with the main points of entry and spread of invasive alien fish species, principally located in western, central, and eastern Romania, i.e., Mures, Crisuri, and Siret watersheds.

Key words: Alien species, invasion hotspots, invasive alien fish, occurrence mapping

Academic editor: Wolfgang Rabitsch
Received: 26 January 2024
Accepted: 19 June 2024
Published: 16 July 2024

Citation: Drăgan O, Rozylowicz L, Ureche D, Falka I, Cogălniceanu D (2024) Invasive fish species in Romanian freshwater. A review of over 100 years of occurrence reports. NeoBiota 94: 15–30. https://doi.org/10.3897/neobiota.94.117313

Introduction

Biological invasions are one of the main drivers of biodiversity decline, coupled with land use changes, overexploitation, climate change, and pollution (IPBES 2023; Roy et al. 2024). The number of invasive alien species is rapidly increasing worldwide, and there is no substantial evidence that the rate of establishment of new invasive species is decelerating (Mormul et al. 2022). Biological invasions often have complex and long-term direct and indirect impacts, and many of these events manifest decades later, when the invaders are well established across large geographic ranges (Pyšek et al. 2020). Invasive alien species, such as the zebra mussel (*Dreissena polymorpha*), cane toad (*Rhinella marina*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Hypophthalmichthys nobilis*), grass carp (*Ctenopharyngodon idella*) and European starling (*Sturnus vulgaris*) have noticeably disrupted ecosystems worldwide, leading to lasting ecological harm (IPBES 2023). Asian carp, for example, outcompetes native fish, reducing biodiversity and disrupting aquatic food webs (Pimentel et al. 2005; Mayer et al. 2021). The negative impact on biodiversity of invasive alien species is accelerating and is expected to increase in the future. Researchers and policymakers proposed several measures to minimise future invasions; however, insufficient funding and limited scale of interventions reduce their efficiency (Seebens et al. 2017; IPBES 2023; Roy et al. 2024).

Invasive alien fish species pose a significant global threat to freshwater, estuarine, and marine ecosystems (Katsanevakis et al. 2013; Nunes et al. 2015), and freshwater ecosystems are considered among the most affected globally by biological invasions (IPBES 2023). Moreover, due to their high economic and social impact, freshwater fish invasions are one of the most documented events, with numerous studies available discussing those invasive alien species, their ecology, invaded regions, pathways, impacts, and management options (FAO 2019; Schneider et al. 2021; Bernery et al. 2022). Most established alien fish species occur in temperate regions of Europe, North America, and South America.

The primary introduction pathways of alien freshwater species in Europe are stocking and aquaculture, where individuals are able to reproduce in their new environment and manage to escape and end up populating entire watersheds (Gherardi et al. 2009). Zieritz et al. (2016) however, outlines various introduction pathways occurring at different periods in Northwestern Europe. Since 2000, there has been a significant increase in accidental introductions compared to deliberate ones. Furthermore, species introduced for aquaculture, research, or ornamental reasons were introduced later in time than those introduced for biological control or leisure. Since the 1960s, the ornamental trade has grown substantially and was accountable for almost all deliberate introductions in Northwestern Europe (Zieritz et al. 2016; Bernery et al. 2022). A recent study on the invasion history of alien fish species in Germany and Austria indicates that, while fisheries (including aquaculture) and the animal trade were responsible for most earlier introductions, waterways were the main pathway for recent invaders (Rabitsch et al. 2013).

Alien freshwater species impact native fish species through predation, competition, hybridisation, and disease propagation (Cucherousset and Olden 2011; Truhlar et al. 2014; Findlay et al. 2015; Tran et al. 2015; Britton 2023). Additionally, they negatively impact native aquatic communities, resulting in economic losses to the fishing and tourism industries and compromising human health and well-being (Tricarico et al. 2016; Haubrock et al. 2022).

In response to the mounting concern posed by invasive alien species (IAS) and the need for supranational coordinated actions, the EU adopted the Invasive Alien Species Regulation (Regulation 1143/2014). This legislation currently includes a list of 88 invasive alien species of EU concern for which Member States are required to initiate measures such as eradication and control. The last updated list contains ten alien fish species of EU concern: *Ameiurus melas*, *Gambusia affinis*, *Gambusia holbrooki*, *Lepomis gibbosus*, *Morone americana*, *Channa argus*, *Perccottus glenii*, *Plotosus lineatus*, *Pseudorasbora parva* and *Fundulus heteroclitus* (European Union 2022).

In Romania, data on the presence and distribution of alien fish species is limited and often outdated. Prior to 1956, alien fish species commonly spread naturally from neighbouring countries. However, after 1956, the implementation of large-scale national stocking programs of alien fish species led to unintentional introductions (Decei 1981; Popa 2002). Salmonids were introduced in mountain lakes, followed by minnows (*Phoxinus* spp.) used as baitfish. Several previous studies (Gavriloaie et al. 2003; Iftime and Iftime 2021) have enumerated or documented the presence and distribution of alien fish species in Romania. However, the available information is generally restricted to a few records, often old. To contribute to the management of alien fish species in Romania and Europe, the objectives of this study are (i) to update the inventory of alien freshwater fish species in Romania and identify the species that meet the criteria for invasiveness; (ii) to map the distribution of invasive alien freshwater fish species in Romanian freshwater and identify the hotspots and pathways to their spread.

Materials and methods

Data collection

We collected occurrence data from various sources published up to end of 2022, namely, scientific and grey literature, social media, public databases, online questionnaires, and our own field data, mainly collected during the 2019–2022 time frame of a national survey project (Ministry of Environment and University of Bucharest 2023).

Literature data were extracted from peer-reviewed articles, conference articles, books, grey literature, doctoral theses, and technical reports regarding the occurrence and distribution of alien fish in Romania. Initially, we used literature collected over the years by the authors. In addition, we searched Google Scholar and Web of Science All Databases collection, using a combination of keywords: alien fish, allochthonous fish, invasive fish, dispersal, distribution, new fish, nonnative fish, non-native fish, AND Danube, Danube Delta, Balkans, Bulgaria, Hungary, Moldavia, Romania, Serbia, Ukraine. We used the names of neighbouring countries as keywords because of the presence of border rivers, such as the Danube River, which is neighbouring Ukraine, Bulgaria and Serbia. The reference sections of the publications identified were further screened for other potentially relevant articles not covered by the search engines used.

Data on alien fish occurrences were also downloaded from the Global Biodiversity Information Facility (GBIF), a validated public online database (https://www.gbif.org/). Additionally, we collected data from social networking platforms (i.e., Facebook), where anglers and other fish-related peers submitted reports regarding invasive species. We only validated Facebook postings that had accompanying photos that allowed accurate species identification. Furthermore, an online survey was sent to fish researchers from different Romanian institutions.

Occurrence records are structured in a database that contains the following attributes: ID, Species, Source, Year of recording, Latitude, Longitude, Toponym, and Habitat. The occurrence database is limited to invasive alien fish species in Romania since the majority of the remaining alien fish species have very few occurrence records.

Data analysis

To enable spatial representation, we first standardised and geo-referenced occurrence reports of invasive alien fish species using a 10 km × 10 km UTM grid. We overlaid the 11 river management areas matching the Romanian Waters Authority (i.e., Crisuri, Somes-Tisa, Siret, Prut-Barlad, Dobrogea-Litoral, Ialomita-Buzau, Arges-Vedea, Olt, Jiu, Banat, Mures) onto the grid map of Romania to conduct the analysis at the catchment level. As a result, each occurrence record was geo-tagged to a specific basin, allowing us to generate river basin statistics. To identify the hotspots, we used the Density Analysis Plugin with the Styled Heatmap function available in QGIS software. This function is integrated into the QGIS Heatmap algorithm (Kernel Density Estimation), automatically styling the layer and enabling users to set the cell size in different units of measure (QGIS.org 2023). Consequently, we selected kilometres as the base unit of measurement and set a kernel radius of 10 km with a uniform kernel shape and a greyscale ranging from 1 (light colour) to 60 (dark colour) to present the number of occurrences inside the radius.

Alien fish species present in Romania

We classified alien fish reported as present in Romania by invasion stage (Blackburn et al. 2011) (Fig. 1) as casual alien species, naturalised alien species, or invasive alien species (Suppl. material 1). Casual alien species (CAS) include alien fish species that have been observed and reported in the wild, sometimes on a single occasion. They are either aquarium fish that possess the ability to survive in a new environment or species that have been accidentally released from fish farms and are unable to reproduce or survive winter. Naturalised alien species (NAS) refer to species that have successfully adapted, forming self-sustaining populations, but which have not spread into new watersheds. The invasive alien species (IAS) category

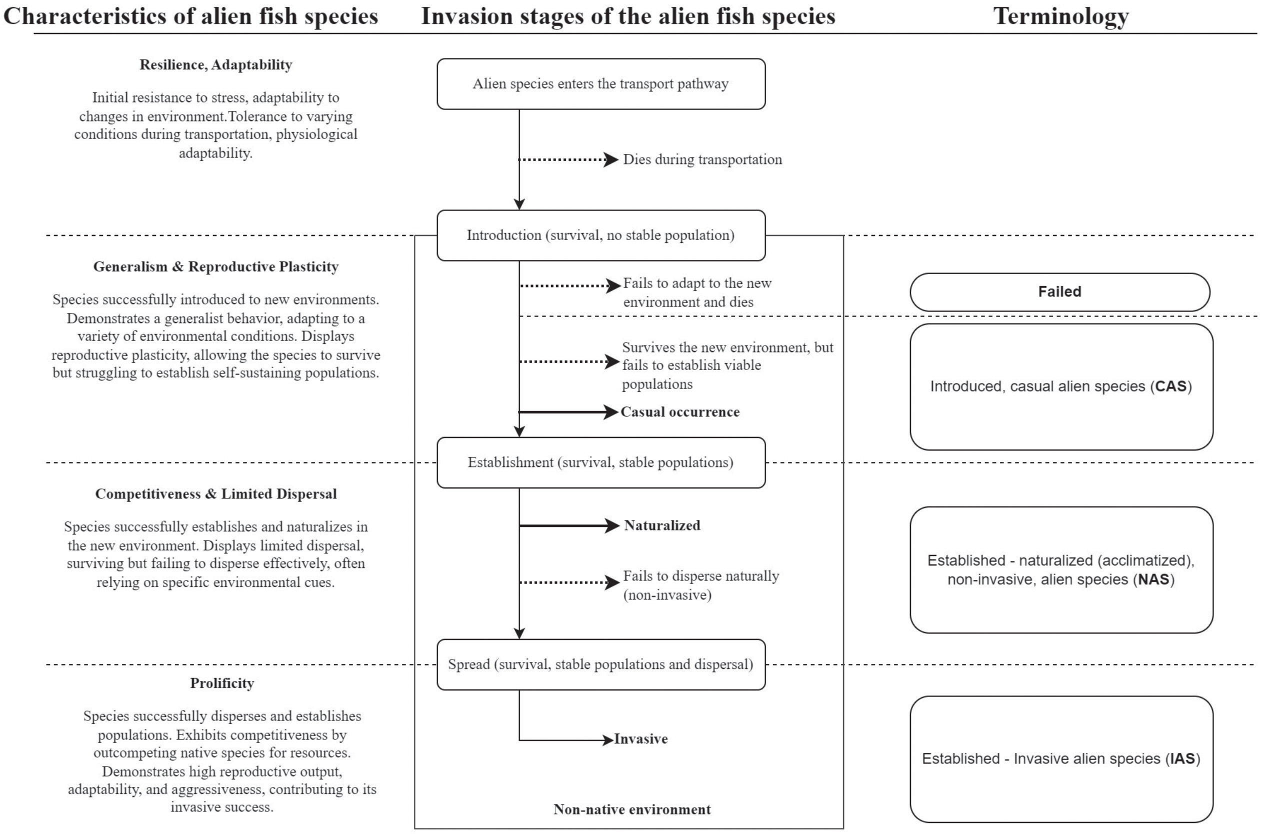


Figure 1. The main steps of the invasion process and the associated alien species status terminology used in this article.

includes species able to survive and outcompete native species, that possess advantageous reproductive features, demonstrate significant tolerance to environmental factors variation, and establish populations capable of spreading into new watersheds and habitats. Apart from these three categories, there are species for which we have limited data regarding their presence and impact (unknown/data deficient alien species) and species that failed to survive after introduction (Failed). We included *Carassius gibelio* in the list of IAS despite its unclear alien status. It was originally considered alien (e.g., Banarescu 1964; Iacob and Petrescu-Mag 2008), although later several authors considered it indigenous not only to Siberia but also to Central and Eastern Europe (Rylkova et al. 2013; Fricke et al. 2021). For Romania, Otel (2019) used historical distribution data to suggest that its natural range included the Romanian sector of the Danube and its main tributaries.

Results

We found 52 alien fish species reported in Romania between 1910 and 2022. Several sources reporting these species also included evidence on the pathway and year of introduction. From the 52 species reported from Romania, 11 fish species (Table 1) fulfil the criteria for invasiveness (IAS), the remaining (Table 2) are CAS (10 species), NAS (4 species), or have an unknown status (3 species). Furthermore, we identified 24 species that have failed to adapt and survive, including tropical aquarium species occasionally reported in the wild and surviving, at least, the summer (Table 2).

Apart from IAS, the rest of the alien fish reported in Romania have few distribution records. Therefore, we focused our study on the distribution of IAS species only (Table 1) and compiled a distribution database with 3107 occurrence records (https://ipt.pensoft.net/resource?r=invasive_fish_species_romania). Most occurrences were recorded starting with the 1990–2000 decade, with a peak in the 2000–2010 decade (Fig. 2). Of these, 2372 occurrence records were from published literature (98 documents, Suppl. material 2), 588 occurrence records were provided by the recent national survey project completed with our own field data, 78 occurrence records were extracted from the GBIF database, 22 occurrence records were extracted from social media, and 47 occurrence records were obtained from online questionnaires.

Table 1. Invasive alien fish species in Romanian freshwater.

Scientific name	Year of introduction	Natural range	Introduced from	Pathway
Ameiurus melas	1997	North America	Hungary	Unaided
Ameiurus nebulosus	1908	North America	Hungary	Unaided
Carassius gibelio	1912	East Asia	Moldavia	Aquaculture, Angling
Ctenopharyngodon idella	1960	South Asia	China	Aquaculture
Hypophthalmichthys molitrix	1960	South Asia	China	Aquaculture
Hypophthalmichthys nobilis	1960	South Asia	China	Aquaculture
Lepomis gibbosus	1929	North America	Hungary	Unaided
Oncorhynchus mykiss	1885	North America	Hungary	Angling
Perccottus glenii	2001	East Asia	–	Unaided
Pseudorasbora parva	1960	North-East Asia	China	Contamination of stocking
Salvelinus fontinalis	1906	North America	Austria	Angling

Table 2. Alien fish species with casual (CAS), failed, naturalised (NAS), and unknown status reported from Romania.

Scientific name	Year of introduction	Natural range	Introduced from	Pathway	Status
Acipenser baerii	2000	North Asia	Former USSR	Aquaculture	CAS
Betta splendens	2009	South-East Asia	–	Ornamental	Failed
Carassius (*auratus*) *auratus*	1920	East Asia	China	Aquaculture, Ornamental	NAS
Chindongo demasoni	2019	Africa	–	Ornamental	Failed
Clarias gariepinus	2002	North Africa	Hungary	Aquaculture	Unknown
Clarias ngamensis	2004	Africa	–	Aquaculture	Unknown
Coregonus albula	1956	North Asia	Former USSR	Aquaculture	CAS
Coregonus lavaretus	1956	North Asia	Former USSR	Aquaculture	
Coregonus peled	1980	North Asia	Former USSR	Aquaculture	NAS
Gambusia holbrooki	1927	North America	Germany	Biological control	NAS
Hemiculter leucisculus	1960	East Asia	China	Accidental	Failed
Ictalurus punctatus	1978	North America	Former USSR	Aquaculture	NAS
Ictiobus bubalus	1978	North America	Former USSR	Aquaculture	CAS
Ictiobus cyprinellus	1978	North America	Former USSR	Aquaculture	CAS
Ictiobus niger	1978	North America	Former USSR	Aquaculture	CAS
Lithochromis rufus	2019	Africa	–	Ornamental	Failed
Macropodus opercularis	–	East Asia	–	Ornamental	Failed
Megalobrama terminalis	1960	East Asia	China	Aquaculture	Failed
Micropterus salmoides	1912	North America	–	Angling	Unknown
Morone saxatilis	2018	North America	–	Angling	Failed
Mylopharyngodon piceus	1960	East Asia	China	Aquaculture	CAS
Ochetobius elongatus	1960	East Asia	China	Accidental	Failed
Oreochromis niloticus	2008	Africa	–	Aquaculture	CAS
Parabramis pekinensis	1960	East Asia	China	Aquaculture	Failed
Pethia conchonius	2008	South Asia	–	Ornamental	Failed
Pethia ticto	2001	South-East Asia	–	Ornamental	Failed
Piaractus brachypomus	2017	South America	–	Ornamental	Failed
Piaractus mesopotamicus	2021	South America	–	Ornamental	Failed
Poecilia reticulata	2008	South America	–	Ornamental	Failed
Poecilia sphenop	2008	South America	–	Ornamental	Failed
Polyodon spathula	1992	North America	USA	Aquaculture	CAS
Pseudobrama simoni	1960	East Asia	China	Accidental	Failed
Pseudolaubuca engraulis	1960	East Asia	China	Accidental	Failed
Pygocentrus nattereri	2022	South America	–	Ornamental	Failed
Salvelinus alpinus	2012	North America	–	Aquaculture	CAS
Squaliobarbus curriculus	1960	East Asia	China	Accidental	Failed
Toxabramis argentifer	1960	East Asia	China	Accidental	Failed
Trichopodus trichopterus	2008	South-East Asia	–	Ornamental	Failed
Xenocypris macrolepis	1960	East Asia	China	Accidental	Failed
Xiphophorus helleri	2008	Central America	–	Ornamental	Failed
Xiphophorus maculatus	2008	Central America	–	Ornamental	Failed

We ranked the invasive alien fish species based on the number of occurrence records as a surrogate index of spread and abundance; the most abundant is *Pseudorasbora parva*, while the least abundant is *Salvelinus fontinalis* (Fig. 3).

The spatial distribution of invasive alien fish species is uneven as most occurrence records were from the Mures River Basin and Cris River Basin, followed by the Siret River Basin and Somes-Tisa River Basin (Fig. 4). The lowest number of records were reported from the Jiu, Olt, and Prut River Basins.

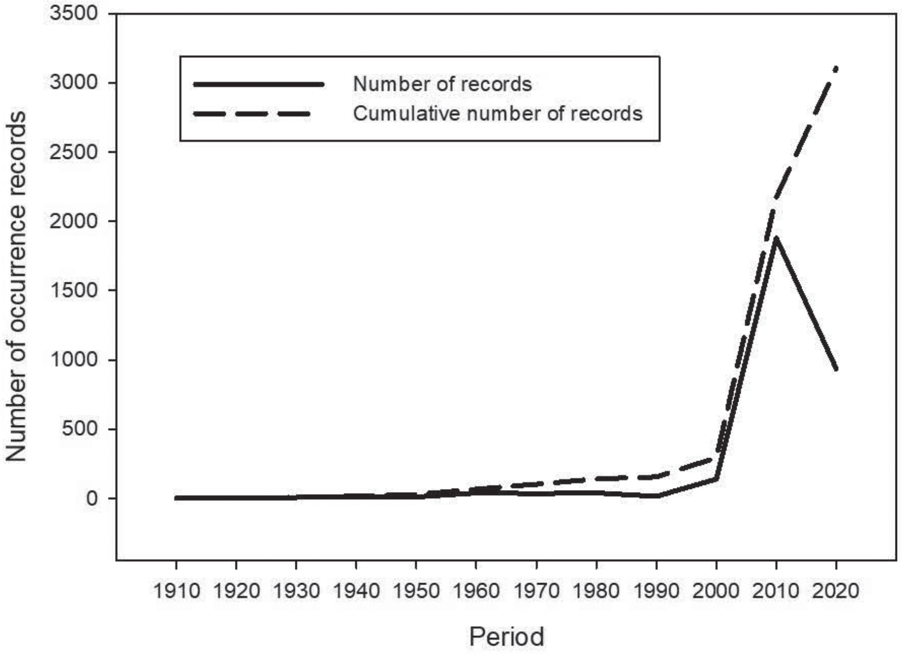


Figure 2. The number of occurrence records reported from Romania by decade (1910–2022).

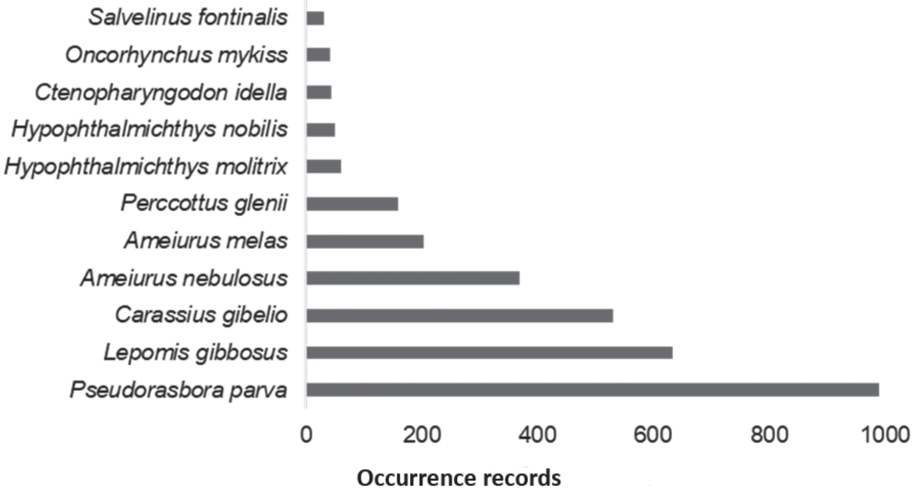


Figure 3. The total number of occurrence records of the 11 invasive alien fish species in Romania.

Spatial distribution of invasive alien fish species in Romania is presented in Fig. 5.

The visualisation of the hotspots highlights clusters of invasive alien fish species occurrences. These include the Danube, Crisuri and Mures rivers in the west and the Siret River and the Danube Delta in the east (Fig. 6).

Discussion

Our review identified 11 invasive alien fish species out of 52 alien fish species reported in Romania from 1910 to 2022. Within the invasive alien species of EU concern, we did not consider *Gambusia holbrooki* as invasive in Romania due to its restricted presence in a few lakes since their first detection decades ago and its inability to disperse naturally in lotic habitats. However, according to EU Regulation 1143/2014, this species should be eradicated if detected.

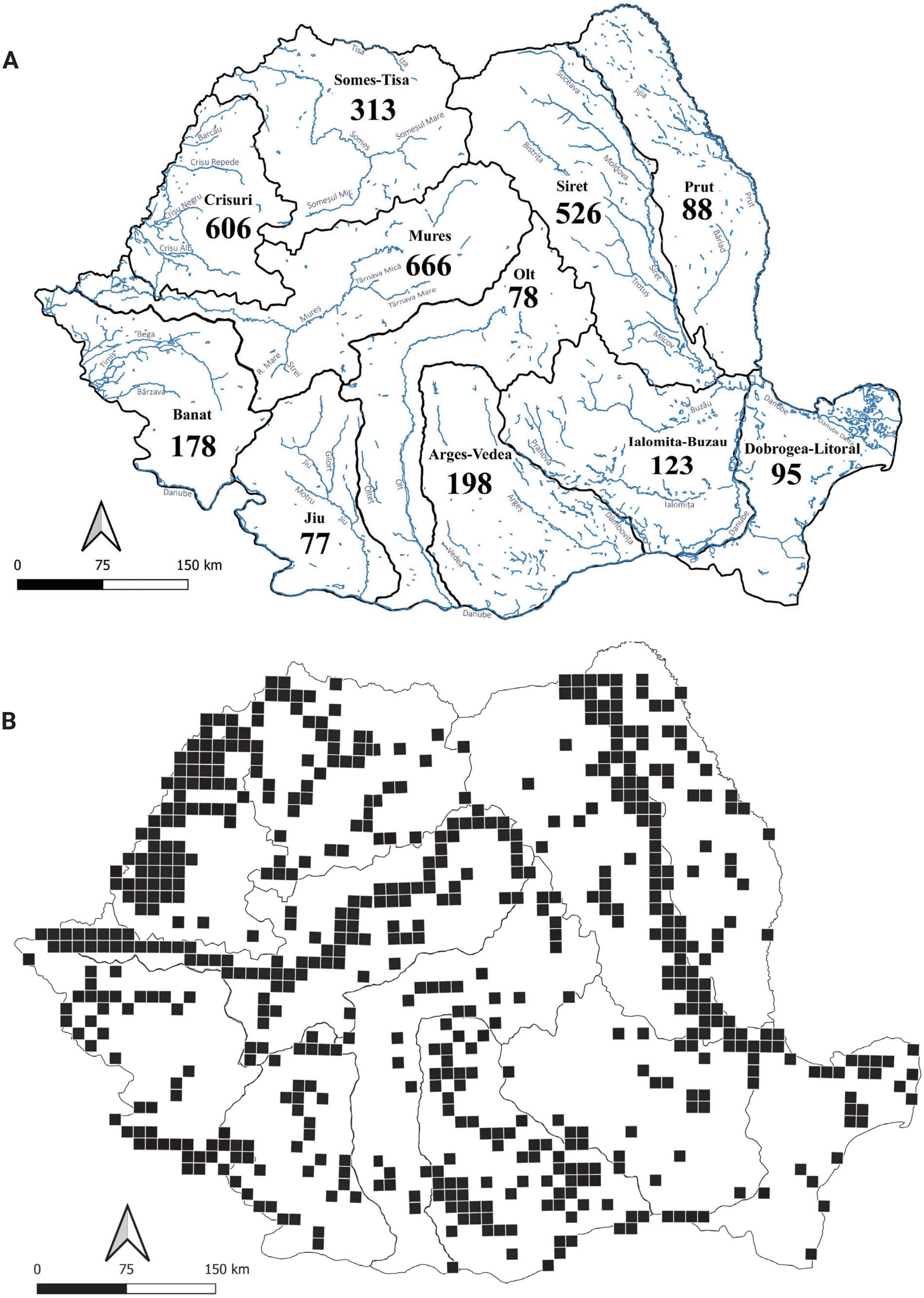
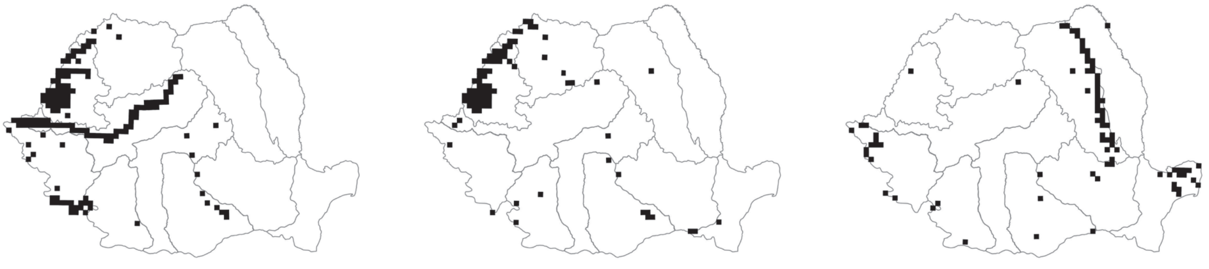
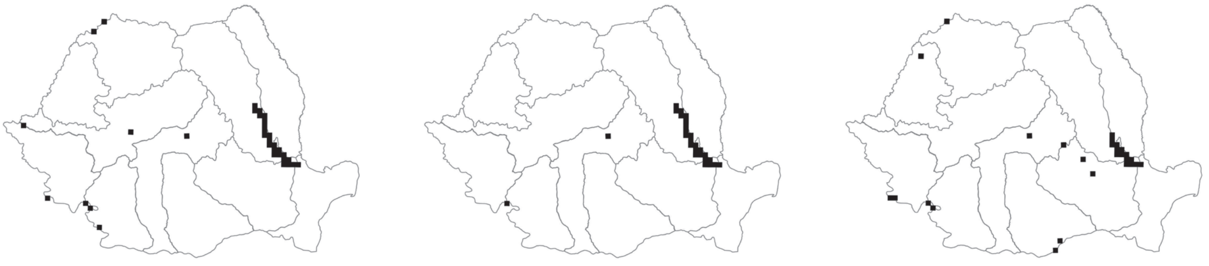


Figure 4. Invasive alien fish species occurrence records by river basin in Romania (**A**), and at UTM 10×10 km grid cell (**B**).

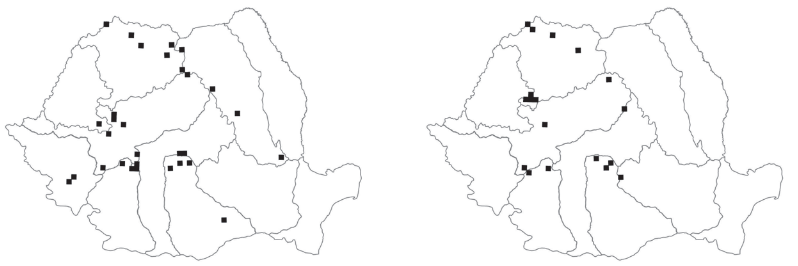
Pseudorasbora parva *Lepomis gibbosus* *Carassius gibelio*



Ameiurus nebulosus *Ameiurus melas* *Perccottus glenii*



Hypophthalmichthys molitrix *Hypophthalmichthys nobilis* *Ctenopharyngodon idella*



Oncorhynchus mykiss *Salvelinus fontinalis*

Figure 5. The occurrence records of invasive alien fish species in Romanian freshwater, by river basin unit, using UTM 10×10 km cells.

While not all alien species evolve to become invasive and have negative economic or ecological impacts, most invasive alien species share common characteristics such as rapid growth, broad environmental tolerance, and a history of invasiveness (Kolar and Lodge 2002; Docherty et al. 2017; Roy et al. 2024). For example, *Carassius gibelio* is probably the most common fish species in many lowland rivers in Romania (ANPA 2024) and it is the most caught species by fishermen, having a high commercial value (Eurofish Magazine 2021). However, at both the European and global levels, it is regarded as an invasive species with traits that impact ecosystems (Kucher et al. 2019; Šmejkal et al. 2024).

By mapping invasive alien fish distribution, we were able to identify several invasion hotspots, mostly along the north-western part of Romania and transboundary rivers, which correspond to the unaided pathway of introduction. We found that very few studies focused on the ecological impact of invasive alien fish species in Romania. This is likely because, until 1989, Romania was primarily focused on the exploitation of

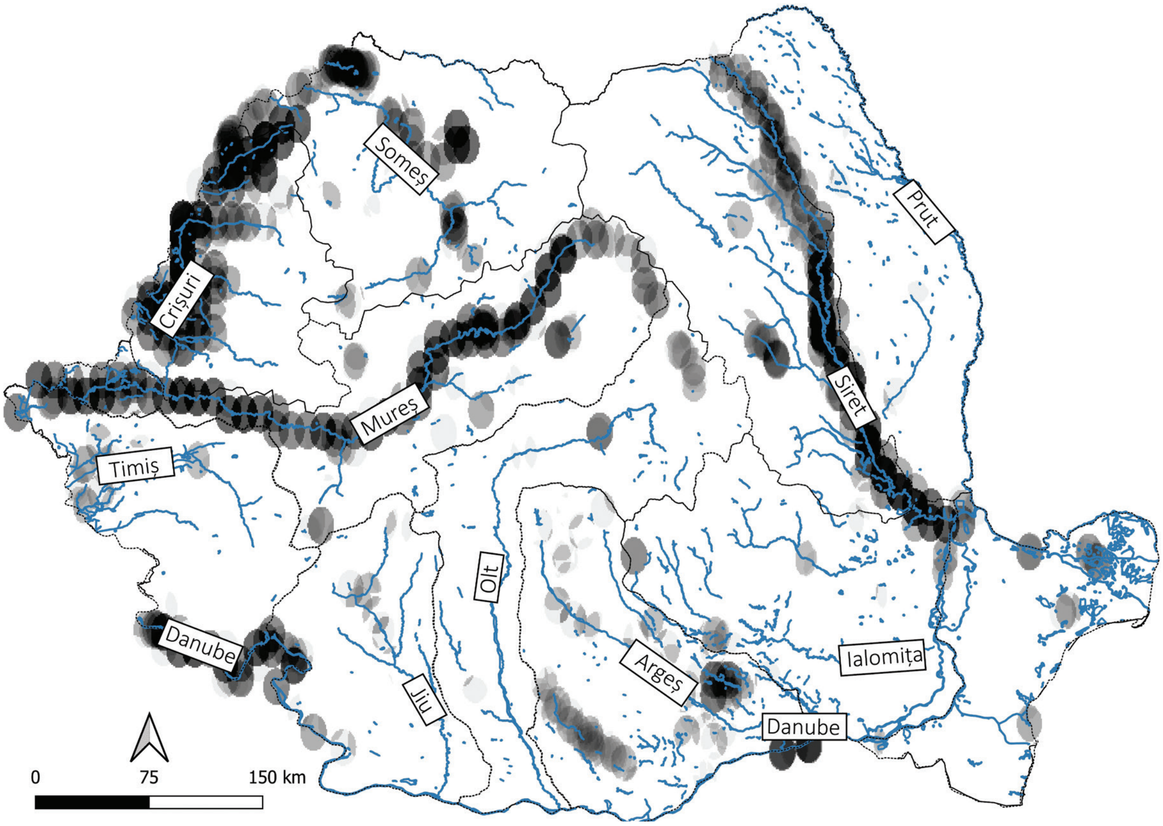


Figure 6. The hotspot map of invasive alien fish species across large rivers in Romania, using a greyscale with values from 1 (light) to 60 (dark) to present the number of occurrences in a 10 km radius.

economically valuable fish species, ignoring the evaluation of the invasive potential of introduced species (Petrisor and Tirziman 2019). The same practices have been reported and observed in neighbouring countries that also started fish introduction and acclimatisation campaigns prior to the 1970s, resulting in a similar timeline in alien species introductions reporting and numbers of alien fish species. For example, 31 alien fish species were reported in Bulgaria (Yankova 2016), while Takacs et al. (2017) reported 59 alien fish species in Hungary, with a large percentage due to aquarium releases, including *Carassius gibelio*, which is now a well-established invasive fish species (Keszte et al. 2021). In Serbia, an ESENIAS country report by Rat et al. (2016) identified 29 fish species as alien within that country, while in contrast, Lenhardt et al. (2011) reported a slightly lower number, citing 22 alien fish species. The primary pathway for the introduction of fish into Serbia is unaided spread through the Danube and Tisza rivers, originating from Romania and Hungary, as detailed by Lenhardt et al. (2011).

The Danube River, which connects approximately one-third of Europe and most Romanian rivers, plays a crucial role in the spreading and distribution dynamics of alien fish species (Bodis et al. 2012). The Balkan Peninsula is considered one of the hotspots of native freshwater fish diversity in Europe (Oikonomou et al. 2014) and, therefore, under significant threat from invasive alien species. The spread of alien freshwater species across natural barriers increased in the Danube basin after the construction of the Main-Rhine-Danube canal (Leuven et al. 2009; Paunovic et al. 2015). The need for coordinated actions resulted in the establishment of the Danube Region Invasive Alien Species (DIAS) Network in 2014 as part of the Priority Area 06 of the EU Strategy for the Danube Region (Rozylowicz et al. 2022).

We observed a spatial bias due to uneven spatial sampling. The data available might not be representative of the actual distribution of IAS in Romania but rather an indicator of the collectors of data, the so-called botanist effect (Moerman and Estabrook 2006; Anastasiu et al. 2024). Thus, the identified hotspots may primarily reflect areas with intensive, opportunistic sampling near major academic and research facilities rather than a systematic coverage. A second source of bias is due to accessibility, with many records originating from areas easily accessible, primarily in lowland or hilly regions. Comparatively, fewer records come from mountainous areas, where accessibility is often limited (Schmeller et al. 2022). To improve the accuracy of species distribution, researchers should systematically sample remote unprotected areas, distant from their research facilities.

Miu et al. (2020) identified protected areas of high priority for fish conservation in Romania, which overlap with the invasion hotspots found in our study. Within these areas, the Iron Gates Natural Park holds significance as the gateway of the Danube into Romania. This site and other important locations on the Romanian Danube shore are designated as RAMSAR sites (Rozylowicz et al. 2022). As designated priority areas for native fish protection, these RAMSAR sites face vulnerability to invasive alien fish species. The hotspots of invasive alien fish species overlap also with the Danube Delta Biosphere Reserve, a protected area hosting a substantial portion of native fish fauna (Rozylowicz et al. 2019).

The updated knowledge base on the distribution of alien fish in Romania is an outline of a complex and dynamic process. As invasive alien species continue to exploit the connectivity of river systems and spread, new areas face the threat of invasion. Furthermore, as climate change intensifies, affecting the hydrological cycle and making water levels increasingly unpredictable, this might eliminate physical barriers, allowing invasive alien fish species to expand and spread into new watersheds (Carosi et al. 2023; Le Hen et al. 2023). The dynamic range of invasive alien fish species due to the high connectivity requires proactive measures to prevent and contain their spread, and joint management strategies and agreements to coordinate efforts across national borders and effectively manage shared river systems (IPBES 2023).

Our study presents an updated occurrence database of invasive alien freshwater fish species in Romania. The database is a baseline for further inventories and monitoring the spread of invasive alien fish and is a valuable resource for conservation. As the threat of invasive alien species continues to grow, comprehensive and updated distribution mapping remains an essential tool for communication and effective environmental management.

Acknowledgements

We are grateful for the assistance of colleagues who contributed by providing data or supporting fieldwork within the project: Florina Stănescu, Alexandra Telea, Sebastian-Theodor Topliceanu, Geanina Fănaru, Sabina Elena Vlad, Marian Tudor, Attila Andras Nagy, Istvan Imecs, and Badilita Alin Marius.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

The study was supported by PN-III-P4-PCE-2021-0818 grant of the Ministry of Research, Innovation and Digitization, the FishME project (ANR-21-BIRE-0002-01, FWF-I-5824, UEFISCDI 276/2022), POIM 2014+ 120008 Appropriate management of invasive species in Romania according to EU Regulation 1143/2014 on the prevention and management of the introduction and spread of invasive alien species, and partially by the EU NextGeneration EU project ReSponSe (PNRR-III-C9-2022 – I5, 760010/30.12.2022).

Author contributions

Conceptualization: DC, OD. Data curation: OD. Formal analysis: OD, DC. Investigation: DU, IF. Methodology: LR, DC. Project administration: DC. Writing – original draft: LR, DC, DU, OD, IF. Writing – review and editing: DU, DC, LR, IF, OD.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Methodology for classification of fish species into casual alien species, naturalized alien species, or invasive alien species

Authors: Ovidiu Drăgan, Laurentiu Rozylowicz, Dorel Ureche, Istvan Falka, Dan Cogălniceanu
Data type: docx

Link: https://doi.org/10.3897/neobiota.94.117313.suppl1

Supplementary material 2

Publications used to compile occurrences distribution of invasive fish species in Romania

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Data type: docx

Link: https://doi.org/10.3897/neobiota.94.117313.suppl2

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 31–56 (2024)
DOI: 10.3897/neobiota.94.119307

Research Article

Effects of earthworm invasion on soil properties and plant diversity after two years of field experiment

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Abstract

Although belowground invasive species are probably equally widespread and as important as their aboveground counterparts, they remain understudied, and their impacts are likely to be stronger when these invaders act as ecosystem engineers and differ functionally from native species. This is the case in regions historically devoid of native earthworms, such as parts of northern North America, which are now experiencing an invasion by European earthworms. Although invasive earthworms have been reported to have multiple consequences for native communities and ecosystem functioning, this knowledge is mostly based on observational studies, and the mechanisms underlying their cascading impacts need to be investigated. Here, we thus investigated the sequence of events, i.e., ecological cascades following earthworm invasion, that have rarely been studied before, in a two-year field experiment. We expected that the changes in soil abiotic properties observed following invasion would coincide with changes in plant community diversity and community trait composition, as well as in alterations in above- and belowground ecosystem functions. To test these hypotheses, we set up a field experiment that ran for two years in a forest in Alberta (Canada) to investigate soil properties and understory plant community composition in response to invasive earthworms.

Our study shows that invasive European earthworms alter several soil abiotic properties (i.e., soil nutrient content, and pH) after two years of experiment. Invasive earthworm effects varied with soil depth for some soil properties (i.e., soil pH, water-stable aggregates, nitrogen, and microbial basal respiration), but we did not find any significant earthworm effect on soil water content, bulk density, or the total soil microbial biomass independently of the soil layer. Moreover, invasive earthworms did not affect plant community composition and only slightly affected community diversity in this short-term experiment. The minor changes observed in plant functional group composition are thus potentially the first signs of invasive-earthworm effects on plant communities.

Our research provides experimental evidence that previously reported observational effects of invasive earthworms on soil properties are indeed causal and already significant after two years of invasion. These changes in soil properties are likely to have cascading effects on plant community composition, functional diversity, and ecosystem functioning, but such effects may take longer than two years to materialize.

Key words: Biological invasion, detritivore, ecosystem engineer, microbes, plant functional traits, soil nutrients

Academic editor: Brad Murray
Received: 22 January 2024
Accepted: 17 May 2024
Published: 26 July 2024

Citation: Thouvenot L, Ferlian O, Horn L, Jochum M, Eisenhauer N (2024) Effects of earthworm invasion on soil properties and plant diversity after two years of field experiment. NeoBiota 94: 31–56. https://doi.org/10.3897/neobiota.94.119307

Introduction

Worldwide biodiversity loss is driven by climate change and anthropic activities, such as habitat fragmentation or pollution, and threatens ecosystem functions and processes (Wardle et al. 2011; Cardinale et al. 2012; Hooper et al. 2012). Biological invasions are also ranked as significant biodiversity threats (Sala et al. 2000; Murphy and Romanuk 2014; IPBES 2019), and there is a growing interest in understanding the effects of invasive species on native biodiversity, the underlying mechanisms of these effects, and in evaluating the costs of their management (Vilà et al. 2011; Simberloff et al. 2013; Vilà and Hulme 2017; Renault et al. 2022; Turbelin et al. 2023).

Although still understudied, belowground invasive species are probably equally widespread and as important as aboveground invasive species (Ehrenfeld and Scott 2001; Hendrix 2006). The effects of these invasive species on native communities and ecosystems are likely to be stronger when the invasive species act as ecosystem engineers (Jones et al. 1994) and when they are functionally dissimilar to the native community (Wardle et al. 2011). A prominent example is European earthworms that invade previously earthworm-free regions of the North-American continent. These earthworm-free regions, such as the eastern and mid-western parts of the USA and the Rocky Mountains in Canada (James and Hendrix 2004; Addison 2009; Hendrix et al. 2008), have been largely devoid of native earthworms since the last glaciation (Bohlen et al. 2004; Hendrix et al. 2008) but are currently facing an ongoing invasion by European earthworms (Bohlen et al. 2004; Hendrix et al. 2008) and, more recently, by Asian jumping worms (Chang et al. 2021). There, invasive earthworms have multiple consequences for native communities and ecosystem functioning (Frelich et al. 2019).

The effects of invasive earthworms on native ecosystems can result from changes in the physical and chemical properties of the soil due to their feeding and burrowing activities. These impacts will depend on the identity and ecological group of the earthworm species, as the different ecological groups have different behaviors (Bouché 1977), and thus on the earthworm community composition (Ferlian et al. 2018, 2020). Invasive European earthworms were shown to remove the soil surface litter and reduce the organic matter in the topsoil horizons (Hale et al. 2005; Resner et al. 2015). Moreover, their activities decrease the soil water content (Larson et al. 2010), but increase soil denitrification (Jang et al. 2022) and the leaching of soil nutrients (Bohlen et al. 2004; Frelich et al. 2006). They consequently change the nutrient availability and distribution in forest soils (Shuster et al. 2001; Bohlen et al. 2004; Resner et al. 2015), with effects potentially depending on if it is the organic or the mineral soil layer considered (Fahey et al. 2013a, 2013b; Ferlian et al. 2020). Another explanation of the impacts of invasive earthworms on ecosystem functioning could be their direct or indirect effects on other above- or belowground organisms (Frelich et al. 2012, 2019; Jochum et al. 2022).

Indeed, soil microbial communities, aboveground and soil fauna, as well as plant communities, were shown to be affected by invasive European earthworms (McLean et al. 2006; Burtis et al. 2014; Craven et al. 2017; Ferlian et al. 2018; Jochum et al. 2021, 2022; Jang et al. 2022), with many studies focusing on plant communities (Craven et al. 2017). For example, several studies showed that invasive earthworms decreased plant species diversity (Hale et al. 2006; Holdsworth et al. 2007; Gibson et al. 2013; Drouin et al. 2016; Craven et al. 2017). Their effects seem to depend on plant species and functional group identity (Drouin et al. 2016; Alexander et al. 2022) but, overall, they promote grass species (Frelich et al. 2006; Drouin et al. 2016; Craven et al. 2017). The changes in plant community diversity and composition could be due to several mechanisms, such as the outcome of earthworm-seed/seedling interactions (Eisenhauer et al. 2009a; Drouin et al. 2014; Clause et al. 2015; Nuzzo et al. 2015; Fleri et al. 2021), or result from changes in the soil properties due to invasive earthworms as mentioned above (Ferlian et al. 2020). Such changes could lead to a modification in plant development and plant functional traits (Dávalos et al. 2013, 2015; Cameron et al. 2014; Dobson et al. 2017; Richardson et al. 2018). The redistribution, mineralization, and elevated availability of nutrients due to earthworm activities could favor grasses (Thouvenot et al. 2021; Schwarz et al. 2024) which are more efficient in taking up soil nutrients and are thus considered as resource-exploitative species (Craine et al. 2001; Freschet et al. 2017) and often possess a high specific leaf area and leaf nitrogen content. This would lead to changes in the dominance structure of different plant functional groups, plant community composition, and thus in the overall taxonomic and functional diversity of the plant community, with consequences for plant community trait composition and ecosystem functions (i.e., "mass-ratio hypothesis, Grime (1998)) like plant community productivity and litter decomposition. For example, the biomass of litter would be lower in the presence of invasive earthworms: this would be explained by a faster decomposition process due to the presence of a litter more easily decomposed, induced by the changes in the litter quality (high nitrogen content) following the dominance of graminoid species in the communities of the invaded area that are efficient in taking up plant-available soil nitrogen.

As previously described, there are diverse hypotheses that have been put forward to explain the impacts of invasive earthworms on native biodiversity and ecosystems (e.g., Hendrix et al. 2008; Eisenhauer et al. 2019), but basically all of what we know is based on observational field studies and lab experiments, and there is a need to establish causal links and to understand the specific mechanisms under field conditions (Eisenhauer et al. 2019). Our study thus aims to fill this knowledge gap, by exploring the mechanisms behind plant community and ecosystem function changes after earthworm invasion and investigating potential cascading effects from altered soil properties to plant community composition. Thus, here, to better understand the short-term effects of earthworm invasions, which have rarely been studied under field conditions, we set up a field experiment that ran for two years in a forest in northern North America. We investigated the effects of invasive European earthworms on the soil structure and nutrient content, as well as on soil microbial activity and plant communities, and the associated consequences for ecosystem functions. We hypothesized to observe changes in soil abiotic properties (H1) that would then be associated with a positive effect of invasive earthworms on the cover and taxonomic diversity of grass species (H2). This would result in modifications of the community-weighted mean plant trait values (i.e., a decrease in plant height and an increase in leaf nitrogen content following grass dominance in the plant community; H3). Such changes were expected to coincide with changes in ecosystem functions like an alteration of plant community productivity and soil microbial activity, and an increase in litter decomposition (H4).

Methods

Study area and experimental design

The field experiment was set up in July 2017 in the still non-invaded area of an aspen forest of the Kananaskis Valley (Eisenhauer et al. 2019), in the front range of the Canadian Rocky Mountains (51°02'06"N, 115°03'54"W, Alberta, Canada), and was terminated in June 2019. The description of the climate in the valley, as well as the soil abiotic parameters of the forest can be found in previous studies (Scheu and Parkinson 1994; Eisenhauer et al. 2007, 2009a; Straube et al. 2009; Jochum et al. 2022). In this forest, trembling aspen (*Populus tremuloides*) and balsam poplar (*Populus balsamifera*) are the dominant tree species. The understory community is composed of different herbs (e.g., *Aster conspicuus*, *Fragaria virginiana*, *Delphinium glaucum*), grasses (e.g., *Calamagrostis rubescens*, *Leymus innovatus*), legumes (e.g., *Lathyrus ochroleucus*, *Vicia americana*), and woody plant species (e.g., *Rosa acicularis*, *Rubus idaeus*, *Symphoricarpos occidentalis*).

Earthworms are currently invading this forest, and the invasion by earthworms has been studied intensively across the last three decades, which is why there is solid empirical evidence for the proceeding invasion of the forest and the moving invasion front (Scheu and Parkinson 1994; Eisenhauer et al. 2007, 2009a; Straube et al. 2009; Jochum et al. 2022; Thouvenot et al. 2024a). The experiment was established in a non-invaded area of the forest that, based on previous work, would be invaded by the earthworm species that had already been present in this forest within a couple of years. This careful and conservative approach is reflected by the fact that some epigeic earthworms could even colonize some of the experimental control enclosures within the duration of our study (see information below). Notably, our experiment was well communicated with local authorities, and we were granted the required permits (Alberta Environment and Parks; Alberta Tourism, Parks and Recreation Division; Permit number 16–139).

In the non-invaded part of the forest, 20 enclosures (1 × 1 m) were randomly established (Eisenhauer et al. 2019), and were on average 47.8 m (±23.7 m (sd)) apart from each other (distance range: from ~10 m to ~128 m; Fig. 1A). The non-invaded part of the forest was defined on the basis of earthworm abundance data from a comprehensive earthworm sampling campaign where the number of individuals was close to zero (the data published in Jochum et al. 2021; Ferlian et al. 2024). The enclosures consisted of metal sheets (60 cm width, 1 m length) encased in the soil: 20 cm of the metal sheets were above and 40 cm below the soil surface to limit earthworm escape or natural colonization (Fig. 1B).

To establish the enclosures, we used an aluminum frame (1 × 1 m) positioned on the soil surface to demarcate the enclosure boundaries. Then, we dug out the topsoil (~10–20 cm deep) around the undisturbed plot area, before cutting the trenches (~40 cm deep) with a soil trencher along the inner edge of the ditches. The four metal shields were then inserted into the soil to reach 40 cm of soil depth. All ditches, grooves, and holes were then filled with the previously removed soil, before being compacted to restore the soil as much as possible. Velcro outdoor tape (hook part) was attached to the inner side of the shields to prevent earthworms from escaping (as earthworms cannot pass the hook-like structure; Lubbers and van Groenigen 2013).

All earthworms used in the field experiment were taken from the local populations of the study site (Alberta Environment and Parks, Permit number 16–139), and we did not introduce any additional earthworm species. Earthworms were collected in the field using the mustard extraction method (Jochum et al. 2022), washed,

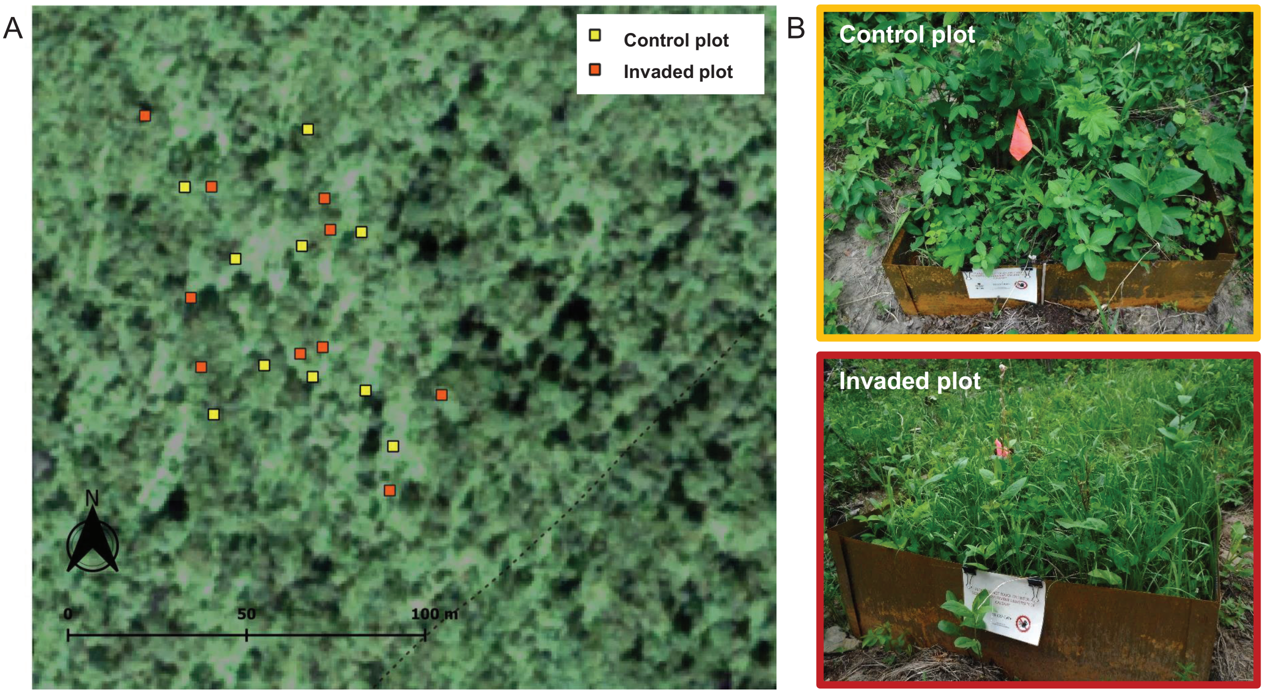

Figure 1. Study site and enclosure set-up. Map (**A**) of the study site located at the north of Barrier Lake, Kananaskis Valley, Alberta, Canada (51°02'N, 115°03'W), with the enclosures (**B**). Colors show the 20 enclosures set-up in the non-invaded area: there are 10 control enclosures (yellow) and 10 invaded enclosures (red). The hiking trail is the black dotted line. Mapping information: coordinate system UTM Zone 11 U, DOP data © government of Alberta 2014, and mapping performed using QGIS 3.30.0 (2023).

and sorted to ecological groups (i.e., epigeic, endogeic, anecic) before being added to the enclosures. We found only four species varying in terms of abundance, and with mainly one species per ecological group, except for epigeic species for which we found two species. This constrained the experimental design/treatment: we thus manipulated the presence *vs.* absence of the invasive earthworm community (i.e., presence *vs.* absence of the three ecological groups of earthworms). This invasion treatment was randomly assigned to each enclosure, with 10 enclosures (replicates) per treatment. Earthworm density added to the enclosures was close to the common medium densities in the area (mean ± sd: 32.9 ± 19.4 g m^{-2} and 42.8 ± 22.7 individuals m^{-2} ; Jochum et al. 2022). We equally distributed the same number of individuals per ecological group and then balanced the total biomass for each enclosure to keep similar biomass among all of the invaded enclosures to control for strong biomass effects observed before (Craven et al. 2017). The earthworm community was composed of the species *Dendrodrilus rubidus* (epigeic), *Dendrobaena octaedra* (epigeic), *Octolasion tyrtaeum* (endogeic), and *Lumbricus terrestris* (anecic). On average, we added ~ 14 anecic individuals (corresponding to a total fresh biomass of ~ 17.6 g m^{-2} on average), 45 endogeic individuals (~ 17.2 g m^{-2}), and 12 epigeic individuals (~ 1.6 g m^{-2}) to each enclosure receiving the earthworm treatment. The earthworms were added on 23rd of July 2017, and we verified the invasion status in all enclosures (i.e., the presence *vs.* absence of the earthworm community) at the end of the field experiment after all measurements by extracting the earthworm community from the 17th to 20th of June, 2019, thus after 694 days of experiment (~23 months). To do so, we assessed the abundance, biomass, and ecological group richness of the earthworm community in one quarter (0.5 × 0.5 m) of each of the enclosures, *via* a combination of hand-sorting and mustard extraction methods

(Ferlian et al. 2022; Jochum et al. 2022). The mustard extraction method is commonly used to estimate earthworm abundance and biomass (Eisenhauer et al. 2007; Straube et al. 2009; Jochum et al. 2022). We found earthworms in all enclosures that received the earthworm treatment but also epigeic earthworms in seven of the control enclosures. This light invasion of the control enclosures could be due to the presence of few individuals, tiny juveniles of epigeic earthworms that occurred at the site before setting up the experiment and are starting to invade the site (as epigeic earthworms are typically the first species to invade a new area). Another explanation would be the natural colonization of the enclosures by epigeic earthworms that are very mobile and might be able to cross the barriers, and/or to the introduction of eggs or individuals by birds or large animals like deer, even if rarely reported. We unfortunately cannot estimate the timing of this invasion of some control enclosures, but despite this colonization, we observed a difference between the two invasion treatments, with a biomass (+11.6%) and ecological group richness (+1.6%) significantly higher (only marginal difference for the earthworm abundance) in the invaded than in the control enclosures (see Suppl. material 1), allowing us to test the effects of earthworm experimental treatment on our focal response variables.

Plant community and plant trait measurements

We visually estimated the cover of each plant species in the twenty enclosures, by using the modified decimal scale from Londo (1976), in June 2019. Thirteen cover categories were defined (i.e., <1%; 1–3%; 3–5%; 5–15%; 15–25%; 25–35%; 35–45%; 45–55%; 55–65%; 65–75%; 75–85%; 85–95%; and >95%), and we used the median values of the categories to calculate the relative abundance of the plant species. Then, plant functional traits were measured on the dominant plant species of each enclosure. In other words, for each enclosures, we listed all the plant species that were the most dominant until reaching a collective minimum total cover of 80% of the enclosure (Pakeman and Quested 2007), and measured traits on these species following standard protocols (Cornelissen et al. 2003; Pérez-Harguindeguy et al. 2016). We measured the vegetative height (cm) of the dominant species in each enclosure, by selecting three individuals, if possible. The height was defined as the shortest distance between the soil and the top leaf. After measuring the height, we assessed the nutrient content of the leaves (carbon and nitrogen) on two individuals per species per enclosure. To do so, we collected and pooled two leaves per individual. However, in cases of low species abundance, we measured the traits on a reduced number of individuals, thus sometimes on only one individual, when this individual represented a high percentage of cover of the enclosure, to avoid missing any important components of the plant community. Leaves were treated as a whole, without separating leaflets or petioles for herbs, legumes, and woody plants; while for grasses, only the laminae were considered. Each pool of leaves was dried individually at 60 °C for at least three days, before being ground, using liquid nitrogen when necessary. Samples were then put into tin capsules prior to the analyses of leaf carbon and nitrogen content (% dry-leaf mass). The nutrient analyses were performed using 3.5 to 5 mg of dry weight by combustion with an elemental analyzer (Vario EL III, Elementar Analysensysteme GmbH, Hanau, Germany).

Additionally, plant functional group-specific biomass was measured in a quarter of each enclosure (0.5 × 0.5 m). Plants were harvested by cutting the shoots at the soil surface level and later sorted in the laboratory according to their functional group (i.e., herbs, grasses, legumes, and woody plants). If not processed immediately, the bags with biomass were stored air-tight in a fridge at 4 °C before being processed (within a maximum of two days after collection). The plant samples were then dried at 60 °C for at least 72 h and weighed to assess plant biomass. We acknowledge that some plant species harvested were not observed during the visual estimation of species-specific plant cover performed earlier, and thus to account for them in the calculation of plant α-diversity indices, we assigned them a cover of 0.5%. Plant community productivity was calculated by summing up the dry biomass of the different plant functional groups.

Soil and ecosystem properties

Soil abiotic and biotic properties were assessed in one quarter next to the one used to measure plant functional group biomass, while litter biomass and canopy openness were measured at the enclosure level.

One soil core (depth 10 cm; diameter 5 cm) was sampled to get information about soil abiotic (e.g., pH, nutrients, water content) and biotic properties (e.g., microbial biomass and basal respiration). Ecological as well as mineralization processes and nutrient contents typically decrease with soil depth, with the strongest decrease typically happening in the upper 10 cm (Chen et al. 2021), and in the organic layer that represents, on average, the first 5 cm in this area (Thouvenot et al. 2024a). Thus, each soil core was split into two depth layers (0–5 cm and 5–10 cm depth). Each layer of soil samples was stored at 5 °C until being processed in the laboratory. Then, they were sieved (2 mm) and stored afterward at -20 °C until further analyses. A sub-sample of this sieved soil was taken, dried at 60 °C for 72 h, and ground. Then, around 20 mg of dry weight per sample was put in a tin capsule for soil nutrient analysis that was performed with an elemental analyzer (Vario EL Cube, Elementar Analysensysteme GmbH, Hanau, Germany).

The fresh soil samples (2 g for the 0–5 cm depth and 10 g for the 5–10 cm depth) were air-dried and then dissolved in 12.5 ml 0.01 M $CaCl_2$ solution for the upper layer and in 25 ml 0.01 M $CaCl_2$ solution for the deeper soil layer. After shaking the solutions, they were left for at least 1 h before the pH measurements were taken. The pH was determined using different subsample sizes as the upper soil layer soaked most of the $CaCl_2$ solution: we had to change the ratio between the soil mass and volume of solution used to be able to measure the pH of the soil solution, and statistical analyses were performed on each soil layer separately.

Soil microbial activity was measured for each soil layer, using 2 to 4 g of fresh soil using an O_2-microcompensation apparatus (Scheu 1992). We measured soil microbial basal respiration (μl O_2 h^{-1} g^{-1} dry soil) every hour for 24 h at 20 °C and calculated microbial biomass (Cmic; μg C g^{-1} dry soil) from the maximum initial respiratory response after the addition of glucose, as done before (e.g., Eisenhauer et al. 2007). Substrate-induced respiration was calculated after measuring the respiratory response to the addition of D-Glucose in excess (i.e., to the addition of 8 mg of glucose per gram of soil dry mass, diluted in 0.25 ml of deionized water).

For measurements of soil aggregate stability, a stable 200 ml container was filled per enclosure with soil from a depth of 0–10 cm. Soil was sampled by carefully digging with a hand-spade and discarding soil particles from the rim of the pile to avoid including soil that was compacted during the procedure. Samples were stored in a cooling bag in the field and dried at 60 °C for 72 h in the lab to terminate microbial processes. Water-stable aggregates were separated from unstable ones using the method described by Kemper and Rosenau (1986). The three measurements of water-stable aggregate per soil layer per enclosure performed were averaged for further analyses.

Soil bulk density was measured in November 2019, in half of the enclosures (5 replicates per treatment), with a 5-cm-diameter soil corer, to a depth of 10 cm. After removal of litter and woody debris, plants were cut off just above the soil surface. Soil cores were then taken, transported to the lab, and weighed fresh before drying them for 24 h at 105 °C and weighing them again to the nearest 0.01 g. Soil bulk density was then calculated as g dry weight per m^3.

Moreover, the litter was collected on the same quarter used to measure plant functional group biomass, and this litter biomass was multiplied by four to represent the whole enclosure. We complemented this litter collection with the litter biomass collected *via* suction sampling to get a measure of total litter biomass per m^2. Suction sampling was performed on the whole enclosure, after plant community trait measurements and aimed to sample vegetation and ground fauna, (unpublished data). Here, we used the litter biomass to get an estimation of the litter decomposition. We estimated the canopy openness (%) i.e., the percentage of open sky, for each enclosure by taking pictures with a cell phone (iPhone 6S Plus+) and an Olloclip FishEye lens, on a tripod at a height of 1.4 m. The hemispheric pictures were processed with the WinScanopy software (Régent Instruments Inc., Québec, QC, Canada) to calculate canopy openness.

Statistical analysis

All statistical analyses and figures were performed with R software version 4.3.1 (R Core Team 2023). The effects of the earthworm invasion treatment were tested on soil abiotic properties (i.e., soil carbon and nitrogen content, soil pH, water-stable aggregates and water content) and soil microbial activity (i.e., basal respiration and microbial biomass) using linear models of the package "stats" with Type III F-tests from the package "car" (Fox and Weisber 2019), with the earthworm invasion treatment and the soil depth as factors tested alone and in interaction. The effect of the earthworm treatment on the bulk density (measured in one soil layer), and on pH (different methods used for each soil layer as explained above) were analyzed using linear models with Anova Type II F-tests, as the model did not include the interaction.

To check for changes in plant community composition in response to our treatment, we performed a non-metric multidimensional scaling (NMDS) analysis, with the function metaMDS from the "vegan" package (Oksanen et al. 2019) using Bray-Curtis distances square root transformed. We tested the difference between earthworm treatments using permutational multivariate analysis of variation (Permanova) after 1000 permutations on the square root transformed distances, using the adonis2 function from the "vegan" package (Oksanen et al. 2019).

To characterize the plant community, three α-diversity indices were calculated for each enclosure and each plant functional group: the species richness, the Shannon diversity and Pielou's evenness. They were calculated using the functions *specnumber* and *diversity* from the "vegan" package (Oksanen et al. 2019). In addition, we calculated the community-weighted means (CWMs) (Lavorel et al. 2008) of the plant height, leaf carbon, and nitrogen content for each enclosure, based on the mean trait values per species per enclosure, weighted by the relative median cover of the plant species in this same enclosure, using the function *weighted.mean* from the "stats" package. The effects of earthworms on α-diversity indices calculated at the community level, on CWM of traits as well as on plant productivity and litter biomass were tested using linear models with Type II F-tests, with the earthworm treatment as a two-level factor and canopy openness as a covariate. The different variables estimated at the plant functional group level were tested using linear models with Type III F-tests: the fixed effects were the earthworm treatment and the plant functional group alone and in interaction, while canopy openness was also specified as a covariate. When the interaction between the plant functional group and earthworm treatment was significant, pairwise comparisons with Holm correction were performed by plant functional group and by earthworm treatment using the package "emmeans" (Lenth et al. 2020).

Model diagnostics were performed using the R base function plot(): the normality of residuals, the homogeneity of variance, and the presence of outliers or influential data points were checked by visual inspection. When necessary, variables were log-transformed ($\log_2$ [x +1]) to meet model assumptions, such as for the plant community productivity, as well as the richness, and relative cover at the plant functional group level, the litter biomass, the soil carbon and nitrogen content, as well as the soil microbial biomass, basal respiration, and dry bulk density. Only soil pH and relative plant functional group biomass were square-root transformed. One data point that stood out in diagnostics plots and with a Cook's distance > 0.5 was removed for soil microbial respiration (Control area, Enclosure 18, soil depth 0–5 cm, Basal respiration value = 0.00). The percentages of change were calculated using estimated marginal means (back-transformed when necessary) from the "emmeans" package. All figures were made with the package "ggplot2" (Wickham 2016).

Use of Artificial Intelligence technologies statement

During the writing process, we used ChatGPT in order to check grammar and spelling, and re-phrase some sentences, but reviewed and edited the content for the manuscript.

Results

Effects of the invasive earthworm treatment on soil abiotic properties

The soil water and carbon content were significantly lower in the deeper soil layer than in the upper soil layer ($F_{1.36}$=32.33, p<0.001, $F_{1.36}$=64.37, p<0.001 respectively, Fig. 2A, B). The earthworm treatment reduced the soil carbon content from 12.2% to 9.6% on average (-21.1%, $F_{1.36}$=4.65, p=0.04), while the soil nitrogen content decreased by 25.8% with the earthworm treatment in the upper soil layer (from ~1.4% of carbon in the control to ~1% in the earthworm treatment) but remained similar in the deeper soil layer (Interaction effect: $F_{1.36}$=4.53, p=0.04, Fig. 2C). Soil pH increased in the deeper soil layer with values ranging from 5.4 in the control treatment to 5.9 in the invasion treatment (+ 8.9%; Earthworm effect: $F_{1.18}$=7.66, p=0.013; Fig. 2D), but it did not change due to earthworm treatment in the upper soil layer (Earthworm effect: $F_{1.18}$=2.05, p=0.17). Moreover, the earthworm treatment did not affect soil bulk density significantly (Earthworm effect: $F_{1.8}$=0.05, p=0.82; Fig. 2E), while the percentage of water-stable aggregates was significantly affected by the interaction between earthworm treatment and soil layer ($F_{1.36}$=5.19, p=0.03, Fig. 2F). The significant difference in soil aggregate stability between soil layers (difference of 11%) in the control treatment was reduced in the

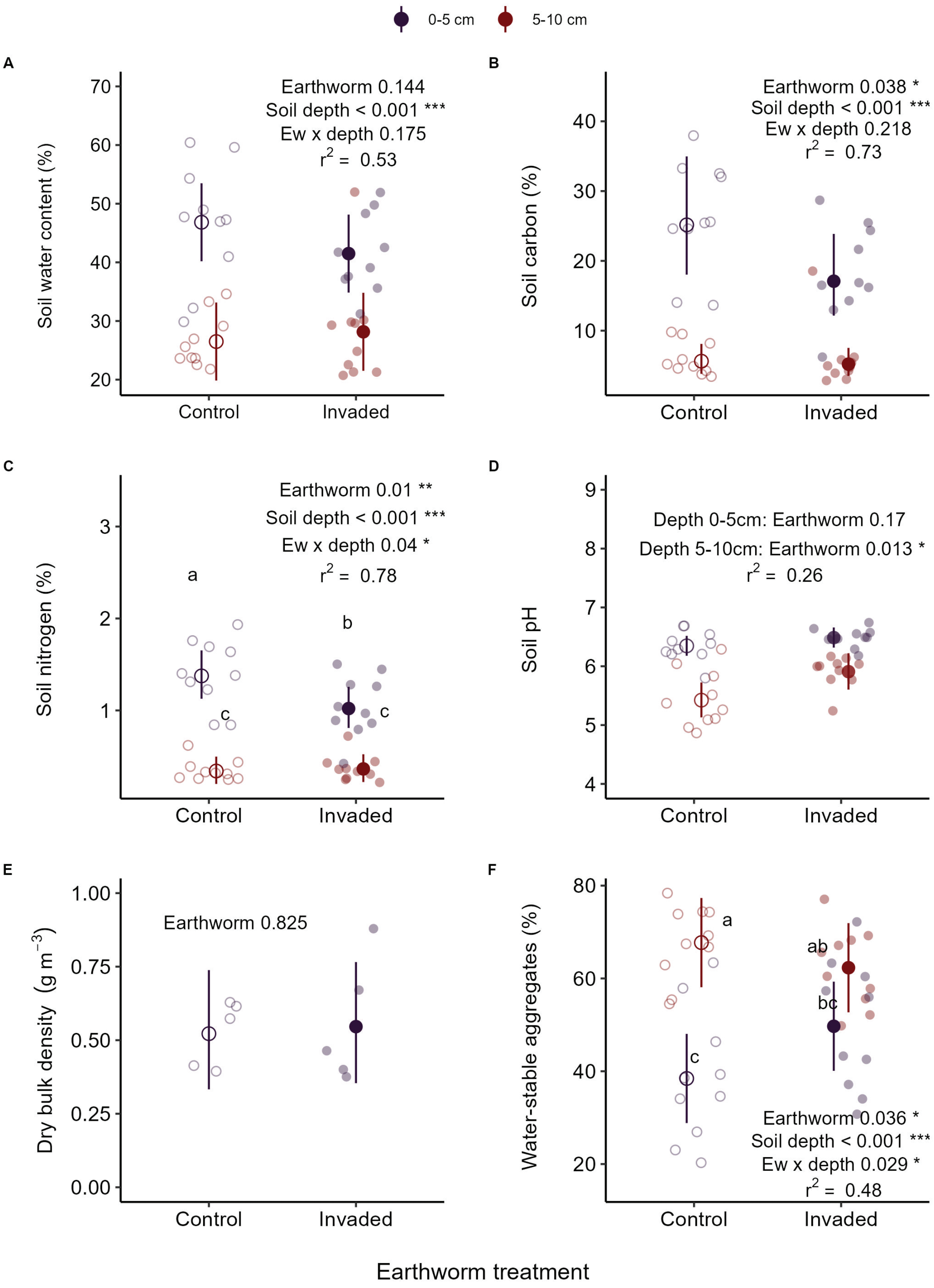

Figure 2. Effect of invasive earthworms on soil abiotic properties. Soil water content (**A**), carbon (**B**) and nitrogen (**C**) contents, pH (**D**), as well as dry bulk density (**E**), soil aggregate stability (**F**), according to the earthworm treatment (control (open circle) vs invaded (filled circle)) and soil depth (0–5 cm (dark gray) vs 5–10 cm (brown), except for the bulk density). Estimated marginal means and confidence intervals $CI_{95\%}$ are shown (after being back-transformed when necessary), while data points are included in the background. The p-values and r^2 are based on linear models. r^2 are given when at least one factor alone or in interaction was significant. Letters correspond to the results of post hoc tests performed when the interaction between earthworm treatment and soil depth was significant: different letters show significant differences between soil depth and earthworm invasion status. Number of observations per earthworm treatment and soil depth: 10 (5 for dry bulk density). Significance codes: ***<0.001; *<0.05.

earthworm treatment (difference of 5% of water-stable aggregates between layers). This effect was probably due to the simultaneous increase of soil aggregate stability in the deeper soil layer (+29.3%), and the decrease (-8%) in the upper soil layer.

Effects of the experimental earthworm invasion on plant community diversity, structure, and trait composition

The Permanova did not show any significant shift in the composition of the plant community in response to the earthworm treatment ($F_{1.18}$=0.86, p=0.76, see Suppl. material 2). Moreover, the α-diversity indices measured at the plant community level were also not affected by the canopy openness, nor the invasive earthworms treatment that did not significantly change plant species richness ($F_{1.17}$=0.001, p=0.97; Fig. 3A), Shannon diversity ($F_{1.17}$=0.37, p=0.55; Fig. 3B), and evenness ($F_{1.17}$=0.84, p=0.37; Fig. 3C) of the community.

Furthermore, our study shows few effects of the earthworm treatment, but significant effects of the plant functional group identity on the plant functional group indices. The plant functional group relative cover ($F_{3.71}$=37.10, p<0.001), richness ($F_{3.71}$=121.36, p<0.001), Shannon diversity ($F_{3.71}$=76.50, p<0.001), and relative biomass ($F_{3.71}$=17.75, p<0.001), but not the evenness ($F_{3.69}$=0.62, p=0.60), were significantly affected by the plant functional group identity. Overall, herbs had the highest relative biomass, cover, richness, and Shannon diversity, while legumes had the lowest. The grasses had similar relative biomass to herbs. Canopy openness increased plant functional group Shannon diversity ($F_{1,71}$=8.02, p=0.006), evenness ($F_{1,69}$=8.39, p=0.005), and slightly the richness ($F_{1,71}$=3.16, p=0.08), but not the relative cover ($F_{1,71}$=2.43, p=0.12) and relative biomass ($F_{1,71}$=0.11, p=0.74). The earthworm treatment alone or in interaction did not affect the relative cover of the plant functional groups ($F_{1.71}$=0.05, p=0.83 and $F_{3.71}$=0.20, p=0.90 respectively, Fig. 4A), nor their relative biomass ($F_{1.71}$=0.04, p=0.85 and $F_{3.71}$=0.02, p=1 respectively, Fig. 4B) or their richness ($F_{1.71}$=0.50, p=0.48 and $F_{3.71}$=1.94, p=0.13 respectively, Fig. 4C). However, the interaction between earthworm treatment and plant functional group marginally affected Shannon diversity ($F_{3.71}$=2.16, p=0.10; Fig. 4D), with this effect mainly driven by a marginally significant increase in the Shan-

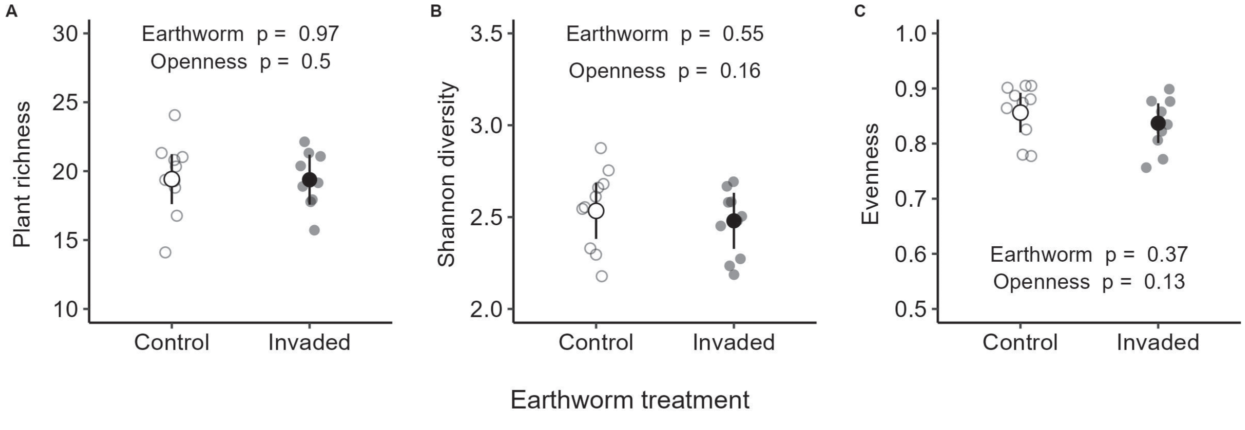

Figure 3. Effect of invasive earthworms on plant community diversity. Comparison of the plant community composition based on the plant richness (**A**), Shannon diversity (**B**), and evenness (**C**) according to the earthworm treatment of the enclosures (control (open circle) versus invaded (filled circle) enclosures). Estimated marginal means and confidence intervals $CI_{95\%}$ are shown (after being back-transformed when necessary), while data points were included in the background. The p-values are based on linear models. Number of observations per earthworm treatment: 10.

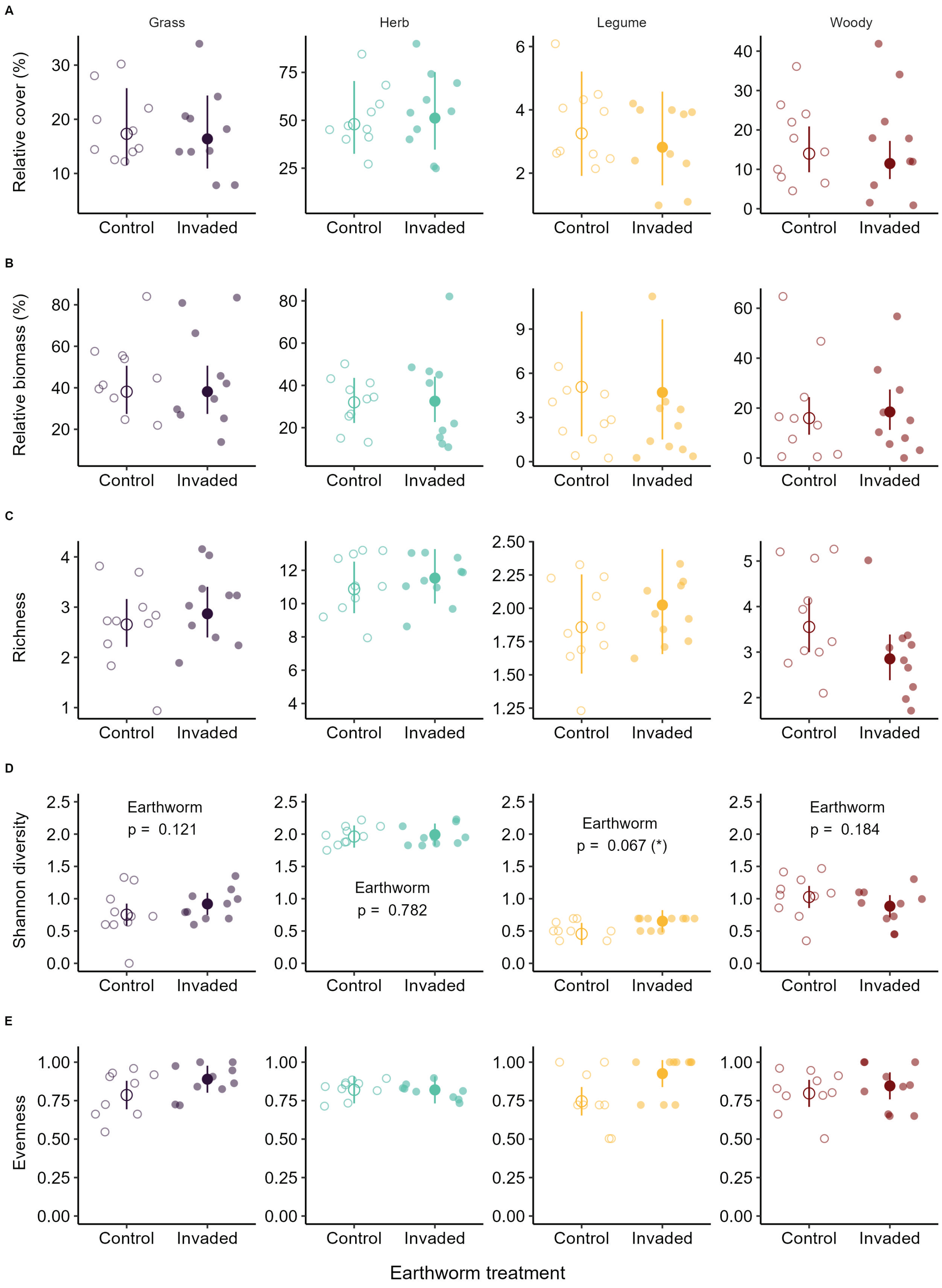

Figure 4. Effect of invasive earthworms on plant functional group productivity and diversity. The impact of invasive earthworms was measured on the relative cover (**A**), relative biomass (**B**), richness (**C**), Shannon diversity (**D**) and evenness (**E**) of the different plant functional groups. Data points (10 observations per earthworm treatment) are included in the background, with open circles for control enclosures and filled circles for invaded enclosures. Estimated marginal means and confidence intervals $CI_{95\%}$ are shown (after being back-transformed when necessary), while data points are included in the background. The p-values corresponded to the results of the post-hoc tests performed by plant functional group, when the interaction between earthworm treatment and plant functional groups was at least marginally significant in the linear models. Significance codes: (*)≤0.10.

non diversity of legumes (+43.3%) in the invaded enclosures. The Shannon diversity of legumes which was on average 0.45 in the control treatment reached 0.65 in the earthworm treatment. Moreover, the earthworm treatment alone marginally affected plant functional group evenness, with an overall higher evenness (+10.5%) in the invaded area ($F_{1,69}$=3.38, p=0.07) for the different functional groups, while there was no significant interaction between earthworm treatment and plant functional group identity ($F_{3,69}$=1.98, p=0.13, Fig. 4E). Plant community trait composition was not affected by the earthworm treatment: the CWM of plant height ($F_{1,17}$=0.07, p=0.79), as well as leaf carbon and nitrogen content ($F_{1,17}$=0.27, p=0.61 and $F_{1,17}$=0.04, p=0.84 respectively; see Suppl. material 3) did not change significantly in response to the invasive earthworm treatment, but the CWM of leaf nitrogen content decreased with an increase of canopy openness ($F_{1,17}$=7.26, p=0.015).

Effects of the invasive earthworm treatment on ecosystem functions

The soil microbial biomass was significantly lower in the deeper soil layer than in the upper soil layer ($F_{1,36}$=96.01, p<0.001, Fig. 5A) and was not affected by the earthworm treatment (alone: $F_{1,36}$=0.18, p=0.68; Interaction effect: $F_{1,36}$=0.51, p=0.48).

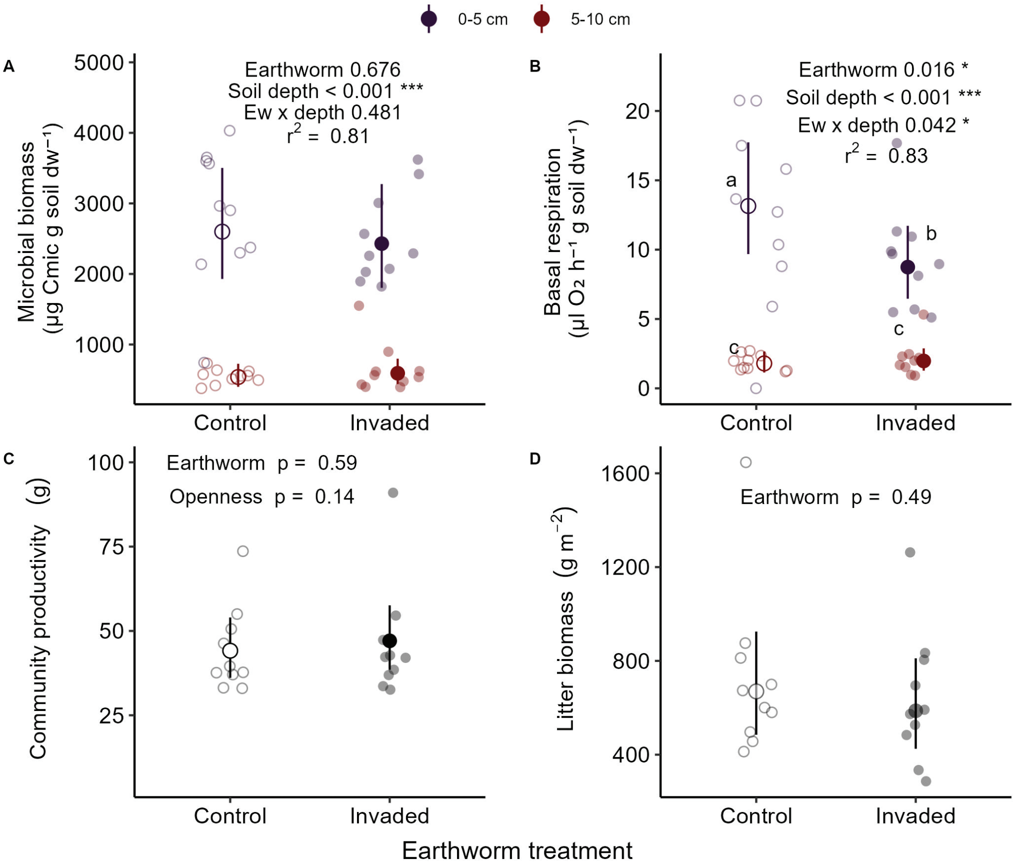

Figure 5. Effect of invasive earthworms on ecosystem functions. Soil microbial biomass (**A**) and basal respiration (**B**) according to earthworm treatment (control (open circle) vs invaded (filled circle)) and soil depth (0–5 cm (dark gray) vs 5–10 cm (brown)), as well as plant productivity (**C**) and litter biomass (**D**) according to earthworm treatment. Estimated marginal means and confidence intervals $CI_{95\%}$ are shown (after being back-transformed when necessary), while data points are included in the background. The p-values and r^2 are based on linear models. r^2 are given when at least one factor alone or in interaction was significant. Letters correspond to the results of post hoc tests performed when the interaction between earthworm treatment and soil depth was significant: different letters show significant differences between soil depth and earthworm invasion status. Number of observations per earthworm treatment and soil depth: 10. Significance codes: ***<0.001; *<0.05.

Conversely, the basal respiration of the microbial community decreased by 33.5% in the earthworm invasion treatment in the upper soil layer with values going from ~13.1 µl O_2 h^{-1} g^{-1} dry soil in the control to 8.8 µl O_2 h^{-1} g^{-1} dry soil in the earthworm treatment, but remained similar in the deeper soil layer (Interaction effect: $F_{1.35}$=4.47, p=0.04, Fig. 5B). Moreover, the earthworm treatment did not significantly affect ecosystem functions like plant community productivity ($F_{1.17}$=0.30, p=0.59, Fig. 5C) and litter biomass ($F_{1.18}$=0.50, p=0.49, Fig. 5D).

Discussion

As one of the first field experiments on the subject, our study shows that invasive earthworms altered soil abiotic properties and soil respiration already two years after the establishment of the experimental treatments. The invasion of earthworms increased soil pH in the deeper soil layer, while it decreased soil nitrogen content in the upper soil layer and decreased soil carbon across soil depths in the invaded enclosures. Furthermore, invasive earthworms reduced the difference in percentage of water-stable aggregates among soil layers that was observed in the control treatment. This change should have affected water flow in the soil (Blouin et al. 2013; Hallam and Hodson 2020), but we did not observe any invasive earthworm treatment effect on the soil water content after two years of experiment. The absence of change in the soil water content with earthworm invasion could be linked to the missing impact on the litter biomass: a high biomass of leaf litter would rather have a protective effect and limit evapotranspiration from the soil, thus keeping the water content high. These results are largely in line with literature based on observational studies. Soil chemical properties are often affected by invasive earthworms due to their feeding and burrowing activities, as highlighted in a recent meta-analysis (Ferlian et al. 2020). The burrowing activities of earthworms could be an explanation for these changes. For example, they often decrease the soil water content and induce a decrease in the soil nutrient content due to the nutrient leaching into deeper soil layers (Bohlen et al. 2004; Frelich et al. 2006; Resner et al. 2015; Richardson et al. 2018). However, we did not find any general homogenization effect of soil abiotic and biotic properties as observed in some meta-analyses (Ferlian et al. 2018, 2020): only the percentage of water-stable aggregates seems to be homogenized across the soil profile.

The earthworm community composition and biomass probably generate variability in our results of soil abiotic properties. Indeed, it is important to note that the difference between the impacts on the diverse soil properties could highlight the effect of the invasive earthworm community composition, as well as the role of the different earthworm ecological groups, as they differ in their feeding and burrowing activities. For example, anecic and endogeic species are more likely to affect the organic soil layer and drive the magnitude of the earthworm community effect, while epigeic earthworms would tend to affect the mineral soil layer (Ferlian et al. 2020). Although the results may depend on soil type, in addition to the earthworm community composition (McLean et al. 2006; Ferlian et al. 2018), the earthworm effect on soil abiotic properties could explain the decrease of soil microbial basal respiration measured in the upper soil layer in the presence of invasive earthworms, which has also been observed in other observational studies (Eisenhauer et al. 2007, 2011). The impact of invasive earthworms on soil microbes which was reviewed in McLean et al. (2006), could be due to the increase of soil pH and nutrient stress in the upper soil layer for instance, that could have further cascading effects on soil microbial community composition and functions (Eisenhauer et al. 2011).

Moreover, the shifts in abiotic and biotic soil properties due to the experimental earthworm invasion were expected to be the reasons for the anticipated changes in plant diversity. Indeed, the decrease in nitrogen content in the upper soil layer could have led to a change in plant community structure and composition by favoring grass species that are more efficient in taking up resources from the soil (Craine et al. 2001; Craven et al. 2017; Freschet et al. 2017). However, our study only found little evidence to support such an effect after two years of this field experiment. There was no significant impact of the experimental earthworm invasion treatment on species richness, Shannon diversity, or evenness of the overall plant community, and these indices, when measured at the plant functional group level, were only slightly impacted by the invasive earthworm treatment.

While we expected a positive effect of invasive earthworms on grass species and a negative one on herb species, our results showed that the earthworm treatment had a marginally significant positive effect on the Shannon diversity of legumes, and on the evenness of all plant functional groups. To our knowledge, few observational studies have explored the effects of invasive earthworms on plant functional group diversity: only Hale et al. (2006) found that invasive earthworm biomass decreased herbaceous plant richness and Shannon diversity in some forests, while it increased it in others. As for soil abiotic and biotic properties, several authors have reported that invasive earthworm species identity and community composition played a role in the changes in the plant community composition. For instance, Hale et al. (2006) found that plant richness varied according to the composition of the earthworm community, with a stronger decrease observed when the community was dominated by the anecic species *L. terrestris.* By contrast, Holdsworth et al. (2007) observed that the presence of the epigeic *D. octaedra* tended to increase plant species richness compared to other earthworm species, even if this effect was mainly attributed by the authors to the effect of *L. terrestris* invasion on *D. octaedra* biomass. Consequently, we can assume that the mechanisms differ by which earthworm species or ecological groups affect plants (Andriuzzi et al. 2016). For example, endogeic earthworms could directly disrupt or benefit the root systems of some species, in particular, due to their higher activity in the top soil layer (Scheu 2003; Capowiez et al. 2021), while anecic earthworms would more likely impact plant species *via* their effect on nutrient re-distribution in the soil, i.e., due to the incorporation of litter nutrients but also nutrient transport into deeper soil layers. Additionally, epigeic species would rather have a limited effect on plant community (Hale et al. 2006) and belowground traits due to their limited mixing effect of mineral and organic layers by feeding on and living in litter material. We thus do not expect the colonization of the control enclosures by epigeic earthworms to have affected the results of our field experiment. This is especially the case because their biomass was rather low compared to that in the earthworm treatment (see Suppl. material 1), and because several meta-analyses (Eisenhauer 2010; Craven et al. 2017; Ferlian et al. 2018, 2020) have shown that invasive earthworm effects increased with biomass, and that epigeic earthworms typically have minor effects under the studied conditions. However, to verify these hypotheses further studies with different earthworm species, ecological groups and plant species and/or functional groups are needed.

The effect of earthworm community composition could also be a potential explanation for the slightly positive effect of invasive earthworms on legume species diversity and plant functional group evenness in the present study. The positive earthworm effect on legumes contradicts literature that mainly showed negative (Eisenhauer et al. 2007) or neutral (Wurst et al. 2003; van Groenigen et al. 2014) effects of (invasive) earthworms on the cover and biomass of legume species in the field. However, some positive effects were reported in laboratory (Eisenhauer and Scheu 2008) and field (Eisenhauer et al. 2009b) experiments using earthworm and plant species co-occurring in Central Europe. For example, Eisenhauer and Scheu (2008) as well as Wurst et al. (2003) found a positive effect of anecic or endogeic earthworms on the biomass and on the total nitrogen content of the legume *Trifolium repens*, when grown without grasses as competitors. As legume species fix atmospheric nitrogen in their nodules (rhizobium symbiosis), they are expected to be rather independent of soil nitrogen (Hirsch et al. 2001; Eisenhauer and Scheu 2008). We consequently expected them to not rely on changes in soil nutrient availability and uptake due to invasive earthworm burrowing and feeding activities. Thus, our results could suggest that the impacts of earthworms on this particular plant functional group may not be directly attributed to their effects on the legume nutrient uptake from soil. Instead, these impacts may be attributed to the decrease in the inter-specific competition resulting from the decrease in the soil nutrient content due to earthworm presence, and specifically to soil nitrogen in the upper soil layer that we observed. Another explanation could be their burrowing and mechanical activities that might alter rooting depth or distribution, promote nodules/nitrogen-fixing bacteria density (Thompson et al. 1993; Doube et al. 1994), and/or influence mycorrhizal colonization of the plants (Lawrence et al. 2003; Paudel et al. 2016). Moreover, the promotion of legume species by invasive earthworms could affect the nitrogen dynamics in the soil and have an indirect facilitative effect on some specific neighboring species via different mechanisms (Temperton et al. 2007). This effect on legumes is maybe a first and transient step toward changes in understory plant community composition and means that, before losing diversity, there might first be a change in the structure of the plant community. However, these results need to be interpreted with caution as they represent only a marginally significant trend, and further long-term experiments (i.e., longer than two years) are needed to investigate the cascading effects of invasive earthworms on plant communities.

Furthermore, after two years of this experiment, invasive earthworms did not significantly impact the relative cover and biomass of the plant functional groups, despite evidence from existing literature (Hale et al. 2006; Paudel et al. 2016; Craven et al. 2017). For instance, studies by Nuzzo et al. (2009) and Holdsworth et al. (2007) reported a decrease in the herb, forb, and woody species cover with an increase of invasive earthworm biomass, while sedge or grass cover increased (Holdsworth et al. 2007; Drouin et al. 2016). The lack of change in plant species cover and biomass in our study could explain the absence of effects of invasive earthworms on the plant community-weighted means of height, leaf carbon, and nitrogen contents after two years of treatment. Indeed, if there is no change in plant community and functional group composition/dominance, it is unlikely to see changes in plant community trait composition, and thus in ecosystem functions like productivity and litter decomposition. We did not observe higher productivity or an accelerated litter decomposition due to changes in plant community traits (such as lower height and carbon content, and higher nitrogen content), attributed to the dominance of grass species in the community as we expected in the presence of invasive earthworms compared to the control enclosures. Additionally, an explanation for missing differences in the litter biomass between treatments could be that the large amount of litter that had accumulated across years before earthworm invasion, is slowly decomposed by the earthworm community, with these effects not yet being visible. These potential effects on plant community composition, traits, and ecosystem functions could thus need more time to materialize. Time-delayed responses from plant functional groups and communities to earthworm invasion were, to our knowledge, not investigated so far. It is probably due to the difficulties to report the time since the establishment of the invasive earthworm community in observational approaches and thus the challenge to investigate potential time lags in the response of the plant community after earthworm community establishment. Despite these difficulties, we stress the need to further investigate the sequence and timing at which changes occur, and when the effects of invasive earthworms on soil properties and their cascading effects on plant communities take place, to better understand the mechanisms behind plant community and ecosystem function changes after the invasion.

Moreover, the time since establishment of the earthworm community itself might have affected our results: the maximum ecological effects of the earthworm community on soil properties, communities, and ecosystem functions might need more time to materialize. Consequently, our study reinforces the idea of a sequence of events, and ecological cascade following earthworm invasion (Frelich et al. 2019), with the first effect of earthworm invasion being to alter soil structure and nutrient availability, as well as microbial activity, mainly in the upper soil layer. Our study also suggests that invasive earthworm effects on plant communities are mainly mediated by changes in soil properties (i.e., indirect effects), while proposed direct effects on plants (e.g., via interactions with seeds and seedlings) may have played a minor role.

Notably, we would like to stress that the present approach of introducing invasive species into an uninvaded area of the forest may slightly facilitate the spread of invasive species. As a word of caution, such work needs to be well planned, should be based on extensive knowledge on the study location and invasive species, and has to be supported by local authorities with the respective permits. Based on careful planning and transparent communication, we received the required permits by local authorities. The invasion of this forest by earthworms has been studied intensively across the last three decades (e.g., Scheu and Parkinson 1994; Eisenhauer et al. 2007; Straube et al. 2009; Jochum et al. 2022), which is why there is solid empirical evidence for the proceeding invasion of the forest and the moving invasion front. Notably, we only selected invasive earthworm species that had already been present in this forest for many years, and set up the experiment in an area of the forest that, based on this previous research, would experience earthworm invasion within a couple of years. As mentioned in the Methods section, some epigeic earthworms even colonized some control enclosures during our experiment, which also reflects the speed of the invasion in this area. Moreover, at the end of the experiment, we returned our research site as much as possible to pre-existing conditions, following Alberta Parks' protocols and recommendations. To do so, we removed the metal sheets of the enclosures using equipment cleaned beforehand to reduce the spread of invasive species. We also replaced/returned native vegetation plots and added native leaf litter on any areas of bare soil to reduce the potential of invasive species/weed establishment.

Conclusions

Our experimental field study shows that invasive earthworms significantly alter soil abiotic properties (i.e., soil nutrient contents, pH, water-stable aggregates) after two years, but without having strong consequences for plant taxonomic diversity, yet. Invasive earthworms slightly affected the Shannon diversity of legumes and the evenness of plant functional groups. These are potentially the first signs of the effects of invasive earthworms on plant communities that have been reported from observational studies (Holdsworth et al. 2007; Nuzzo et al. 2009; Alexander et al. 2022). However, in our two-year field experiment, invasive earthworms did not affect the relative cover and biomass of particular plant functional groups, yet, which has to be linked to the lack of changes in the plant community-weighted mean trait values, and the ecosystem functions studied, i.e., plant community productivity and litter decomposition that remained unaffected after two years of invasion. Our study suggests that the impacts of invasive earthworms on plant communities and ecosystem functioning are likely to become apparent after two years of invasion. These findings underscore the significant impacts of invasive earthworms on soil abiotic and biotic properties (Ferlian et al. 2020) that may then cascade to influence biological communities above and below the ground (Frelich et al. 2019; Jochum et al. 2022) and highlight the time lags in the response of the plant community to the establishment of invasive earthworms.

Acknowledgments

We are grateful to the Government of Alberta (Canada) and Alberta Environment and Parks for permitting (permit no. 16–139) us to perform our field experiment in the Barrier Lake's forest in the Kananaskis Valley (Alberta, Canada). We thank Ulrich Pruschitzki, Madhav P. Thakur and Tom Künne for helping with the set-up of the experimental enclosures; Ian Macdonald for his help with plant species identification; Sophia Findeisen, Romy Zeiss, Morgan Blieske, Michelle Ives, Adrienne Cunnings, Alfred Lochner, Ulrich Pruschitzki, and Anja Zeuner for their help in the field and/or in the lab for soil property and plant trait measurements. We also thank Ryan Ingham and Arc Ridge Ltd for their help with the restoration of the plots. We are also grateful to Julius Quosh for processing the fisheye pictures to measure canopy openness, as well as to Svenja Haenzel and Adrienne Cunnings for their administrative support in the planning of this field work.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

This project received support from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no. 677232 to NE), and funding from DFG (Ei 862/18-1, to NE and LT; Ei 862/29-1, Ei 862/31-1). Further support came from the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, funded by the German Research Foundation (FZT 118, 202548816). The authors also acknowledge support from the Open Access Publishing Fund of Leipzig University supported by the German Research Foundation within the program Open Access Publication Funding.

Author contributions

Conceptualization: LT, NE. Methodology: LT, NE. Investigation: LT, LH, OF, MJ. Formal analysis: LT, LH. Resources: NE. Data curation: LT. Writing- original draft: LT. Writing-review & editing: LT, NE, MJ, OF, LH. Visualization: LT. Supervision: LT, NE. Funding acquisition: LT, NE.

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Data availability

The data and code are publicly available on the data repository Zenodo https://doi.org/10.5281/zenodo.11395032 (Thouvenot et al. 2024b).

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Supplementary material 1

Description of the invasive earthworm treatment

Authors: Lise Thouvenot, Olga Ferlian, Lotte Horn, Malte Jochum, Nico Eisenhauer

Data type: docx

Link: https://doi.org/10.3897/neobiota.94.119307.suppl1

Supplementary material 2

Plant community composition

Authors: Lise Thouvenot, Olga Ferlian, Lotte Horn, Malte Jochum, Nico Eisenhauer

Data type: docx

Link: https://doi.org/10.3897/neobiota.94.119307.suppl2

Supplementary material 3

Plant community traits

Authors: Lise Thouvenot, Olga Ferlian, Lotte Horn, Malte Jochum, Nico Eisenhauer

Data type: docx

Link: https://doi.org/10.3897/neobiota.94.119307.suppl3

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 57–77 (2024)
DOI: 10.3897/neobiota.94.122496

Research Article

Stable isotope analysis reveals diet niche partitioning between native species and the invasive black bullhead (*Ameiurus melas* Rafinesque, 1820)

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Abstract

The introduction and spread of alien fish species pose a major threat to native communities and ecosystem functioning in freshwaters. Black bullhead is one of the most successful invaders in European waters with several detrimental effects on native biota and ecosystems. In this study, we used stable isotope analysis to compare the body size and season-dependent diet, trophic position, isotopic niche size, and niche overlap of the invasive black bullhead with two native fish species (roach and European perch) in Lake Balaton, Hungary. We found that black bullhead could be characterized by invertivore-piscivorous feeding habit with a high rate of fish consumption. The rate of fish predation by invasive black bullhead increased with body size, while no seasonal differences were observed in fish consumption. Contrary to our hypothesis, little evidence of actual feeding competition was found between black bullhead and native fishes. Our results suggest that the studied species assimilate distinct energy resources in different proportions leading to a substantial amount of niche partitioning among them. We conclude that black bullhead may represent a threat for native, small-sized fishes primarily through predation and recommend urgent management actions (e.g. selective removal of the species) to minimize its adverse impacts on native communities.

Key words: Biotic interactions, fish, non-native, piscivory, predation, trophic position

Academic editor: Nicola Smith
Received: 7 March 2024
Accepted: 5 June 2024
Published: 26 July 2024

Citation: Czeglédi I, Specziár A, Preiszner B, Boros G, Bánó B, Mozsár A, Takács P, Erős T (2024) Stable isotope analysis reveals diet niche partitioning between native species and the invasive black bullhead (*Ameiurus melas* Rafinesque, 1820). NeoBiota 94: 57–77. https://doi.org/10.3897/neobiota.94.122496

Introduction

The occurrence and spread of invasive species are leading drivers of biodiversity loss and pose major threats to ecosystem integrity. Adverse impacts of invasive species can be various and may occur at multiple ecological levels from single prey-predator relationships and competition (Bergstrom and Mensinger 2009; Seiler and Keeley 2009; Remon et al. 2016; Somogyi et al. 2023) to shifts in ecosystem functioning (Dietrich et al. 2006; Crowl et al. 2008; Linders et al. 2019). Freshwaters, and especially lakes, are often subject to high rates of species introductions, despite these ecosystems being known to be particularly vulnerable and susceptible to human activities and biological invasions (Gozlan et al. 2010; Mandrak and Cudmore 2010; Miró and Ventura 2013). Lakes cover only a limited area worldwide but support diverse species, including endemic taxa, and contribute substantially to ecosystem services (Dudgeon et al. 2006; Heino et al. 2021). However, habitat degradation, climate change and biological invasions have led to a considerable decline in species richness in these ecosystems occurring at a faster rate than in terrestrial and marine counterparts (Johnson et al. 2008; Strayer and Dudgeon 2010).

Several non-native fish species have been introduced into European freshwaters during the past decades mainly to support for commercial purposes, sport fishing or the ornamental market (Holčík 1991; Gozlan 2008). These species adversely affect the structure and functioning of native fish assemblages (Cucherousset and Olden 2011; van der Veer and Nentwig 2015) by altering natural aquatic food webs and competing for food resources with native species (Khan and Panikkar 2009; Britton et al. 2010; Bezerra et al. 2018). Black bullhead (*Ameiurus melas* Rafinesque, 1820) is one of the most successful invaders in European waters with detrimental ecological and socio-economic impacts (Leunda et al. 2008; Copp et al. 2016; Ferincz et al. 2016). The species was introduced to Europe from North America in the late 19th and early 20th centuries. Its fast spread was supported by physiological and life-history traits such as high fecundity, parental care, omnivory and tolerance to water pollution and low oxygen concentration (Ribeiro et al. 2008; Novomeská and Kováč 2009).

Black bullhead is an effective competitor and predator of native fish species. For example, Jaćimović et al. (2021) highlighted the opportunistic generalist feeding of the species with fish as a main prey item in its diet, followed by aquatic invertebrates. Similarly, Leunda et al. (2008) and Ruiz-Navarro et al. (2015) indicated that the species consumed co-occurring fish beside plant material and invertebrates affecting negatively the native ichthyofauna through both direct predation and competition. Additionally, Kreutzenberger et al. (2008) found the reduced predatory efficiency of pike (*Esox lucius* Linnaeus, 1758) in the presence of black bullhead and emphasized the potential adverse impact of this invasive species on native communities. However, there is a dearth of information on the position and specific role of black bullhead in the food web in its introduced range. While general knowledge suggests that black bullhead is an omnivorous species, there is less information about the seasonal and body size-dependent variation of its diet. In addition, the lack of detailed information on diet-based niche overlap between black bullhead and native fishes may hinder the planning of effective management strategies to control the negative impacts of this invasive species.

Stable isotope analysis (SIA) of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) is a powerful method to examine the general feeding habits of fish (Vander Zanden et al. 1999; Britton et al. 2010; Dominguez Almela et al. 2021). Unlike gut-content analysis, which provides information about the recently consumed preys and is biased towards the underestimation of the importance of easily digestible food items, SIA provides longer-term and time-mediated information about a species' diet and trophic niche (Balzani et al. 2020; McCue et al. 2020). In fact, stable nitrogen isotope ratio is an appropriate measure of consumer trophic position as it becomes enriched by 3–4‰ between prey and predator tissues (Cabana and Rasmussen 1994; Vander Zanden et al. 1999). Stable carbon isotope ratios exhibit little or no trophic level enrichment and are similar between the prey and the consumer providing a useful indicator of nutrient and energy source (Vander Zanden et al. 1999; Cucherousset et al. 2012). Stable isotope analysis thus can allow inferences regarding competition and niche partitioning among species and has been shown to be a useful method in determining the ecological impacts of alien fishes (Post 2002; Cucherousset et al. 2012).

The objective of this study was to compare the diet and the isotopic niche size and niche overlap of the invasive black bullhead and two native fish species. Since previous gut content analysis-based studies suggested that black bullhead is typically omnivorous with a dominance of invertebrate and fish preys in its diet (Leunda et al. 2008; Jaćimović et al. 2021), we chose another omnivorous cyprinid fish, the roach (*Rutilus rutilus* Linnaeus, 1758) and an invertivore-piscivorous percid fish, the European perch (*Perca fluviatilis* Linnaeus, 1758, perch hereafter) to infer potential competition between black bullhead and the native fish species in Lake Balaton, Hungary. We were especially interested to explore the seasonal and/or body size-dependent pattern in niche size and niche overlap among the species. Our predictions were as follows: (i) black bullhead will exhibit omnivorous feeding (i.e., broad niche size) with a preference to animal prey (ii) it will occupy an intermediate trophic position between perch and roach, and therefore acts as a strong competitor of both native fishes, (iii) the rate of predation on fish by black bullhead will increase with body size, and (iv) the adverse effects of black bullhead will be permanent throughout the year, that is the level of potential competition (niche overlap) with native fishes and the rate of fish in diet will not show seasonal differences.

Methods

Study area

Lake Balaton is the largest lake (surface area: 593 km^2; mean depth: 3.2 m) in Central Europe situated at 46°42'–47°04'N, 17°15'–18°10'E and 104.8 m above sea level. The lake is typically turbid and mesotrophic (Istvánovics et al. 2007). About 40% of the littoral zone is covered by common reed [*Phragmites australis* (Cav.) Trin. ex Steud.] (Specziár et al. 2013), but the rest of the shoreline is anthropogenically modified (mainly riprap sections, beaches, and harbours). Recently, 34 fish species occur in the lake, from which 9 species are invasive. Beside black bullhead, the following invasive fishes occur in Lake Balaton: Asian carp species and their hybrids (silver carp, *Hypophthalmichthys molitrix* Valenciennes, 1844 and bighead carp, *Hypophthalmichthys nobilis* Richardson, 1845) pumpkinseed (*Lepomis gibbosus* Linnaeus, 1758), monkey goby (*Neogobius fluviatilis* Pallas, 1814), tubenose goby (*Proterorhinus semilunaris* Heckel, 1837), Prussian carp (*Carassius gibelio* Bloch, 1782), topmouth gudgeon (*Pseudorasbora parva* Temminck & Schlegel, 1846), grass carp (*Ctenopharyngodon idella* Valenciennes, 1844). Detailed information on the limnology and fish fauna of the lake can be found in Bíró (1997), Istvánovics et al. (2007) and Takács et al (2017).

Studied fish and collection of organisms

Fish were collected by fyke netting and electrofishing in Lake Balaton during three sampling periods in 2022: (1) spring (from 26 May to 28 May), (2) summer (from 15 August to 25 August), and (3) autumn (from 29 October to 3 November). Fyke nets were inspected and emptied every day. The net frame had a length of 80 cm with an easily expanding 15 cm throat size. Mesh size of the net was 8 mm. Electrofishing was performed using a backpack electrofishing gear (IG200/2B, PDC, 50–100 Hz, 350–650 V, max. 10 kW; Hans Grassl GmbH, Germany) from a rubber boat in the littoral zone of the lake during night-time. Two size groups of each species were collected in each season. The smaller size group included 1+ year old individuals with a size of 8–11 cm standard body length (SL), while the larger size group contained 3+ years old individuals with a size of 17–23 cm SL. Altogether, 15 individuals were collected of each species in each season and size group. Collected individuals were immediately transported into the Balaton Limnological Research Institute where they were stored frozen (-20 °C) until the laboratory processing (within 1–2 weeks).

The most common potential food items in Lake Balaton suggested by Specziár (2010) were also collected. These were benthic chironomid larvae (*Chironomus balatonicus* Dévai, Wülker & Scholl, 1983), mussel (*Dreissena bugensis* Andrusov, 1897), snail (*Theodoxus fluviatilis* Linnaeus, 1758)), crayfish (*Faxonius limosus* Rafinesque, 1817), amphipod (*Dikerogammarus spp*.), benthic filamentous green algae (*Cladophora glomerata* Kützing, 1843) and a mixture of submerged aquatic macrophytes (*Ceratophyllum demersum* Linnaeus, 1753; *Myriophyllum spicatum* Linnaeus, 1753; *Najas marina* Linnaeus, 1753; *Potamogeton perfoliatus* Linnaeus, 1753). Sediment samples were collected using an Ekman grab sampler and chironomid larvae were separated from the sediment by washing the samples through a 0.25 mm mesh sieve. Crayfish individuals were caught by fyke netting with the same type of fyke net used for fishing. Mussels, snails, amphipods, and filamentous green algae were collected by hand from the surface of large rocks. Aquatic macrophytes were also collected by hand and washed with lake water to remove the periphyton and deposited inorganic particles. Small fish were also considered as a potential food item. For this, we used the isotopic signal of a common species in the lake (1+ year old individuals of roach with 5–6 cm SL) collected by electrofishing beside the studied fish. Five samples of each potential food item were collected. Each sample of chironomid larvae, amphipod, mussel and snail represented 10–30 individuals per sample to have sufficient biomass for SIA.

All procedures involving the handling and treatment of animals were in accordance with Hungarian law and the permit for the delivery and use of aquatic animals for scientific purposes (permit reg. no.: VE-I-001/01890-3/2013, valid between 22 August 2013 and 21 August 2023, issued by the Food-Security and Animal Health Directorate, Governmental Office of Veszprém County, Hungary).

Stable isotope analysis

We used $\delta^{13}C$ and $\delta^{15}N$ ratios to examine the diet and provide estimates of niche overlap among black bullhead and native fish species. Dorsal muscle tissues were taken from fish for SIA. All samples were dried to a constant weight at 50 °C before grinding into a fine powder with a mortar and pestle. Stable isotopes were measured with a Thermo Scientific™ EA IsoLink™ IRMS System coupled to a Thermo Finnigan DeltaPLUS XP continuous-flow isotope ratio mass spectrometer. Stable isotope ratios are reported as ‰ with the δ notation as follows:

$$\delta X\ (‰) = [(\text{R sample}/\text{R standard}) - 1] \times 1000$$

where X is ^{13}C or ^{15}N and R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratios. The reference materials used were secondary standards of known relation to the international standards of Vienna

Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen. Each sample was measured at least in three replicates for each stable isotope. Standard deviations of individual $\delta^{15}N$ and $\delta^{13}C$ measurements were ± 0.1 ‰ and ± 0.1 ‰, respectively.

Data analysis

All data analyses were performed in the R environment (R Core Team, 2021). $\delta^{13}C$ and $\delta^{15}N$ ratios were compared among species, seasons and size groups with an aligned rank transform analysis of variance (ART ANOVA) in the package ARTool 0.11.1 (Kay et al. 2021). ART ANOVA is a non-parametric approach to factorial ANOVA which is useful when the data are not normally distributed. A post hoc contrast test (i.e. ART-C) was performed when significant differences were found (Elkin et al. 2021).

For visualization, isotopic niches of species for each season and size group were determined as standard ellipse areas (SEA) using the package SIBER 2.1.6 (Jackson et al. 2011). Standard ellipse area contains approximately 40% of the data and it is the recommended method for comparing core aspects of a population's niche (Layman et al. 2012). Ellipse areas were calculated with small sample size correction (SEAc) (Jackson et al. 2011).

For calculating isotopic niche sizes (SEAc) and niche overlaps between black bullhead and native fish species we used the package nicheROVER 1.1.0 (Lysy et al. 2021). This package uses a probabilistic method to calculate niche size and pairwise niche overlap in a Bayesian framework, accounting for uncertainty due to sample size producing elliptical projections of core isotopic regions (Swanson et al. 2015). To test whether one group's ellipse is smaller or larger than another one, we calculated the probability that its posterior distribution is smaller or larger. We considered differences to be significant if the probability was more than 0.95. For estimating isotopic niche overlap, directional pairwise overlaps (i.e. the probability that an individual of one species fall within the niche of another species in $\delta^{13}C$ and $\delta^{15}N$ bivariate space) were calculated from the posterior distribution of species μ and Σ (Lysy et al. 2021). For exploring the effects of black bullhead on roach and perch, we estimated the proportion of isotopic area of native species overlapped by the isotopic area of black bullhead. Calculations for niche sizes and overlaps were conducted for each season and size group separately, with 10,000 Monte Carlo draws.

We further applied Hotelling T^2 test statistic to examine whether native fishes and black bullhead occupied unique isotopic niches. This test uses a permutation procedure and evaluates the null hypothesis that the Euclidian distance between each pair of centroids does not differ from zero (Turner et al. 2010).

The relative contribution of different food sources was estimated using Bayesian stable isotope mixing models (package MixSIAR 3.1.12) (Stock et al. 2018). The models included the variation of stable isotope values of consumers and its potential food sources as well as variation in the trophic fractionation (i.e. the difference in isotopic composition between a consumer and its diet). We used an uninformative prior. Trophic fractionation values incorporated in the models were 0.4 ± 1.3 ‰ SD for $\delta^{13}C$ and 3.4 ± 1.0 ‰ for $\delta^{15}N$ (Post 2002). Markov chain Monte Carlo sampling was implemented with the following parameters: number of chains = 3; chain length = 100,000; burn in = 50,000; thin = 50 (Stock and Semmens 2016). To test the differences in the rate of fish consumption between the size groups of black bullhead, we calculated the probability that large individuals consumed fish in a higher proportion than small ones from the posterior distribution of MixSIAR model outputs for each season. Seasonality in black bullhead fish consumption were also explored by comparing the probability that the proportion of fish in the diet is higher in one season than in another one. Similarly to the niche size tests, we considered differences to be significant if the probability was more than 0.95.

For quantifying the relative contribution of littoral and pelagic carbon sources to the diet of each consumer, we carried out two source-one biotracer ($\delta^{13}C$) Bayesian mixing models. For this, we chose one consumer from both the littoral (snail) and the pelagic zone (mussel) of the lake. Snails and mussels are widely used organisms for quantifying the importance of littoral and pelagic carbon sources (Post 2002; Vander Zanden and Vadeboncoeur 2002; Williams and Trexler 2006; e.g. Pingram et al. 2014). Each MixSIAR model was conducted separately for each season and size group of consumers.

Finally, we used a two-baseline model from the package tRophicPosition 0.8.0 (Quezada-Romegialli et al. 2018) to estimate the fish species trophic position in the food web based on the posterior distribution of the model output. Snails and mussels were used as littoral and pelagic sources in the model, respectively. Trophic fractionation values suggested by Post (2002) were used, similarly to MixSIAR models.

Results

ANOVA showed that $\delta^{13}C$ differed significantly among species ($F_{2,252}$ = 116.80, $P < 0.0001$). Generally, black bullhead was more depleted in $\delta^{13}C$ than perch and roach (contrast tests: $P < 0.0001$) (Fig. 1), while there was no significant difference between the two native species (contrast test: $P = 0.3711$). Although, species:season ($F_{4,252}$ = 9.52, $P < 0.0001$) and species:size group ($F_{2,252}$ = 23.04, $P < 0.0001$) interactions proved to be also significant, black bullhead $\delta^{13}C$ was the lowest throughout the year and in both size groups (Fig. 1). $\delta^{13}C$ varied also among seasons ($F_{2,252}$ = 3.92, $P = 0.0211$) as spring samples had significantly higher values than summer samples (contrast test: $P = 0.0255$). $\delta^{15}N$ differed significantly among species ($F_{2,252}$ = 262.36, $P < 0.0001$), seasons ($F_{2,252}$ = 21.40, $P < 0.0001$) and size groups ($F_{1,252}$ = 81.57, $P < 0.0001$). Although species:season ($F_{4,252}$ = 4.76, $P = 0.0010$) and species:size group ($F_{2,252}$ = 15.24, $P < 0.0001$) interactions were also significant, perch had the highest, while roach had the lowest $\delta^{15}N$ throughout the year and in both size groups (contrast test: black bullhead vs. perch: $P = 0.0017$, black bullhead vs. roach: $P < 0.0001$, perch vs. roach: $P < 0.0001$) (Fig. 1). Regarding seasonality, summer samples were generally more enriched while spring samples were more depleted in $\delta^{15}N$ than autumn samples (contrast test: spring vs. summer: $P < 0.0001$, spring vs. autumn: $P = 0.0005$, summer vs. autumn: $P = 0.0201$) except for black bullhead where autumn samples were more enriched than summer and spring samples. Finally, large size group of species were generally more enriched in $\delta^{15}N$ than small size group in each season ($F_{1,252}$ = 81,57, $P < 0.0001$), except for large roach which was slightly more $\delta^{15}N$ depleted than small one.

Core niche size of black bullhead was significantly (>0.95 probability) smaller in spring for the small and in autumn for the large size group, compared to other seasons (Fig. 1, Appendix 1). Regarding native species, only the small size group of perch showed seasonal changes in niche size; it was largest in autumn and smallest in summer with an intermediate value in spring. Roach had larger niche size than black bullhead in each season and in each size group, although this difference was not significant in spring and summer in the large size group (Fig. 1, Appendix 1). Niche size variability between black bullhead and perch did not show a clear pat-

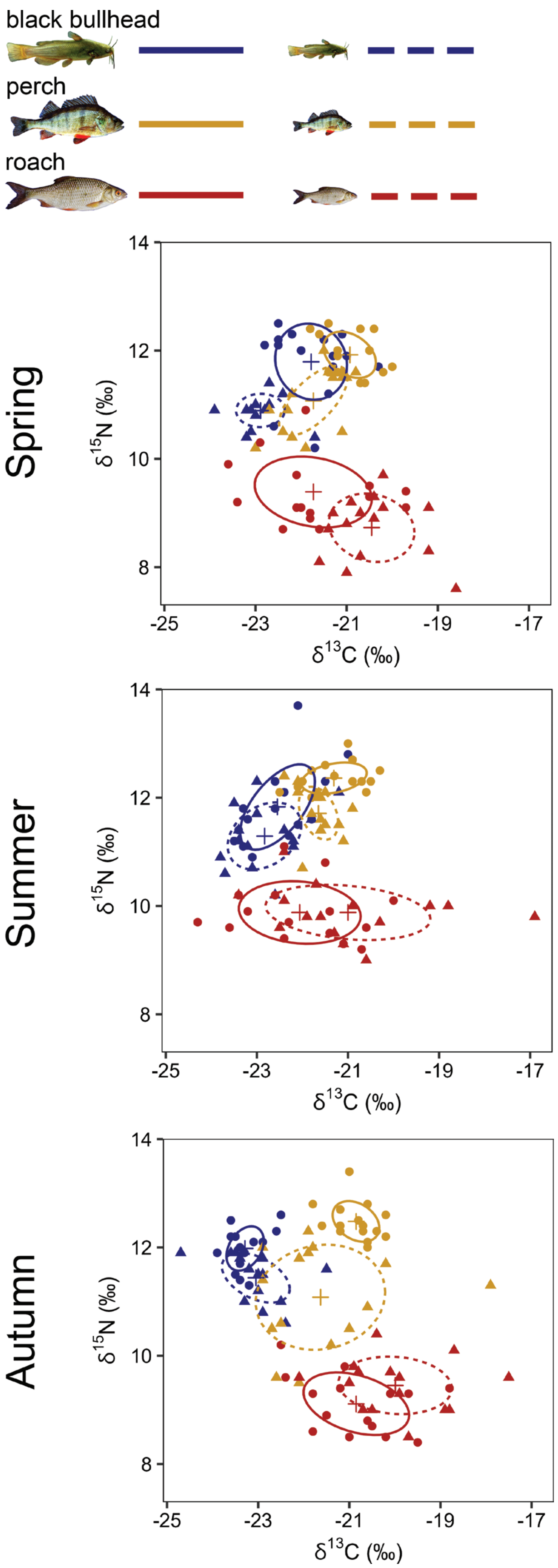

Figure 1. Isotopic niches (SEAc) and their centroids (+) of large and small size groups of black bullhead (*Ameiurus melas*), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) collected in Lake Balaton (Hungary) in spring (from 26 May to 28 May), summer (from 15 August to 25 August) and autumn (from 29 October to 3 November) of 2022. Axes show the $\delta^{13}C$ and $\delta^{15}N$ values of individuals.

tern. In spring and summer large black bullhead had a significantly larger niche size than large perch, while in autumn, their niche sizes were similar. In small size groups, black bullhead had significantly larger niche size in summer, while significantly smaller niche size in spring and autumn than perch. Overlap between core niches of black bullhead and perch varied among seasons and size groups (Figs 1, 2A, B). Diet niche of both large and small perch was slightly overlapped with large black bullhead showing a decreasing pattern throughout the year with a negligible overlap in autumn (Fig. 2A, B). Diet niche of small perch was also minimally overlapped with small black bullhead in each season (Fig. 2B). Overlap between the core niches of black bullhead and roach was negligible (Fig. 2C, D).

Centroid locations of fish species in the isotopic space varied significantly ($P < 0.05$) in each season, suggesting that species occupy unique trophic niches relative to each other throughout the year. Size groups within species were also separated significantly except for summer black bullhead and summer roach for which Euclidian distance between centroids of their large and small size groups were not significantly different from zero (Fig. 1, Appendix 2).

The most important dietary component of large black bullhead and large perch was fish in each season (Fig. 3). As secondary diet, large individuals of both species

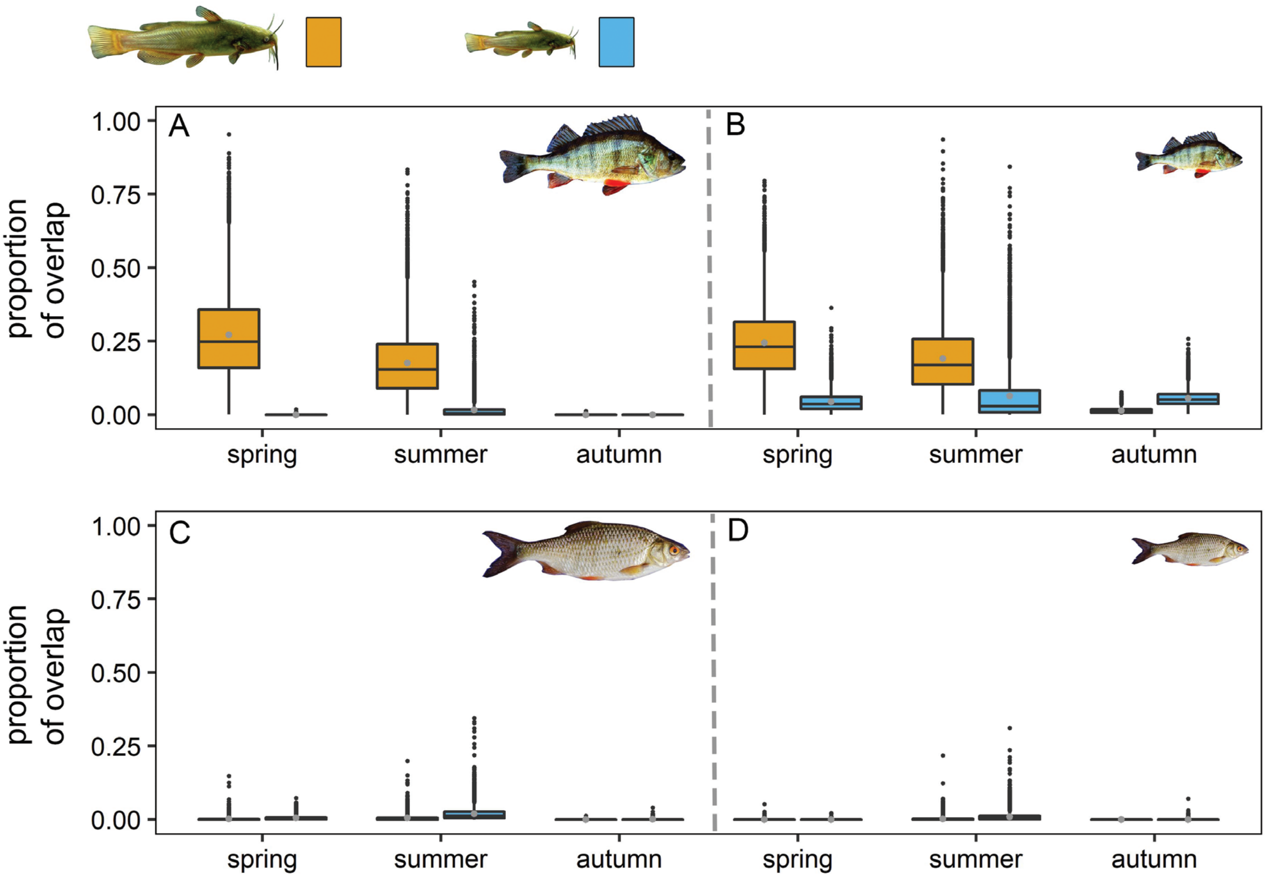

Figure 2. The proportion of isotopic area of large perch (*Perca fluviatilis*) (**A**), small perch (**B**), large roach (*Rutilus rutilus*) (**C**) and small roach (**D**) overlapping with the isotopic area of large and small black bullhead (*Ameiurus melas*) collected in Lake Balaton (Hungary) in spring (from 26 May to 28 May), summer (from 15 August to 25 August) and autumn (from 29 October to 3 November) of 2022. Axes show seasons and the proportion of diet overlap. Whiskers indicate 95% credible intervals with outliers (black dots). Boxplots indicate 25th, 50th and 75th percentiles. Grey dots indicate the mean proportion of overlap. Each value was calculated from model posterior distributions (see Materials and Methods for details).

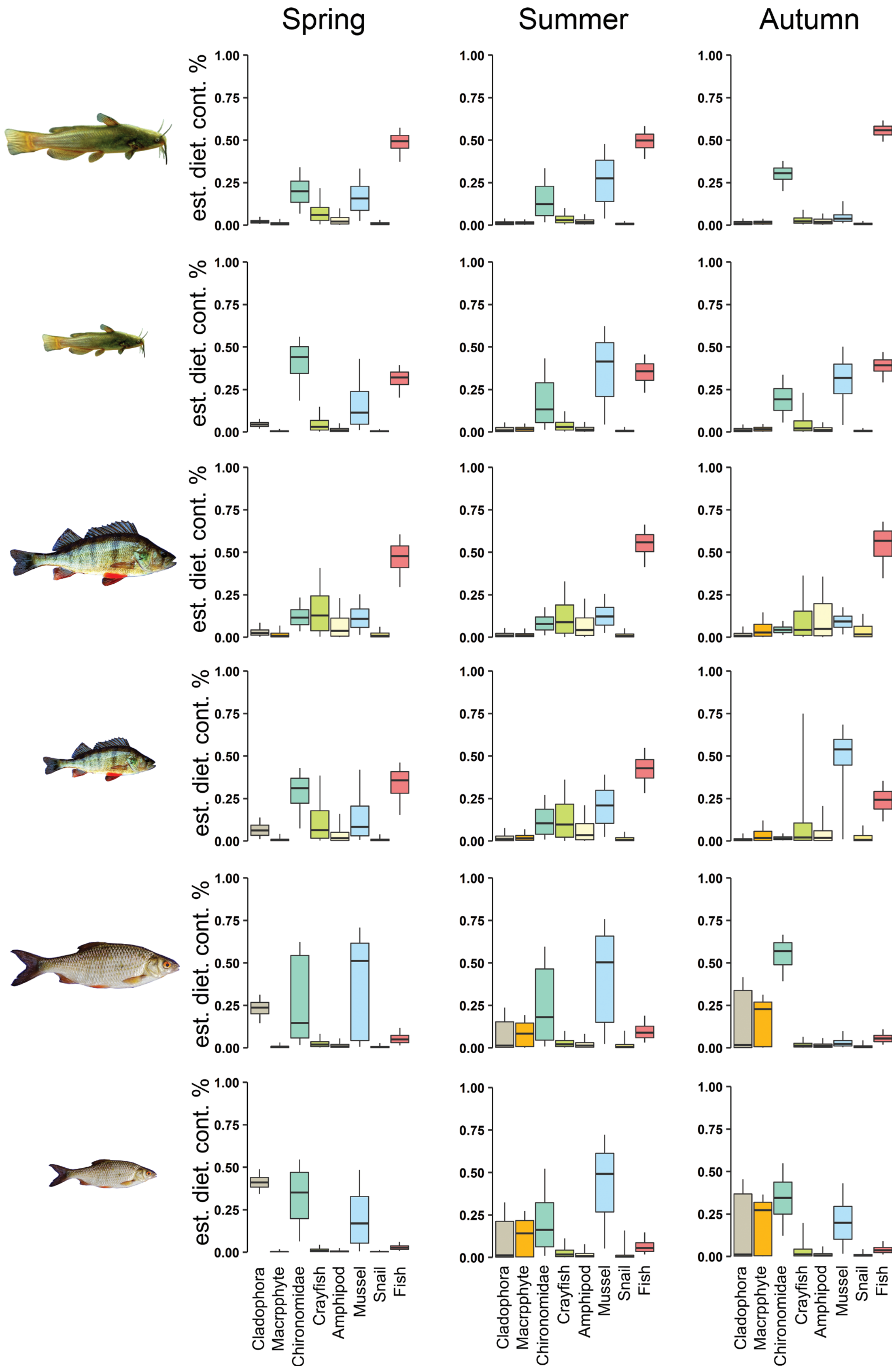

Figure 3. Estimated seasonal contributions of food sources to the diet of large and small size groups of black bullhead (*Ameiurus melas*), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) based on Bayesian stable isotope mixing models. Fish were collected in Lake Balaton (Hungary) in spring (from 26 May to 28 May), summer (from 15 August to 25 August) and autumn (from 29 October to 3 November) of 2022. Axes show different food sources and their estimated contributions to the diet. Whiskers indicate 95% credible intervals. Boxplots indicate 25th, 50th and 75th percentiles. Each value was calculated from model posterior distributions (see Materials and methods for details).

consumed macro invertebrates; diet of black bullhead contained mainly chironomid larvae and mussels, while the proportion of different macro invertebrate components distributed more equally in the diet of perch. Diet of small individuals of both species included both macro invertebrates and fish as main dietary components. Similarly to large conspecifics, small black bullhead fed mainly on mussels and chironomid larvae, while small perch fed on various macro invertebrates throughout the year. Proportion of fish in the diet of large black bullhead was significantly higher than in the diet of small black bullhead in each season (>0.95 probability), while there were no significant differences between seasons (spring vs. summer: probability = 0.56; spring vs. autumn: probability = 0.85; summer vs. autumn: probability = 0.77). Roach diet comprised both plant-based components and macro invertebrates in both size groups. The main macro invertebrates consumed by roach were chironomid larvae and mussel in each season. Regarding plants, only cladophora was consumed by roach in spring, while in summer and autumn, when developed aquatic macrophytes became abundant in the lake, roach shifted its plant-based diet to this food item. Although each studied fish species relied predominantly on the pelagic production in each season (77–95%, Appendix 3), the two source-one biotracer ($\delta^{13}C$) Bayesian mixing models indicated some differences in the use of primary carbon sources between black bullhead and the studied native fishes. Specifically, black bullhead utilized pelagic-based food web at a higher rate than native species independently of size group and season.

Large perch occurred in the highest trophic position in each season followed by large black bullhead (Fig. 4), but these differences were not significant (spring: probability = 0.62, summer: probability = 0.83, autumn: probability = 0.85).

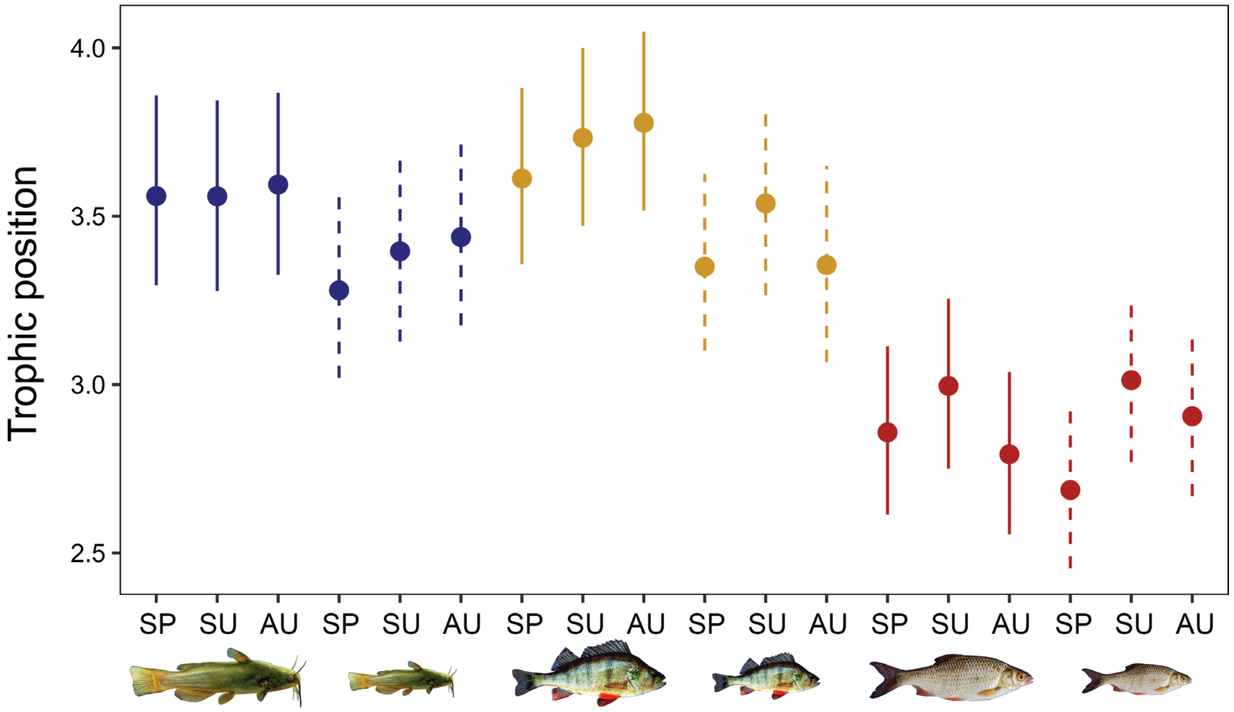

Figure 4. Estimated posterior trophic position of large and small size groups of black bullhead (*Ameiurus melas*), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) collected in Lake Balaton in spring (SP, from 26 May to 28 May), summer (SU, from 15 August to 25 August) and autumn (AU, from 29 October to 3 November). Axes show seasons and the trophic position of species in different size groups. Each value was calculated from model posterior distributions (see Materials and Methods for details).

Similarly, trophic position of small black bullhead and small perch did not differ significantly (spring: probability = 0.66, summer: probability = 0.79, autumn: probability = 0.68), while large perch had a significantly higher trophic position (>0.95 probability) than small black bullhead and small perch in each season and in autumn, respectively. Both species had a significantly higher trophic position than roach in each season and size group.

Discussion

The most prominent impact of invasive black bullhead on native fish and ecosystems is that it can act as a predator and a competitor of food resources simultaneously (Leunda et al. 2008; Ruiz-Navarro et al. 2015; Jaćimović et al. 2021). In this study we found that black bullhead in Lake Balaton could be characterised by invertivore-piscivorous feeding habits with a substantial rate of fish consumption. Large black bullhead consumed fish in a higher proportion than small ones and the rate of fish consumption was independent of the season, confirming our predictions. However, contrary to our hypothesis, substantial food niche partitioning occurred among black bullhead and native fish species suggesting that they assimilate distinct energy sources in different proportions and thus occupy different trophic positions in the food web. In sum, two (iii and iv) out of our four predictions (see Introduction for details) were supported, while two (i and ii) were not supported by our results.

Niche partitioning facilitates the local coexistence of native and alien species that divide available resources by separating along one or more niche dimensions (e.g., diet, habitat, activity time) (Schoener 1974). According to our results, core niche segregation of black bullhead and native fishes occurred along both the $\delta^{13}C$ and $\delta^{15}N$ axes indicating differences in the diet source and that they obtain resources at different trophic levels. Black bullhead consumed mussels and chironomid larvae in a high proportion. Since these invertebrates filter various suspended materials from the whole water column (Oliver 1971; Pinder 1986; MacIsaac et al. 1995; Spooner and Vaughn 2006), their assimilations could shift black bullhead's isotopic signal towards a more negative direction along the $\delta^{13}C$ axis. In contrast, the diet of perch was more heterogeneous regarding macro invertebrates, while the diet of roach included considerable amount of plant material, which contributed to the more enriched $\delta^{13}C$ values of these species (Post 2002; Guinan Jr et al. 2015; Yu et al. 2016).

Variation in the foraging areas could also contribute to the variability of $\delta^{13}C$ values. For example, Coulter et al. (2019) highlighted that niche partitioning of native and alien planktivorous fishes might have been driven by the distinct rate of using pelagic vs. littoral/benthic food sources. Similarly, Mumby et al. (2018) emphasized the role of habitat partitioning in the very limited extent of isotopic niche overlap in an offshore fish assemblage. However, our studied species relied predominantly on the pelagic production of the lake indicating little importance of foraging migration in niche partitioning. The dominance of pelagic-based food components in the diet could be unexpected considering that the studied fishes inhabit mainly the littoral zone (Specziár, 2010; Specziár et al. 2013). However, Lake Balaton is a shallow and turbid lake, and these properties facilitate the homogenization of the materials produced in different zones of the lake. This suggests that the isotopic signature of species living in the littoral zone can be affected by food components produced in the pelagic area (Monroy et al. 2014). Apparent niche segregation of perch and black bullhead from roach along $\delta^{15}N$ axis was driven mainly by the distinct rate of piscivory. Although we found significant differences in δ^{15}N values between black bullhead and perch, too, they still occupy the same trophic position, at least within size groups. These findings highlight that although different resource-consumer dynamics exist between black bullhead and perch, they play similar functional roles in the food web.

Isotopic areas of native species overlapped only slightly or negligibly with the isotopic area of black bullhead throughout the year and in each size group suggesting relatively low potential for competition among them. It is important to note that our results do not rule out that black bullhead competes for food with other fishes in the lake. It does highlight, however, that the species' omnivorous or invertivore-piscivorous feeding habit detected in other ecosystems do not necessarily predetermine that black bullhead would compete with co-occurring native fishes from similar feeding groups (Leunda et al. 2008; Jaćimović et al. 2021). The potential lack of competition among the studied species may emerge through different mechanisms. Firstly, black bullhead probably exploits open resource niches, which may exist in anthropogenically modified and intensively utilized ecosystems like Lake Balaton (see e.g. Czeglédi et al. 2019), or obtains resources that are underused by native species (Coulter et al. 2019). Alternatively, given that the first occurrence of the black bullhead in Lake Balaton dates back to the 1980s, it is possible that the native species has already undergone competition-induced niche shifts during their co-existence. For testing these assumptions, exploring historical isotopic signatures of preserved specimens from the pre-invasion period, and using a finer taxonomic resolution of potential prey items would be necessary (e.g. complement our SIA with gut-content analyses). However, niche size of black bullhead was significantly smaller than that of roach and did not show a clear distinction with the niche size of perch, of the three studied species, black bullhead was the only one that showed significant seasonal niche size variability in both size groups. Based on these results, we assume that black bullhead does not have a permanently wide foraging niche by feeding on a variety of prey items in Lake Balaton, which can be an advantageous strategy in the invasion process (Tonella et al. 2018), but instead varies its foraging niche size temporally. Plasticity in niche size also supports the avoidance of competition with native fish and may facilitate the fast spread and wide establishment of black bullhead, similarly to other successful invaders (Almeida et al. 2012; Pettitt-Wade et al. 2015; Dominguez Almela et al. 2021).

Although black bullhead was predicted to occupy intermediate trophic position between perch and roach, the preference towards animal-based food items raised the species into the same level where the invertivore-piscivorous perch occurs. By taking into consideration all fishes in Lake Balaton, only piscivore apex predators such as pike, pikeperch (*Sander lucioperca* Linnaeus, 1758), European catfish (*Silurus glanis* Linnaeus, 1758), and asp (*Leuciscus aspius* Linnaeus, 1758) are in a higher position in the food web (Specziár, 2010). The substantial rate of fish consumption revealed by this study suggests that black bullhead is likely to have a large impact on native fish assemblage through predation. Our results thus are in accordance with Ruiz-Navarro et al. (2015), who found no isotopic niche overlap between black bullhead and roach in the invaded area and emphasized the importance of direct predation as the main adverse effect of black bullhead on native fish. Similarly to Leunda et al. (2008), who also highlighted the potential detrimental effects of black bullhead on local native fish fauna through predation, we found an ontogenetic diet shift between the species' size groups with an increasing rate of fish consumption with body size. For larger individuals, capturing protein-rich fish prey is probably more energetically profitable than choosing macro invertebrates with hardly digestible calcium carbonate shells and chitin exoskeleton. Here, it is important to note that neither SIA nor gut content analyses allow to ascertain the origin (i.e., live or carcass) of fish preys and, in recent laboratory experiments, Preiszner et al. (2020, 2024) revealed that black bullhead may exhibit scavenging behaviour. Thus, it is possible that some of the fish consumed by black bullhead were carcasses, although carcass feeding has been suggested to be rather a complementary foraging strategy and not pivotal in their diet (Preiszner et al. 2020).

In conclusion, we compared the diet of black bullhead with an omnivorous and an invertivore-piscivorous fish species and found proof for intensive fish predation but revealed little evidence of actual feeding competition with native fish in Lake Balaton. The high proportion of fish in the diet of black bullhead might raise concerns in its invaded range. For example, in Lake Balaton, its population size suddenly increased (relative abundance of the species was <1% between 1996 and 2018 and 14.5% in 2022, unpublished standard monitoring data), and thus the species may represent a threat for native, small-sized fishes. Its predation can be especially significant during the spawning period, in spring and early summer when juvenile native fish are recruiting in the lake. Moreover, current human-induced alterations in the environmental characteristics of the lake (e.g. establishing wave-free harbours where dense submerged macrovegetation can develop) may further facilitate the spread and population growth of black bullhead (Jenkins 1957; Copp et al. 2016). In light of our compelling evidence of potential risks posed by the black bullhead, in ecosystems where its population expands, we strongly recommend urgent management actions, such as the selective removal of the species (see e.g., Jaćimović et al. 2023) to minimize its adverse impacts on the native fish fauna.

Acknowledgements

We thank Ingrid Győri, Bendegúz Márton Tóth, Krisztina Krassován, Hajnalka Horváth and Izabella Battonyai Tátrainé for their assistance in laboratory works. István Czeglédi was supported by the OTKA PD 138296 grant (National Research, Development and Innovation Office – NKFIH). Péter Takács was supported by the OTKA FK 140902 project (National Research, Development and Innovation Office – NKFIH). The research presented in this article was supported by the Széchenyi Plan Plus program (RRF 2.3.1-21-2022-00008 project) and the Sustainable Development and Technologies National Programme of the Hungarian Academy of Sciences (FFT NP FTA).

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

This work was supported by the OTKA PD 138296 grant (National Research, Development and Innovation Office – NKFIH), the Széchenyi Plan Plus program (RRF 2.3.1-21-2022-00008 project) and the Sustainable Development and Technologies National Programme of the Hungarian Academy of Sciences (FFT NP FTA).

Author contributions

Conceptualization: IC, TE. Data curation: IC, AS. Formal analysis: IC. Funding acquisition: IC, TE. Investigation: IC, TE, AS, PT, BB, GB, AM, BP. Methodology: IC, BB, TE, AM, PT, AS, BP, GB. Project administration: IC. Resources: IC, TE. Supervision: IC. Visualization: IC. Writing - original draft: IC. Writing - review and editing: AS, TE, BB, BP, GB, AM, PT.

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Data availability

All of the data that support the findings of this study are available from the corresponding author upon reasonable request.

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Appendix 1

Table A1. Seasonal niche size differences within species and size groups. Values indicate probability values from posterior distributions.

Black bullhead							
small size group				**large size group**			
	spring	summer	autumn		spring	summer	autumn
spring	NA			spring	NA		
summer	>0.95	NA		summer	0.57	NA	
autumn	>0.95	0.89	NA	autumn	>0.95	>0.95	NA
Perch							
small size group				**large size group**			
	spring	summer	autumn		spring	summer	autumn
spring	NA			spring	NA		
summer	>0.95	NA		summer	0.69	NA	
autumn	>0.95	>0.95	NA	autumn	0.76	0.58	NA
Roach							
small size group				**large size group**			
	spring	summer	autumn		spring	summer	autumn
spring	NA			spring	NA		
summer	0.88	NA		summer	0.58	NA	
autumn	0.62	0.80	NA	autumn	0.78	0.72	NA

Table A2. Niche size differences between species. Values indicate probability values from posterior distributions.

Spring							
small size group				**large size group**			
	Black bullhead	Perch	Roach		Black bullhead	Perch	Roach
Black bullhead	NA			Black bullhead	NA		
Perch	>0.95	NA		Perch	>0.95	NA	
Roach	>0.95	0.91	NA	Roach	0.86	>0.95	NA
Summer							
small size group				**large size group**			
	Black bullhead	Perch	Roach		Black bullhead	Perch	Roach
Black bullhead	NA			Black bullhead	NA		
Perch	>0.95	NA		Perch	>0.95	NA	
Roach	>0.95	>0.95	NA	Roach	0.85	>0.95	NA
Autumn							
small size group				**large size group**			
	Black bullhead	Perch	Roach		Black bullhead	Perch	Roach
Black bullhead	NA			Black bullhead	NA		
Perch	>0.95	NA		Perch	0.66	NA	
Roach	>0.95	>0.95	NA	Roach	>0.95	>0.95	NA

Appendix 2

Table A3. Results of Hotelling T^2 test. Upper matrix indicates Hotelling T^2 values, while lower matrix indicates P values.

Spring							
		Black bullhead		Perch		Roach	
		large	small	large	small	large	small
Black bullhead	large	NA	51.10	14.74	11.08	112.16	193.76
	small	<0.0001	NA	217.41	30.84	80.72	233.43
Perch	large	0.0030	<0.0001	NA	25.93	216.15	310.86
	small	0.0105	<0.0001	0.0001	NA	60.06	167.44
Roach	large	<0.0001	<0.0001	<0.0001	<0.0001	NA	17.58
	small	<0.0001	<0.0001	<0.0001	<0.0001	0.0012	NA
Summer							
		Black bullhead		Perch		Roach	
		large	small	large	small	large	small
Black bullhead	large	NA	4.97	22.85	20.99	78.25	91.96
	small	0.108	NA	60.06	32.25	54.51	67.23
Perch	large	0.0003	<0.0001	NA	25.84	265.79	317.83
	small	0.0005	<0.0001	0.0001	NA	111.44	119.02
Roach	large	<0.0001	<0.0001	<0.0001	<0.0001	NA	4.02
	small	<0.0001	<0.0001	<0.0001	<0.0001	0.1612	NA
Autumn							
		Black bullhead		Perch		Roach	
		large	small	large	small	large	small
Black bullhead	large	NA	13.78	257.56	39.31	310.47	355.38
	small	0.0042	NA	259.66	15.57	177.34	181.49
Perch	large	<0.0001	<0.0001	NA	35.37	476.71	380.15
	small	<0.0001	0.0023	<0.0001	NA	55.03	52.4
Roach	large	<0.0001	<0.0001	<0.0001	<0.0001	NA	10.08
	small	<0.0001	<0.0001	<0.0001	<0.0001	0.0150	NA

Appendix 3

Table A4. The relative contribution of littoral and pelagic carbon sources to the diet of fish species.

Season	Species	Size group	Source of carbon	Mean	SD	2.50%	5%	25%	50%	75%	95%	97.50%
Spring	Black bullhead	large	pelagic	0.928	0.031	0.866	0.875	0.907	0.930	0.951	0.978	0.984
			littoral	0.072	0.031	0.016	0.022	0.049	0.070	0.093	0.125	0.134
		small	pelagic	0.927	0.029	0.869	0.877	0.907	0.929	0.949	0.973	0.980
			littoral	0.073	0.029	0.020	0.027	0.051	0.071	0.093	0.123	0.131
	Perch	large	pelagic	0.864	0.035	0.799	0.807	0.838	0.864	0.887	0.923	0.935
			littoral	0.136	0.035	0.065	0.077	0.113	0.136	0.162	0.193	0.201
		small	pelagic	0.861	0.030	0.803	0.811	0.840	0.861	0.882	0.910	0.920
			littoral	0.139	0.030	0.080	0.090	0.118	0.139	0.160	0.189	0.197
	Roach	large	pelagic	0.850	0.032	0.790	0.799	0.829	0.850	0.872	0.903	0.913
			littoral	0.150	0.032	0.087	0.097	0.128	0.150	0.171	0.201	0.210
		small	pelagic	0.846	0.030	0.790	0.798	0.825	0.847	0.867	0.896	0.906
			littoral	0.154	0.030	0.094	0.104	0.133	0.153	0.175	0.202	0.210
Season	**Species**	**Size group**	**Source of carbon**	**Mean**	**SD**	**2.50%**	**5%**	**25%**	**50%**	**75%**	**95%**	**97.50%**
Summer	Black bullhead	large	pelagic	0.941	0.026	0.886	0.894	0.924	0.943	0.961	0.981	0.985
			littoral	0.059	0.026	0.015	0.019	0.039	0.057	0.076	0.106	0.114
		small	pelagic	0.935	0.028	0.878	0.887	0.916	0.937	0.956	0.977	0.983
			littoral	0.065	0.028	0.017	0.023	0.044	0.063	0.084	0.113	0.122
	Perch	large	pelagic	0.869	0.031	0.810	0.818	0.847	0.869	0.891	0.921	0.930
			littoral	0.131	0.031	0.070	0.079	0.109	0.131	0.153	0.182	0.190
		small	pelagic	0.856	0.030	0.797	0.806	0.836	0.855	0.876	0.906	0.914
			littoral	0.144	0.030	0.086	0.094	0.124	0.145	0.164	0.194	0.203
	Roach	large	pelagic	0.879	0.030	0.821	0.830	0.858	0.879	0.900	0.929	0.939
			littoral	0.121	0.030	0.061	0.071	0.100	0.121	0.142	0.170	0.179
		small	pelagic	0.866	0.032	0.807	0.815	0.844	0.866	0.888	0.919	0.931
			littoral	0.134	0.032	0.069	0.081	0.112	0.134	0.156	0.185	0.193
Season	**Species**	**Size group**	**Source of carbon**	**Mean**	**SD**	**2.50%**	**5%**	**25%**	**50%**	**75%**	**95%**	**97.50%**
Autumn	Black bullhead	large	pelagic	0.945	0.022	0.899	0.906	0.931	0.946	0.961	0.977	0.981
			littoral	0.055	0.022	0.019	0.023	0.039	0.054	0.069	0.094	0.101
		small	pelagic	0.942	0.022	0.894	0.903	0.927	0.944	0.958	0.975	0.980
			littoral	0.058	0.022	0.020	0.025	0.042	0.056	0.073	0.097	0.106
	Perch	large	pelagic	0.832	0.027	0.780	0.788	0.814	0.832	0.850	0.875	0.884
			littoral	0.168	0.027	0.116	0.125	0.150	0.168	0.186	0.212	0.220
		small	pelagic	0.824	0.025	0.775	0.783	0.808	0.825	0.841	0.866	0.873
			littoral	0.176	0.025	0.127	0.134	0.159	0.175	0.192	0.217	0.225
	Roach	large	pelagic	0.776	0.025	0.727	0.735	0.760	0.776	0.792	0.816	0.824
			littoral	0.224	0.025	0.176	0.184	0.208	0.224	0.240	0.265	0.273
		small	pelagic	0.766	0.025	0.718	0.725	0.749	0.767	0.783	0.808	0.816
			littoral	0.234	0.025	0.184	0.192	0.217	0.233	0.251	0.275	0.282

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 79–100 (2024)
DOI: 10.3897/neobiota.94.125227

Research Article

Differential survival and feeding rates of three commonly traded gastropods across salinities

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Abstract

Increasing rates of biological invasions pose major ecological and economic threats globally. The pet trade is one major invasion pathway, and environmental change could mediate the successful establishment and impact of these released or escaped non-native species (NNS). Salinity regime shifts are a pervasive but often overlooked environmental change in aquatic ecosystems. This study investigates the establishment and impact risks posed by three readily available, traded snail species – *Melanoides tuberculata*, *Tarebia granifera* and *Anentome helena* – by assessing their survival and feeding responses across a spectrum of salinity levels (0.2–16 g/kg). Survival differed among the species, with *M. tuberculata* showing close to 100% survival across the salinity range, *T. granifera* exhibiting heightened mortality at 16 g/kg, and *A. helena* displaying no survival at salinities above 12 g/kg. In feeding experiments assessing the more resilient *M. tuberculata* and *T. granifera*, the former had greater consumption rates towards both plant- (spinach) and animal-based (daphniid) resources. While salinity and density effects did not affect animal consumption, they both had significant effects on plant consumption, with feeding suppressed for both consumers under a salinity of 8 g/kg relative to freshwater conditions. When combining proportional survival and resource consumption for *M. tuberculata* and *T. granifera*, *M. tuberculata* demonstrated higher impact potential towards both plant and animal resources, highlighting its potential to exert higher ecological impacts. Studies have overlooked the importance of salinity for invasion success and the impact of pet trade species. We therefore propose that these methods provide a screening tool to assess the potential risks of traded species establishing and exerting impacts, and we encourage future studies to account for a broader range of abiotic stressors.

Key words: *Anentome helena*, feeding rates, *Melanoides tuberculata*, Relative Impact Potential, risk assessment, salinity, survival, *Tarebia granifera*

Academic editor: Jaimie T. A. Dick
Received: 11 April 2024
Accepted: 12 June 2024
Published: 26 July 2024

Citation: Renk E, Dickey JWE, Cuthbert RN, Kazanavičiūtė E, Briski E (2024) Differential survival and feeding rates of three commonly traded gastropods across salinities. NeoBiota 94: 79–100. https://doi.org/10.3897/neobiota.94.125227

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Introduction

Non-native species (NNS) are a major global threat to ecosystems and biodiversity, often causing substantial economic costs (Molnar et al. 2008; Cuthbert et al. 2021a; Diagne et al. 2021; IPBES 2023). An increasingly globalised world, with novel trade and transport routes, has facilitated the spread of NNS via means such as ship ballast water (Smith et al. 1999; Drake and Lodge 2004; Briski et al. 2012), the construction of new corridors like the Suez or Panama Canals (Balzani et al. 2022), and the horticultural and pet trades (Chucholl 2013; Lockwood et al. 2019; Dickey et al. 2023b). Indeed, the aquatic pet trade alone has enabled the establishment of freshwater and marine species in new habitats, with well-known examples including goldfish (*Carassius auratus* Linnaeus, 1758), pond slider terrapins (*Trachemys scripta* Thunberg, 1792) and various crayfish species, which have exerted negative impacts on recipient ecosystems (Vodovsky et al. 2017; Dickey et al. 2018; Britton 2022). In some cases, the species are deliberately released into the wild (Shiu and Stokes 2008; Maceda-Veiga et al. 2019), while in others escapes occur (Patoka et al. 2017). Further, incidental "hitch-hiker" species, like zebra mussels (*Dreissena polymorpha* Pallas, 1771), can be transported via the pet trade (Patoka and Patoková 2021; Dickey et al. 2023c).

Species must be able to withstand broad biotic and abiotic conditions during the invasion process, namely transport, introduction and establishment stages, to become invasive NNS (Blackburn et al. 2011). Indeed, successful invaders often show a heightened tolerance to abiotic stressors, as well as fast somatic growth and high fecundity (Côté and Smith 2018). One important constraining abiotic factor for aquatic invertebrates is salinity, which affects mortality, fecundity, growth and community composition (Zalizniak et al. 2009) and acts in combination with other environmental changes (Cuthbert et al. 2019). Indeed, this stressor poses a threat to biodiversity, and can be a result of agricultural land use, mining discharge, aquaculture effluent, fracking, road salt run-off, as well as rising sea levels (Cunillera-Montcusí et al. 2022; Barrios-Figueroa and Urbina 2023). Broad salinity tolerance facilitates establishment and may lead to greater spread and competitive advantages (Lockwood and Somero 2011). For example, the red lionfish (*Pterois volitans* Linnaeus, 1758), most likely introduced to the Atlantic Ocean through an aquarium spill (Whitfield et al. 2002; Hixon et al. 2016), is expanding its range into lower salinity estuaries of North America (Schofield et al. 2015). As well as helping to predict whether species can establish in freshwater, brackish and marine habitats, understanding the role of salinity can also offer insights into tolerance and performance under freshwater salinisation. Determining the ability of NNS to survive and exert impacts under such abiotic stressors is an important concern for conservationists, and is a crucial consideration for predicting and proactively preventing potentially damaging invasions.

Aquatic snails have frequently established and exerted negative impacts in novel environments after introductions via the pet trade (Preston et al. 2022). One example is the golden apple snail (*Pomacea canaliculata* Lamarck, 1822) in Asia. Native to South America, specimens were originally imported as aquarium pets, as well as food sources and for use in commercial aquaculture (Joshi 2007, de Brito and Joshi 2016). The snail spread rapidly and consequently generated vast economic losses to crops, while also posing a threat to human health as intermediate hosts of zoonotic nematode parasites (Xu et al. 2016; Djeddour et al. 2021). While such high impact species can be regulated and thus become absent from the pet trade, other species with invasion histories remain readily available, such as *Melanoides tuberculata* (Müller, 1774), *Tarebia granifera* (Lamarck, 1822) and *Anentome helena* (von dem Busch, 1847) (Dickey et al. 2023b). Both the Afro-Asiatic freshwater snail *M. tuberculata* and the South-East Asian *T. granifera* have established non-native populations in tropical and subtropical areas worldwide, partly due to the pet trade (Vaz et al. 1986; Duggan 2010; Work and Mills 2013; Coelho et al. 2018), as well as through deliberate introductions as biocontrol agents (Pointier and Jourdane 2000). Both species have broad generalist diets and abiotic tolerances (Miranda et al. 2010; Weir and Salice 2012; Raw et al. 2016). Indeed, these species have been found in freshwater and estuarine conditions within their non-native ranges (Miranda et al. 2010; Farani et al. 2015). In contrast, the carnivorous "assassin snail" *A. helena*, with a native distribution including Cambodia, Indonesia, Thailand, Vietnam, Laos and Malaysia (Ng et al. 2016), has only a limited non-native range to date, despite its popularity in the pet trade for controlling outbreaks of pest species (Karmakar et al. 2022; Dickey et al. 2023a). Part of one of the few freshwater, stenohaline, potamodromous genera within the almost entirely marine Nassariidae family (Galindo et al. 2016), its only reported non-native occurrence to date has been from a freshwater reservoir in Singapore (Ng et al. 2016).

In recent years, many approaches have been developed to investigate the probability of invasion success and the magnitude of impact (Ruiz et al. 2000; Geller et al. 2010; Dickey et al. 2020; McCard et al. 2021). In this study, we tested the tolerance of three commonly traded snail species (*M. tuberculata*, *T. granifera* and *A. helena*) to salinity changes. We also determined their feeding ability and preferences under those abiotic conditions. First, we examined the survivability of the three study species over a range of salinities, from freshwater to brackish, to mimic pet trade release events (i.e. without any acclimation period). Second, we tested the effect of salinity on the *per capita* feeding rates of two of these species which showed the highest survival, *M. tuberculata* and *T. granifera*, using a method similar to functional response experiments (i.e. the rate of resource consumption by a consumer in relation to resource density), which are regularly used to predict the potential ecological impact a species might exert (Dick et al. 2014; Faria et al. 2023). Finally, we combined survival and feeding rates through the Relative Impact Potential metric to quantify and compare how salinity affects the potential ecological impacts of *M. tuberculata* and *T. granifera* (Dick et al. 2017; Dickey et al. 2020).

Materials and methods

Species acquisition and husbandry

Our three study species (Suppl. material 2: fig. S2) were ordered from an online vendor (i.e. garnelen-direkt.de): *M. tuberculata* was ordered on May 23rd 2022, *T. granifera* on July 26th 2022, and *A. helena* on October 1st 2022 (280 individuals ordered per species; mean shell lengths of 1.69cm, 1.42cm and 1.77cm, respectively). All three species arrived within 24 hours and were transported in moist, shockproof containers. After arrival, the snails were placed in a climate chamber at GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany, in a 56 L glass aquarium containing constantly oxygenated freshwater per the vendor's recommendation. All individuals of each species were placed in a separate aquarium.

The temperature of the climate chamber was maintained at 18 °C (± 0.6 °C) with a 16:8h light and dark regime and the water temperature was kept at 19 °C (± 0.8 °C). The two detritivorous species (i.e. *M. tuberculata* and *T. granifera*) were fed *ad libitum* with "Veggi Wafers" (Pleco Tetra, USA), while the carnivorous *A. helena* were fed frozen *Mysis* sp. (Vivantis aqua, Germany).

Survival experiments

Animals were acclimated for at least two weeks before experimentation. The survival of snails was determined in eight different salinities: 0.2, 0.6, 1, 2, 5, 8, 12, and 16 g/kg. This range was chosen to represent a spectrum from freshwater - the recommended conditions for all three species in the pet trade - to brackish water representative of estuarine conditions or those of Kiel Fjord in the Baltic Sea. Baltic Sea water was diluted with freshwater or mixed with artificial salt (Aquarium Systems Instant Ocean, France) to reach the desired salinities, as needed. The experiments started by placing ten snails in each 2 L aerated aquarium under the experimental salinity conditions, without prior adaptation, with experiments replicated three times per species and salinity (Suppl. material 2: fig. S2). Salinity was measured and adjusted as needed twice per week, and snails were fed *ad libitum* daily; *M. tuberculata* and *T. granifera* with spinach, and *A. helena* with thawed *Mysis* sp.. Uneaten food was removed once per week to prevent degression of water quality. Three criteria were applied to determine viability of snails, which can prove difficult due to their ability to retreat into their shells. First, snails were assessed visually for control of movement or attachment to the tank wall. Second, non-moving snails were lightly poked with tweezers, and if they reacted, were deemed living. Third, any snails that did not react to poking were put in freshwater for 60 minutes and if no signs of movement were observed within this time, they were considered dead and removed from the experiment. Live snails were placed back into the same experimental aquarium that they were taken from. The survival status was assessed daily, with the experiments lasting for 30 days. The snails were kept in their respective tanks for an additional 14 days (as per the methods of Casties et al. 2019; Paiva et al. 2020), under the same salinity and feeding regimes, after the survival experiment to determine potential delayed onset mortality.

Proportional food consumption experiments

Proportional consumption experiments, designed in the style of functional response trials, tested the feeding rates of the two detritivorous snails, *T. granifera* and *M. tuberculata*, over three salinities. The carnivorous *A. helena* was not tested in these experiments, as it demonstrated high mortalities in higher salinities and was thus deemed low risk under these conditions (see the results section below). One set of trials assessed consumption of a plant-based food resource and the other of an animal-based resource. The experiments were performed in open and aerated plastic bottles (550 ml) in the same climate chamber in which the survival experiments were conducted. All snails had been previously used for the survival experiment and therefore had acclimated to the salinities for at least two months. For *M. tuberculata*, 0.2, 8, and 16 g/kg were chosen as experimental salinities, while for

T. granifera, due to its high mortality at 16 g/kg, the salinities 0.2, 8, and 12 g/kg were chosen. Note that we were primarily interested in how the feeding rates of the two species compared at 0.2 and 8 g/kg, with the 12 and 16 g/kg conditions tested for intraspecific comparisons at their highest respective "survivable" salinity. Five different resource densities were offered to the tested individuals. Each experimental salinity and resource density was replicated five times, resulting in 75 trials for both species under both resource types.

The snails were fed *ad libitum* for at least two weeks and starved for four days before the experiments commenced to standardise hunger levels. The trials ran for five days (120 hours) for both species towards both resources. Spinach was provided as a plant-based food source, and offered in discs, prepared with a hole punch to ensure uniformity (average area 0.210 cm^2) (Fig. 3). To ensure that the different experimental salinities did not affect the leaf disc area, controls were run in which spinach discs were kept in the salinities for five days without a consumer. The numbers of spinach discs offered to the gastropods were 0.5, 1, 2, 4, and 8. Spinach was always added to the containers first, followed by one snail each. To determine consumption, all spinach discs were photographed and the area consumed was determined by subtracting the remaining area (measured using ImageJ: Abràmoff et al. 2004) from the average unconsumed leaf disc area (see Suppl. material 2: fig. S3 for image of consumed and unconsumed leaf discs). For the animal resource, frozen *Daphnia* sp. (Vivantis Aqua, Germany) was chosen and offered at densities of 1, 4, 8, 16, and 24 per container. Except for the food source and density, the trial was done identically to the plant-based approach. After the trial, snails were removed and the remaining *Daphnia* sp. counted. The number of *Daphnia* sp. consumed was measured by subtracting the remaining numbers from the initially supplied densities.

Statistical analysis and visualization

Sigmoidal mortality curves were constructed for each species for each salinity treatment, described by the following equations (Briski et al. 2008, 2011; Paiva et al. 2018):

$$y = 100 / [1+e^{-Z(s-Q)}] \tag{1}$$

$$y = 100 / [1+e^{-Z(t-Q)}] \tag{2}$$

where y is the proportional mortality, Z is the mortality rate and Q is the onset of mortality. In Eqn. 1, s represents salinity (used for Fig. 1) and in Eqn. 2, t represents time in days (used for Fig. 3). All curves were constructed using S-Plus 6.1 (S-Plus ® 6.1, 2002; Insightful Corp., Seattle, WA, USA). Raw data are available as Suppl. materials 1, 2.

All further statistical analyses were performed with R v4.0.3. A Cox proportional hazards model was fitted to analyse the survival data by determining the hazard ratio. This ratio is commonly used for survival analysis and compares mortality rates under different conditions (in this case, our salinity levels). Hazard ratios of one indicate no effect of the variable on mortality rate, with those less than one indicative of reduced mortality rate and those greater than one indicating increased mortality rate. The analysis was conducted using the 'survival' package (Therneau 2023).

To model the proportional consumption (species, salinity and resource density used as independent variables in the full model) at experimental salinities of 0.2 and 8 g/kg – i.e. the two common salinities for *M. tuberculata* and *T. granifera* – the package 'glmmTMB' (Brooks et al. 2017) for fitting generalized linear models (ordered beta family for data with zeros and ones) was applied, with non-significant terms removed step-wise. To determine the shapes of the feeding curves (i.e. whether a decelerating hyperbola Type II-esque curve, or a sigmoidal Type III-esque curve: see Dick et al. 2014), polynomial logistic regression was used by analysing proportional resource consumption across densities for each species, salinity, and resource type (Pritchard et al. 2017). While a positive first-order term indicates a Type III functional response, a negative first-order term characterises Type II functional response. Seven different self-starter models were then fitted using the 'devtools' package (Wickham et al. 2002), with asymptotic regression, negative exponential, power curve (Freundlich function; fixed so that $0 < b < 1$), logarithmic and Michaelis-Menten used as Type II curves, and 3-parameter Gompertz and logistic sigmoidal used as Type III curves. Akaike Information Criterion (AIC) values were used to determine the best fit.

The potential ecological impact of a NNS under context-dependencies can be quantified using the Impact Potential (IP) metric (Dick et al. 2017; Dickey et al. 2020), typically calculated as the product of consumer maximum feeding rate (FR) - typically the asymptote of the functional response curve or the feeding rate at a fixed, abundant resource density (Dickey et al. 2020) - and some measure of numerical response (NR) to determine population impact.

$$\mathrm{IP} = \mathrm{FR} \times \mathrm{NR} \tag{3}$$

Here, for each experimental salinity, we used "proportional consumption at maximum resource density" (i.e. consumption per the given area of 8 spinach discs, or per 24 *Daphnia* sp.: FR) derived from our proportional food consumption experiments for FR, and "proportional survival" at the corresponding salinity, employing the results from the preceding survival experiments for NR.

Results

Survival experiments

The survival experiment indicated clear differences in salinity tolerance among the three species (Tables 1–3, Figs 1–3). Mortality rose rapidly in *A. helena* with increasing salinity, whereas less immediate and severe effects were found in *M. tuberculata* and *T. granifera* (Fig. 1). The multivariate Cox proportional hazards model showed a significant effect of salinity ($p < 0.05$; HR = 3.37) and species ($p < 0.05$) and on mortality rate, with *M. tuberculata* and *T. granifera* demonstrating reduced mortality rates relative to *A. helena* (HRs of 0.0047 and 0.0073 respectively, relative to *A. helena*) (Fig. 2).

The highest survival rate was observed for *M. tuberculata*, with four deceased snails out of 240 overall (Figs 1–3). These deaths occurred at various salinity levels of 0.6, 2, 8 and 12 g/kg. No deaths were recorded at the highest salinity (16 g/kg) within the experimental 30-day or the 14-day post-trial observation period (Table 1). *Tarebia granifera* experienced slightly higher mortality than

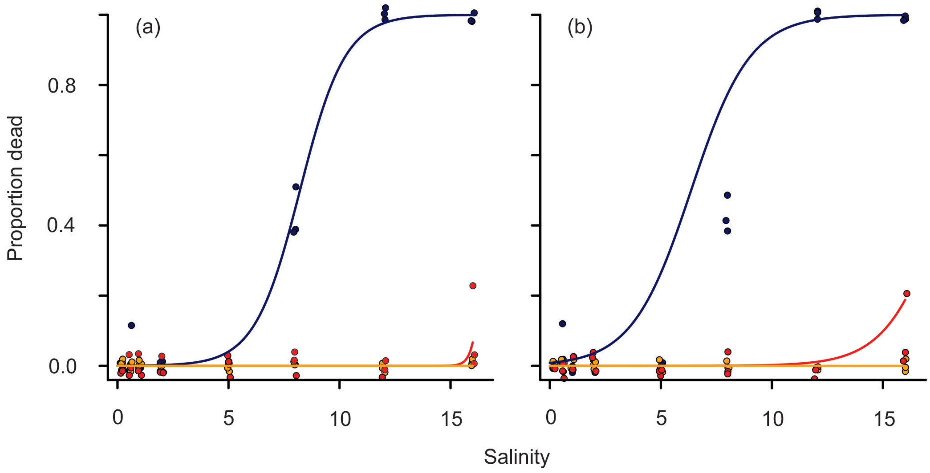

Figure 1. Survival curves in regard to salinity (g/kg) for the three species after (**a**) 15 and 30 days (**b**). *Melanoides tuberculata*, *Tarebia granifera* and *Anentome helena*, are displayed in yellow, red and navy respectively.

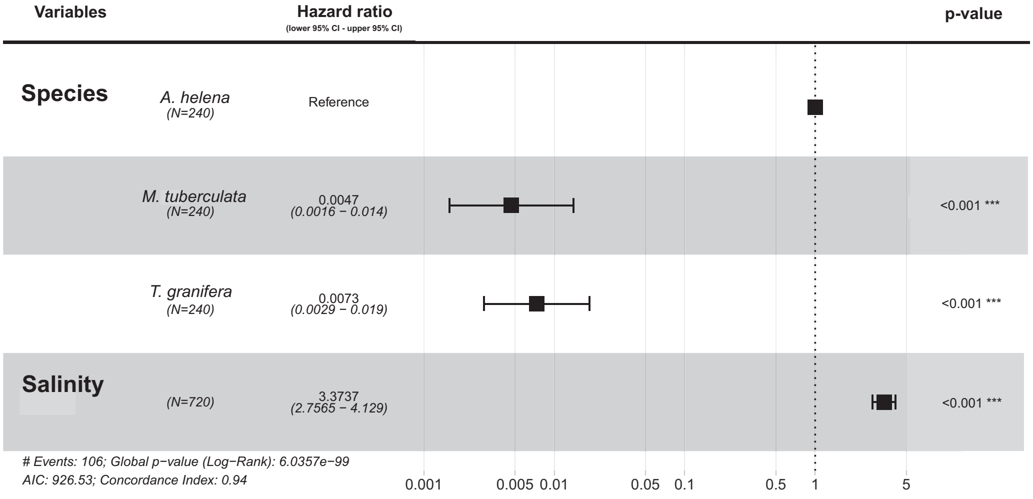

Figure 2. Forest plot based on the Cox proportional hazards regression model, with species and salinity as covariates. Hazard ratios, the ratios of the mortality rates under our experimental salinities, are shown by black squares with 95% confidence intervals by solid horizontal lines (note also stated numerically in "Hazard ratio" column). Hazard ratios, HRs, greater than one (i.e. to the right of the dashed line) indicate that the covariate is associated with increased risks of mortality, with those less than one (i.e. to the left of the dashed line) associated with decreased risks of mortality. We see significant effects of species and salinity on survival, and using the freshwater *Anentome helena* as our reference, we see that *M. tuberculata* and *Tarebia granifera* have reduced risks of mortality relative to the reference species, with HRs of 0.0047, and 00073, or 99.53% and 99.27% less, respectively. The concordance index of 0.94 suggested good predicative accuracy of the model on survival outcomes. Survival is based on results at 30 days under experimental salinity conditions.

M. tuberculata, but only within the 16 g/kg treatment. There were seven deceased snails recorded at this experimental salinity, with additional deaths recorded during the 14-day post-trial observation period, resulting in twelve snails out of the initial 30 from the 16 g/kg treatment (Table 1). For *A. helena*, all snails in the 12 and 16 g/kg treatments died within the first 24 hours of the experiment, whereas three snails survived the 30-day trial period at 8 g/kg. However, all of these snails in the 8 g/kg treatment ultimately died within the 14-day post-experiment observation period. For this species, only the 0.2 g/kg treatment had 100% survival during the initial 30-day experiment, with three additional snails dying in freshwater during the 14-day post-experiment observation period (Table 1).

Table 1. Mortality recorded for a) *Melanoides tuberculata*, b) *Tarebia granifera* and c) *Anentome helena* during the initial 30-day experimental period and then the 14-day observation period after survival trials across salinities.

Species	Salinity	Day 30		Day 44	
		Mean survival	**Standard deviation**	**Mean survival**	**Standard deviation**
a) *M. tuberculata*	0.2ppt	10	0	10	0
	0.6ppt	9.667	0.577	9.667	0.577
	1ppt	10	0	10	0
	2ppt	9.667	0.577	9.667	0.577
	5ppt	10	0	10	0
	8ppt	9.667	0.577	9.667	0.577
	12ppt	9.667	0.577	9.667	0.577
	16ppt	10	0	10	0
b) *T. granifera*	0.2ppt	10	0	10	0
	0.6ppt	10	0	10	0
	1ppt	10	0	10	0
	2ppt	10	0	10	0
	5ppt	10	0	10	0
	8ppt	10	0	10	0
	12ppt	10	0	10	0
	16ppt	8.667	2.309	4	5.196
c) *A. helena*	0.2ppt	10	0	9	1
	0.6ppt	8.667	1.155	8.667	1.155
	1ppt	9.667	0.577	9	1.732
	2ppt	9.667	0.577	9.333	0.577
	5ppt	9.667	0.577	9.667	0.577
	8ppt	1.333	2.309	0.667	1.154
	12ppt	0	0	0	0
	16ppt	0	0	0	0

Proportional consumption experiments

In the control trials, the surface area of the plant-based food source was unaffected and therefore all consumption was solely attributed to snail consumption. For the two common salinities assessed, proportional consumption was significantly affected by salinity, species and resource density, however, no significant interactions were found (Suppl. material 2: table S1). Consumption was significantly higher for *M. tuberculata* than for *T. granifera* ($z = 4.065$, $p < 0.001$), with significantly reduced consumption at 8 versus 0.2 g/kg ($z = 7.841$, $p < 0.001$). A significant negative effect of density on proportional consumption was also found ($z = 2.557$, $p = 0.01$).

For *M. tuberculata*, those in the 8 g/kg treatment exhibited the lowest consumption, consuming in total 2.27 cm^2 out of the 16.31 cm^2 offered across each treatment (Suppl. materials 1, 2). This was followed by the 16 g/kg treatment, where snails consumed 7.90 cm^2, and the 0.2 g/kg treatment, where they consumed 9.66 cm^2. Negative first order terms were found at all three experimental salinities for *M. tuberculata*, with a negative exponential model having the best fit in the 0.2 g/kg treatment, and power curves having the best fit for the 8 and 16 g/kg treatments based on lowest AIC values (Fig. 4a, Suppl. materials 1, 2).

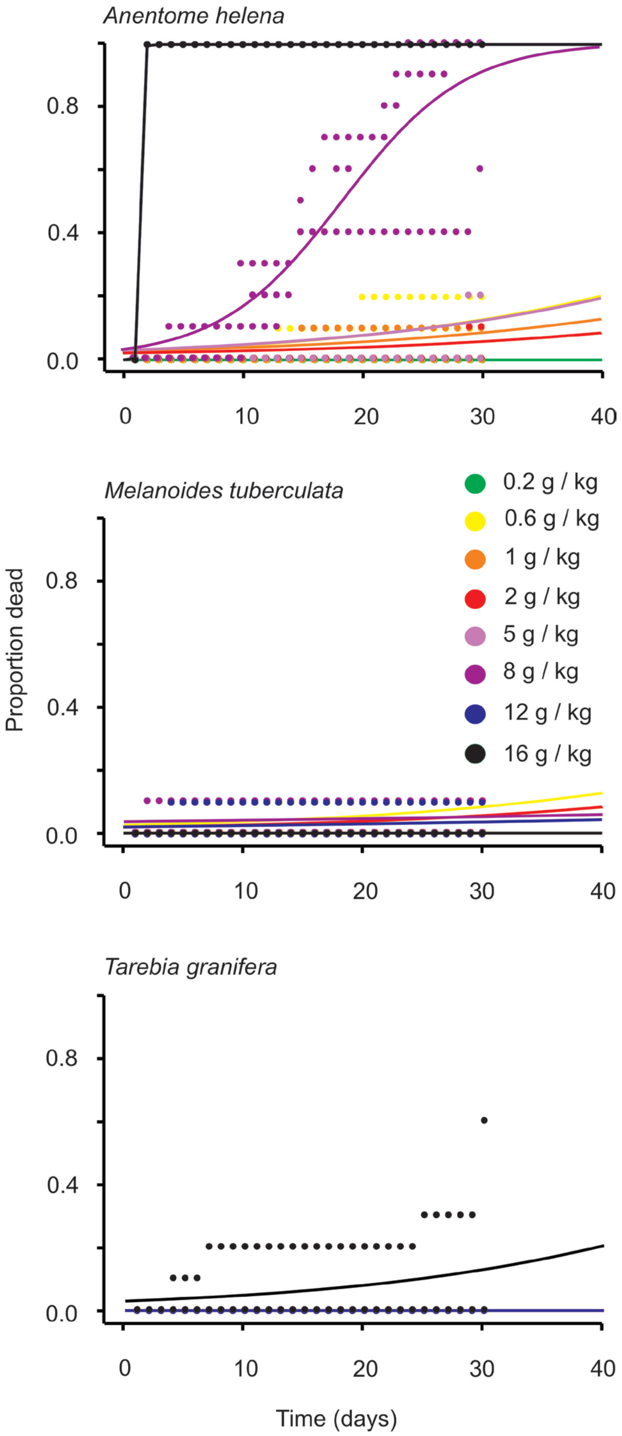

Figure 3. Survival curves for *Melanoides tuberculata*, *Tarebia granifera* and *Anentome helena* with proportional deaths for all tested salinities.

For *T. granifera*, the 12 g/kg treatment snails exhibited the lowest consumption with 0.47 cm^2 out of the 16.31 cm^2 offered (Suppl. materials 1, 2). This was followed by the 8 and 0.2 g/kg treatments, where snails consumed 1.35 cm^2 and 4.96 cm^2, respectively. Negative first order terms were found for all proportional consumption trials with spinach. The model with the best curve fit for *T. granifera* spinach consumption at 0.2 g/kg was the negative exponential, at 8 g/kg the logarithmic model and at 12 g/kg the power curve (Fig. 4b, Suppl. materials 1, 2).

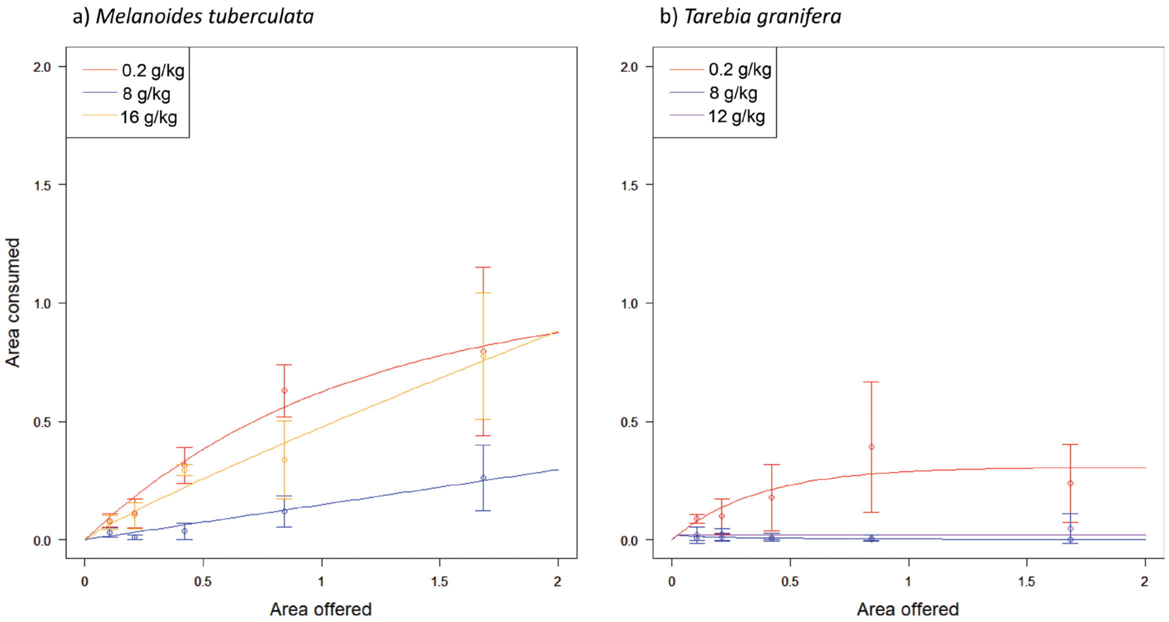

Figure 4. Plant-based proportional consumption curves for *Melanoides tuberculata* and *Tarebia granifera*. Consumption rates were measured for the salinities 0.2, 8 and 16 g/kg for *M. tuberculata* and 0.2, 8 and 12 g/kg for *T. granifera*.

For the *Daphnia* sp. trials, after stepwise removal of non-significant terms, there was only a significant effect of species on the proportional consumption, with *M. tuberculata* consuming more than *T. granifera* (z = 5.368, p < 0.001; Suppl. material 2: table S1). *Melanoides tuberculata* exhibited the highest *Daphnia* sp. consumption at 8 g/kg, consuming 225 out of 275 *Daphnia* sp. offered (Suppl. materials 1, 2). This was followed by the 0.2 g/kg treatment, with 187 out of 275 *Daphnia* sp. consumed, and the 16 g/kg treatment, with 152 out of 275 consumed (Suppl. materials 1, 2). There was a negative first order term for *M. tuberculata* feeding on *Daphnia* sp. at 0.2 g/kg, while 8 and 16 g/kg treatments displayed positive first order terms. The Michaelis-Menten model best fit the data for 0.2 g/kg treatment and the 3-Parameter Gompertz model for both 8 and for 16 g/kg (Fig. 5a, Suppl. materials 1, 2).

For *T. granifera* feeding on *Daphnia* sp., consumption across all tested salinities displayed negative first order terms. At 0.2 and 8 g/kg power curves had the best fit, with negative exponential model at 12 g/kg treatment (Fig. 5b, Suppl. materials 1, 2). *Tarebia granifera* consumed 97 out of 275 *Daphnia* sp. at 0.2 g/kg, 68 out of 275 at 8 g/kg and 135 out of 275 at 12 g/kg (Suppl. materials 1, 2).

Relative impact potential

At the two compared salinities, (i.e. 0.2 and 8 g/kg) the impact potential for spinach consumption of *M. tuberculata* was higher than for *T. granifera*. Within species, spinach consumption of *M. tuberculata* at 0.2 g/kg showed the highest impact potential, closely followed by 16 g/kg, with the lowest impact score at 8 g/kg (Table 2). For *T. granifera* spinach consumption, 0.2 g/kg also had the highest impact potential, with reduced impacts at 8 and 12 g/kg (Table 2).

In the case of *Daphnia* feeding trials, *M. tuberculata* also exerted higher impact potential than *T. granifera* at matched salinities. *Melanoides tuberculata* had the highest impact potential at 8 g/kg, followed by 16 and 0.2 g/kg (Table 3). *Tarebia granifera* showed the highest impact potential at 12, followed by 0.2 and 8 g/kg (Table 3).

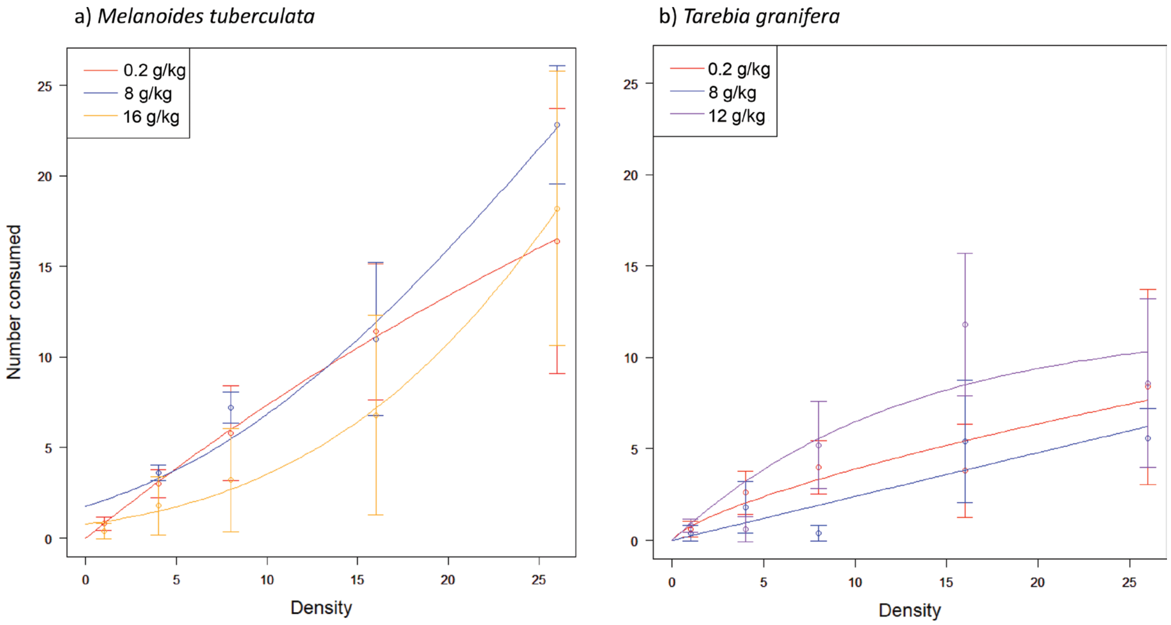

Figure 5. Animal-based proportional consumption curves for *Melanoides tuberculata* and *Tarebia granifera*. The consumption of *Daphnia* sp. was measured for the salinities 0.2, 8 and 16 g/kg for *M. tuberculata* and 0.2, 8 and 12 g/kg for *T. granifera*.

Table 2. Impact potential calculations for *Melanoides tuberculata* and *Tarebia granifera* based on survival and consumption of a plant-based resource.

Species	Salinity	Survival (%)	Survival standard deviation	Survival 95% confidence intervals	Spinach consumption (%)	Spinach consumption standard deviation	Spinach 95% confidence intervals	Impact Potential (% consumption * % survival)
M. tuberculata	0.2	1	0	0	0.472	0.271	0.237	**0.472**
T. granifera	0.2	1	0	0	0.141	0.125	0.109	0.141
M. tuberculata	8	0.967	0.058	0.065	0.155	0.105	0.092	**0.150**
T. granifera	8	1	0	0	0	0	0	0
M. tuberculata	16	1	0	0	0.460	0.203	0.178	0.460
T. granifera	12	1	0	0	0.028	0.047	0.041	0.028

Table 3. Impact potential calculations for *Melanoides tuberculata* and *Tarebia granifera* based on survival and consumption of an animal-based resource.

Species	Salinity	Survival (%)	Survival standard deviation	Survival 95% confidence intervals	Daphnia consumption (%)	Daphnia consumption standard deviation	Daphnia consumption 95% confidence intervals	Impact Potential (% consumption * % survival)
M. tuberculata	0.2	1	0	0	0.631	0.358	0.314	**0.631**
T. granifera	0.2	1	0	0	0.323	0.262	0.229	0.323
M. tuberculata	8	0.967	0.058	0.065	0.877	0.160	0.140	**0.848**
T. granifera	8	1	0	0	0.215	0.080	0.070	0.215
M. tuberculata	16	1	0	0	0.700	0.371	0.325	0.700
T. granifera	12	1	0	0	0.331	0.225	0.197	0.331

Discussion

There is a pressing urgency for invasion scientists to develop methods of effectively predicting, and in turn, proactively preventing damaging NNS introductions into novel ecosystems. Here, focusing on three readily available gastropod species within the pet trade, each with invasion histories to date, we employed methods determining survival and feeding rates under sudden exposure to ecologically-relevant experimental salinities. We found clear differences in survival rates across our experimental salinities, with *M. tuberculata* exhibiting close to 100% survival, *T. granifera* showing mortality at 16 g/kg and *A. helena* experiencing 100% mortality at salinities above 12 g/kg. Assessing *per capita* consumption towards plant and animal-based resources, *M. tuberculata* demonstrated higher feeding rates than *T. granifera* for the common experimental salinities of 0.2 and 8 g/kg. This ultimately gave *M. tuberculata* higher impact potential values, and suggests that this species warrants prioritization based on our experimental conditions.

Survival experiments

The survival experiments demonstrated distinct salinity tolerance differences between the three study species. While *M. tuberculata* and *T. granifera* have had documented occurrences in estuarine habitats (Miranda et al. 2011; Farani et al. 2015), we sought to determine the ability of individuals sourced from the pet trade to withstand sudden introduction to different salinities, mimicking release events. *Melanoides tuberculata* proved to be the most robust across our experimental salinity spectrum, whereas *T. granifera* survived low and intermediate experimental salinities, but exhibited higher mortality than *M. tuberculata*, notably in the 16 g/kg salinity treatment. Indeed, although two *T. granifera* tanks held at 16 g/kg and comprising 20 individuals survived the initial 30-day trial period, almost all of them ultimately died within 14 days subsequently. This result thus cautions inferences from relatively short-term survival trials in experiments, as results can rapidly change with additional time points. These findings are consistent with shorter term studies featuring wild-caught individuals, with Farani et al. (2015) finding high adult and juvenile *M. tuberculata* survival after 96 hours in 9 and 18 g/kg salinity, and higher adult survival rates at these salinities (94% and 92%, respectively) than in freshwater conditions (68%). Da Silva and Barros (2015) also found survival above 90% after 48 hours in salinities up to 25. Further, Miranda et al. (2010) found *T. granifera* capable of surviving and reproducing in salinities up to 20, and even surviving levels from 30 to 40 for just under a month. Both of these species possess a number of adaptations to survive brief periods of saline stress, such as reducing activity, retreating into their shells and closing their operculum, and through brood pouches which provide protection to developing young prior to birth (Ben-Ami and Hodgson 2005; Miranda et al. 2010). While it is important to acknowledge that tolerances to abiotic stressors can change over time (Spence and Tingley 2020), and that gradual acclimation could enhance survivability, there is a clear need to account for "prior adaptation" (Hufbauer et al. 2012) when assessing the abilities of traded species to survive and exert impacts in novel ecosystems, especially since the pet trade can artificially select for successful invaders (Briski et al. 2018; Gippet and Bertelsmeier 2021).

While *M. tuberculata* and *T. granifera* demonstrated tolerance of most study salinities, *A. helena* experienced mortality at all experimental salinities above 0.2 g/kg, with 100% mortality within the first 24 hours of the trial in 12 and 16 g/kg tanks, supporting the assertion of the genus *Anentome* being stenohaline (Galindo et al. 2016). With salinity stress shown to affect vital functions such as movement, feeding, respiration, excretion, and growth (Pourmozaffar et al. 2020; Barrios-Figueroa and Urbina 2023), there are various avenues through which our mortality end-point may have occurred. This lack of tolerance to salinity change, as well as predatory performance being reduced at low temperatures (Dickey et al. 2023a) may partly explain the currently limited non-native range of *A. helena*, despite its prevalence in the pet trade. Indeed, with the temperature maintained at a constant level throughout this experiment (approximately 19 °C), representing a relevant summer temperature in temperate regions, it was at the lower end of tolerance for all three study species. Therefore, it may be that the combination of stressors from salinity and temperature affected survival, and under higher temperatures, *A. helena* in particular may have experienced lower mortality (see, for example, Cuthbert et al. 2021b).

Consumption rates

All species have a range of salinities at which energy expenditure is optimized, but at elevated levels, gastropods need to invest more energy in osmoregulation via the ATP-fuelled active pumping of ions from the environment (Barrios-Figueroa and Urbina 2023). This energetically costly process likely influences food choice for species with generalist diets, as well as the rate of intake. Here, the proportional consumption experiments provided insights into the feeding behaviours of *M. tuberculata* and *T. granifera* when exposed to two food resources under three experimental salinities. *Melanoides tuberculata* tended to show higher feeding rates than *T. granifera* for both resources, at the two common experimental salinities, and consumed the most spinach in the 0.2 and 16 g/kg treatments, with lower consumption at the intermediate salinity.

For *Daphnia* sp. consumption, *M. tuberculata* again consumed significantly more than *T. granifera* over the two common experimental salinities of 0.2 and 8 g/kg. *Melanoides tuberculata* showed the greatest consumption at 8 followed by 16 and 0.2 g/kg. Interestingly, *M. tuberculata* displayed feeding curves that resembled Type III forms for *Daphnia* sp. consumption at 8 and 16 g/kg, indicating proportionately lower rates of consumption at low resource densities. With the maximum feeding rate also highest at 8 g/kg, this may indicate that salinity has a greater influence on movement, rather than consumption and digestion for *M. tuberculata.* It is worth noting that the intermediate salinity level of 8 g/kg had the highest *Daphnia* sp. consumption for *M. tuberculata* but also the lowest spinach consumption, with a similar pattern shown for *T. granifera* at 12 g/kg. These findings may suggest preferences for animal-based food resources at these salinities, but this requires further testing. Indeed, *Daphnia* sp. may offer greater energy return for investment under conditions of salinity stress for both species. However, it remains unclear why spinach consumption remained high for *M. tuberculata* at the highest experimental salinity. Future research could specifically study this by presenting both resources simultaneously, such as via invader “prey switching” studies (Cuthbert et al. 2018; Joyce et al. 2019). Further, the provided food resources in this study were chosen to determine the broad effects of salinity on the consumption of plant- and animal-based resources for our study species, with spinach and *Daphnia* sp. known to be readily consumed (based on pet ownership websites). However, we encourage future studies to focus on a particular study system and include site-specific resources that the species could encounter if released. For example, while our experimental salinities included those relevant to the Baltic Sea, food resources from this area could be used, such as *Fucus* sp. as an ecologically relevant, brown algae resource. Nevertheless, this study represents one of the few assessing the role of salinity on pet trade gastropod diets. Salinity and diet effects on fitness have been shown for other taxonomic groups however, and in a study on juvenile Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758), a two-way salinity and diet treatment interaction (plant-based or containing both animal and plant ingredients) was discovered on growth performance, with individuals on a plant-based diet showing reduced final body weight, average daily gain, total length and standard length at higher salinities (Gonçalves et al. 2022). Another study, on killifish (*Fundulus heteroclitus* Linnaeus, 1766), demonstrated a preference for higher salinity after consuming high levels of dietary calcium than when unfed (Bucking et al. 2012).

Impact potential

Combining survival and feeding rates, *M. tuberculata* had a higher impact potential than *T. granifera* for both food resources at the two common salinities of 0.2 and 8 g/kg, while also possessing a broader salinity tolerance. While our survival study was focused on mimicking release events, questions remain surrounding the effects that longer periods of acclimation, and indeed adaptation over multiple generations, might have for both survival and feeding rates under the combined stressors of temperature and salinity. While *M. tuberculata* has demonstrated a broader salinity tolerance, *T. granifera* has been shown to be tolerant of temperatures between 0 and 47.5 °C, which may give it a greater potential for establishment in temperate zones, with a likely optimum for physiological activities at around 30 °C (Miranda et al. 2010). A more constrained temperature range of 16 °C to 37 °C has been shown for *M. tuberculata* (Okumura and Rocha 2020), and accordingly, the presence of *M. tuberculata* has only been recorded in thermally polluted habitats in Western Europe to date, such as in the Gillbach in Germany (Emde et al. 2016) and the Ebro in Spain (Oscoz et al. 2010). However, much like freshwater seepage zones in South Africa for *T. granifera* (Miranda et al. 2010), these habitats may allow adaptation to cooler conditions over time. Like salinity, temperature may also play a role in the dietary choice. For example, in a study on the diets of opaleye fish (*Girella nigricans* Ayres, 1860), more algal material was consumed at higher temperatures (Behrens and Lafferty 2012), however, this pattern was not shown for the freshwater gastropod, *Lymnaea stagnalis* Linnaeus, 1758 (Zhang et al. 2018).

Another interesting avenue for further research centres on the interactions between these two species with native species, and with each other. Both species have been used as effective biocontrol agents against gastropods which are hosts to harmful parasites, driven by their abilities to rapidly colonize waterbodies and reach high abundances (Pointier and Jourdane 2000). With *T. granifera* shown to exude a chemical cue causing negative taxis responses in other gastropod species, including *M. tuberculata* (Raw et al. 2013), questions remain with regards to the role of abiotic stressors on its competitive ability, and whether European native species could be similarly affected. Further, the shells of *T. granifera* have also been shown to be more resistant to shell-crushing predators in South Africa (Miranda et al. 2016), which may also prove advantageous against, for example, crayfish species in Europe (Renai and Gherardi 2004; Mathers et al. 2022). Understanding the roles of such abiotic stressors on reproduction of these species is also vital and can be incorporated into the impact potential metric, as both *M. tuberculata* and *T. granifera* can reproduce by parthenogenesis (Berry and Kadri

1974). During this study, we observed cases of reproduction for both *M. tuberculata* and *T. granifera*, however, this was not quantified formally. Reproduction was noted at the salinities of 0.2, 8 and 16 g/kg for *M. tuberculata* and at 0.2 and 12 g/kg for *T. granifera*. Further studies should also test the effects of these stressors on "dispersal enhancing" behavioural responses (Dickey et al. 2022; McGlade et al. 2022).

Conclusions

The pet trade is a highly dynamic, global industry and every species sold has the potential to be released, or to escape, into the wild. There are still many knowledge gaps concerning species in the trade (Dickey et al. 2023b), especially in terms of their abilities to survive upon release into novel environments and exert impacts following inherent anthropogenic selection. While future studies need to incorporate other abiotic stressors, and also assess how trophically-analogous native species may be affected under similar conditions, we propose that the methods implemented here highlight *M. tuberculata* as a species highly tolerant of salinity, and potentially more capable of establishing and exerting impacts across a spectrum of habitats from freshwater to brackish than other readily available species. Such methods offer potential for prioritising "risky" pet trade species, facilitating intervention to limit their availability, and in turn help to inform the creation of low-risk species lists (Simberloff 2006; Patoka et al. 2018; Dickey et al. 2023b). However, efforts to address the problem of pet abandonment at its source, through the education of potential pet owners, will continue to warrant prioritisation.

Acknowledgements

We would like to thank Gregor Steffen for helping with the experiments. Thanks also to the constructive comments from an anonymous reviewer who undoubtedly helped to improve the manuscript.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

JWED acknowledges financial support from the Alexander von Humboldt Foundation and the Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB), and RNC by the Alexander von Humboldt Foundation as well as the Leverhulme Trust (ECF-2021-0001).

Author contributions

JWED, ER and EB conceived the study. ER and EK conducted the experiments. ER, JWED, RNC and EB contributed to the statistical analysis and prepared the initial manuscript. All authors provided valuable input to the development of the final manuscript and have given approval for publication.

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Data availability

Raw data supporting the findings of this study are available as Supplementary Information. There was no custom code or mathematical algorithm used in the study.

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Supplementary material 1

The results from survival and feeding experiments

Authors: Elisabeth Renk, James W. E. Dickey, Ross N. Cuthbert, Elžbieta Kazanavičiūtė, Elizabeta Briski

Data type: xlsx

Explanation note: **table S1.** Survival of 3 snail species during the 30-day trial period. **table S2.** Spinach consumption *Melanoides tuberculata*. **table S3.** Spinach consumption *Tarebia granifera*. **table S4.** First order terms for both species, spinach consumption. **table S5.** Model fitting for both species, spinach consumption. **table S6.** Daphniid consumption *Melanoides tuberculata*. **table S7.** Daphniid consumption *Tarebia granifera*. **table S8.** First order terms for both species, daphniid consumption. **table S9.** Model fitting for both species, daphniid consumption.

Link: https://doi.org/10.3897/neobiota.94.125227.suppl1

Supplementary material 2

Supplementary information

Authors: Elisabeth Renk, James W. E. Dickey, Ross N. Cuthbert, Elžbieta Kazanavičiūtė, Elizabeta Briski

Data type: pdf

Explanation note: **fig. S1.** Experiment species *Melanoides tuberculata* (a), *Tarebia granifera* (b) and *Anentome helena* (c). Photographed by Gregor Steffen, Geomar Kiel 2023. **fig. S2.** Experimental set up for the survival experiments. **fig. S3.** Examples of uneaten(a) and partially consumed (b) spinach discs.

Link: https://doi.org/10.3897/neobiota.94.125227.suppl2

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 101–125 (2024)
DOI: 10.3897/neobiota.94.122939

Review Article

Strangers in a strange land; freshwater fish introductions, impacts, management and socio-ecological feedbacks in a small island nation – the case of Aotearoa New Zealand

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Abstract

Invasive non-native species (INNS) are key drivers of global biodiversity loss. This is particularly evident in freshwater ecosystems, where the rates of both vertebrate biodiversity loss and biological invasion exceed those of marine and terrestrial systems. Aotearoa New Zealand (henceforth Aotearoa) like many other island nations, has a troubled history with NNS. However, it is also unique, as the main islands were the last major landmasses on Earth to remain uninhabited by humans. The endemic fauna had evolved in isolation from any anthropogenic influence or introduced NNS, until the mid-thirteenth century with the arrival of Māori, the first people to inhabit Aotearoa. Centuries later, following European colonisation, many non-native freshwater fish were deliberately introduced by acclimatisation societies. Currently, most of the native freshwater fish species of Aotearoa are at risk of extinction, despite almost 90% of these being found nowhere else on earth. Many of these species are highly valued by the indigenous people of Aotearoa, who have repeatedly highlighted biases towards NNS in freshwater fish management. With the rate of biological invasions increasing, it is timely to address interconnected issues concerning the history, impacts, management and current / future policy directions, including those involving biosecurity, for non-native freshwater fish in Aotearoa. We do this by applying a social-ecological systems (SES) lens, with a focus on causal-loop relationships and feedbacks to improve understanding of the dynamics of drivers, mechanisms and impacts of such invasions. We highlight the tensions that have resulted from managing some NNS as 'pests' threatening native biodiversity, while simultaneously promoting a tourism and recreational fishery resource for specific NNS. This has generated extremely polarized views on the 'status' of non-native freshwater fish species and given rise to contradictory and divergent goals for their management. We show how a disjointed and often incoherent policy landscape has contributed to legal 'anomalies' for NNS, including policy misalignments and gaps, hampering effective use of resources, while also entrenching contradictory management programmes for different stakeholders. Our study shows how these interconnected issues have been manifested in social-ecological feedback loops on core aspects of NNS policy and management, past and present. Consequently, there is a need for increased comprehension of the diverse array of potential impacts of NNS for different environments, stakeholders and Māori while developing coherent and practical management methods to reduce such impacts and improve social-ecological resilience. We conclude that adopting a SES approach will aid this endeavour.

Key words: Biosecurity, causal-loops, fish introductions, freshwater fish policy, non-native species, social-ecological system

Academic editor: Shana McDermott
Received: 13 March 2024
Accepted: 14 June 2024
Published: 26 July 2024

Citation: MacNeil C, Holmes R, Challies E, McFarlane K, Arnold J (2024) Strangers in a strange land; freshwater fish introductions, impacts, management and socio-ecological feedbacks in a small island nation – the case of Aotearoa New Zealand. NeoBiota 94: 101–125. https://doi.org/10.3897/neobiota.94.122939

Introduction

Invasive non-native species (INNS) are intrinsically linked with many stakeholder actions (governments and public) throughout the world, to affect human well-being via negative or positive social-economic and social-ecological impacts. An INNS is a non-native species (NNS) which has expanded its range beyond its point of introduction and has had a measurable impact on factors such as ecology, ecosystem services, economics and human health (Sinclair et al. 2020). They can be key drivers of social-ecological regime shifts, which are large-scale, potentially irreversible changes in social-ecological systems (SES) (Shackleton et al. 2018) and are either solely or partially responsible for 60% of recorded global animal and plant extinctions (IPBES 2023). Such biodiversity loss is particularly pronounced in freshwater ecosystems, where the rate of decline in vertebrate biodiversity is higher than in either marine or terrestrial systems (Grooten and Almond 2018; Tickner et al. 2020). This is partially a result of freshwater systems experiencing a higher rate of biological invasions than other ecosystems (Gallardo et al. 2016; Tickner et al. 2020; McFadden et al. 2023). Understanding the impacts of INNS on different recipient environments, ecosystem services and stakeholder values, and focussing on those INNS capable of causing social-ecological regime shifts, are increasing priorities for governments and the research community (Shackleton et al. 2018; MPI 2023).

Aotearoa New Zealand (henceforth Aotearoa) like many other island nations, has had a troubled history with NNS, stretching back several centuries (King 2019). Accidental introductions such as the Norwegian rat (*Rattus norvegicus*) and domestic cat (*Felis catus*) have had devastating impacts on naïve native species (King 2019), while in 2023 the freshwater Gold-Asian clam (*Corbicula fluminea*) was first detected, which could completely ‘re-engineer’ the country’s river and lake ecosystems (MPI 2023). Many freshwater fish such as trout and salmon species, were deliberately introduced for aquaculture, sport and tourism (McDowall 1990). Such deliberate fish introductions, like accidental introductions, have had profound impacts on ecology, stakeholders and Māori (McDowall 1990, 2011; Collier and Granger 2015).

The historical context of biological invasions and introductions in Aotearoa is globally unique (Champion 2018). The main islands were the last major landmasses on Earth to remain uninhabited by humans and until the mid-thirteenth century, the endemic fauna had evolved in isolation from any introduced NNS (King 2019). Despite 88% of freshwater fish species in Aotearoa being found nowhere else on earth (Department of Conservation (DOC) 2020), almost the same percentage (76%) are currently either facing extinction or at risk of being threatened with extinction (StatsNZ 2023). The country’s freshwater ecosystems are also increasingly prone to invasion, due to global trade and climate change (Champion 2018). Many of these threatened native fish are also taonga (treasured) species for Māori and central to mahinga kai (the traditional value of food resources and their ecosystems, as well as the practices involved in their production, harvesting and protection) (Harmsworth et al. 2016; Rainforth and Harmsworth 2019).

Although there is a complex interplay among drivers of freshwater fish invasions (Milardi et al. 2022), humans can, to a degree, influence invasion processes and outcomes by planning for and responding to the detection, transport, introduction and spread of NNS (Sinclair et al. 2020; Milardi et al. 2022). It has been shown that a SES approach which focusses on causal-loop relationships and feedbacks can provide a valuable insights on how INNS management strategies work in practice (Shackleton et al. 2018). Feedbacks occur when the initial interaction between two or more elements 'feed-back' to the initiating process with positive (reinforcing) or negative (dampening) effects (see Figs 1, 2 for fish examples and definition of terms; Sinclair et al. 2020).

These feedbacks provide a useful starting point from which to build a SES approach to understand interdependent systems, such as governance institutions and goals for development, biodiversity and ecosystem services (Reyers and Selig 2020). For example, Aotearoa has adopted increasingly stringent biosecurity measures within the country and at the border, including measures applicable to freshwater fish (Champion 2018). When effective, these measures generate negative feedback loops that work to stabilise or reduce invasions through detection, prevention, elimination or management of specific INNS designated as 'unwanted organisms' by the government (Champion 2018; Shackleton et al. 2018; Sinclair et al. 2020).

The identification and strengthening of negative feedback loops associated with biosecurity, is also increasingly important in an era where climate change is accelerating the rate of biological invasion (Tickner et al. 2020). Research shows that the range expansion of many physiochemically tolerant aquatic NNS is favoured by higher temperatures and changing precipitation regimes (Rowe and Wilding 2012; Copp et al. 2021). There is also the potential for non-native 'sleeper' species, whose population size and range in a country such as Aotearoa is currently limited by climate, to become more widespread and problematic as climate change progresses (Hulme 2017). Despite these growing concerns, Aotearoa remains relatively free from freshwater NNS compared to Europe and North America (Champion 2018). This means NNS still remain a significant unrealised threat to freshwater ecosystems in Aotearoa, with a high degree of latent impact that may manifest in the future (Gluckman 2017). Given the increasing threats of invasion and climate change, we feel it is it is very timely to address several interconnected core issues concerning the history, impacts, management and current policy directions for non-native freshwater fish in Aotearoa, and to identify the social-ecological feedback loops underlying these.

Over recent decades, concerns about the ecological impacts of freshwater NNS have existed alongside a drive for economic expansion of aquaculture and sports-fishing / angling tourism, shaping government policies. The tensions that have resulted from managing some NNS as 'pests' threatening native biodiversity, while simultaneously promoting a tourism / fishery resource for other NNS, have unsurprisingly generated highly polarized views on the 'status' of introduced freshwater fish species (Chadderton 2003; Jellyman et al. 2018; Tadaki et al. 2022). This promotion of contradictory and divergent goals for introduced fish species has resulted in fisheries policies, legal status and terminology that function differently for different species or even the same species in different regions of the country (Chadderton 2003). Our study will show how this has been associated with 'branding' of NNS with more loaded terms, including negatively as 'pest', 'noxious' and 'unwanted', or positively as 'sports fish' (Dean 2003).

It is increasingly argued that there is a need to 'strike the right balance' between native and non-native species in fisheries management in Aotearoa (Chadderton 2003).

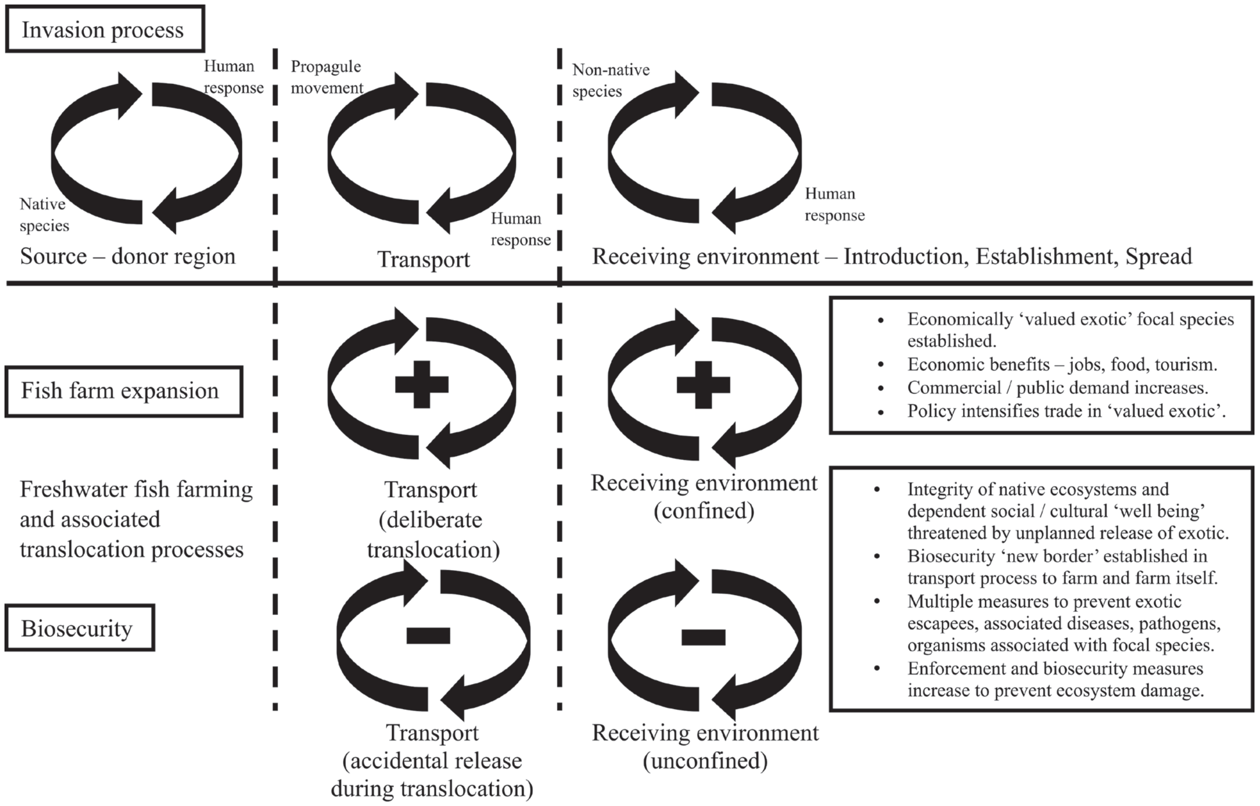


Figure 1. Different stages of biological invasion (adapted from Colautti et al. 2006 and Sinclair et al. 2020), focussed through human-natural systems and positive/negative feedback loops associated with fish farming, translocation and biosecurity. A social-ecological system can be defined as a complex, interconnected network of people and the environment, highlighting the interactions between human society and the ecosystems that support it. In a social-ecological system, both social and ecological factors influence and are influenced by one another, and changes in one element of the system can have cascading effects on other parts of the system (Shackleton et al. 2018). A feedback loop can be defined as a self-regulating system in which the output of the system has an impact on the input of the system, which then modifies the output. Feedback loops can be positive or negative, depending on whether the output of the system reinforces or counteracts the input.

This means managing NNS in a context dependent way, based on the interacting needs of stakeholder groups and Māori at a 'local' or regional scale. This balancing act has not always been achieved and arguably is still not being achieved in many cases. This has contributed to current legal 'anomalies' in the governance of NNS, including policy misalignments, gaps and duplications, which hamper effective management and efficient use of resources. This has also entrenched divergent management programmes and stakeholder and Māori interests. Our study will document these interconnected issues and show how these have been manifested in social-ecological feedback loops, past and present, involving introductions, biosecurity, aquaculture, possession, translocation and fish passage. By clearly identifying both loops and drivers, we aim to show how an SES approach to non-native freshwater fish management can inform more coherent, effective policies in the future, despite the legacies of a confused legislative landscape still manifest in present-day Aotearoa.

A social ecological systems approach to examining NNS management

A social-ecological systems (SES) approach can provide insights into the drivers, mechanisms and impacts of biological invasions (Shackleton et al. 2018). It is crucial to recognise the multiple socioecological feedback loops within the invasion process

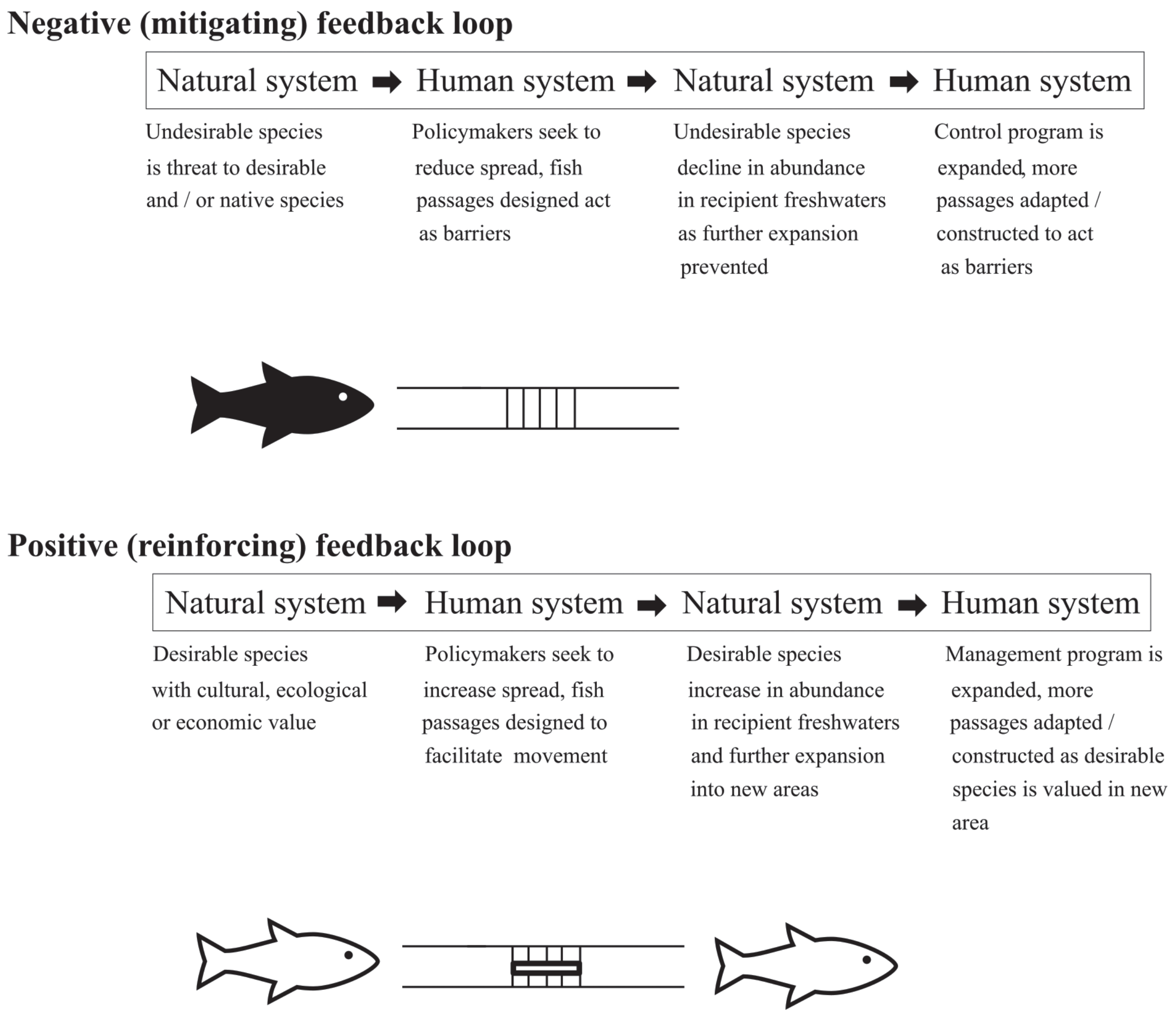


Figure 2. Current Aotearoa policy for in-stream structures such as fish passages to simultaneously act as barriers to 'undesirable' species spread and facilitators of 'desirable' species spread focussed through human-natural systems and simultaneous negative (mitigating) and positive (reinforcing) feedback loops for 'undesirable' and 'desirable' species respectively.

that highlight the role that humans can play, be it by deliberate introductions, management actions and biosecurity measures, or other interventions (Fig. 1). For instance, while the invasion process can be positively driven by economic interests such as non-native fish farming, biosecurity measures work negatively on any undesirable species spread. The source loop encompasses the human response in the NNS source (donor) region. Here, collection of desired freshwater fish is approved of, then the transport loop associates people with the introduction / translocation process where desired species are in established ongoing and future transport mechanisms (i.e. air, ocean and land freight) from the donor to recipient regions (Figs 1, 2). Concurrent with the transport loop, the risks associated with non-focal species which may be inadvertently caught up in the transport mechanism have to be managed and minimised. The recipient loop encompasses the way humans and ecosystems interact with the arrival, establishment and spread of non-native fish, for example via aquaculture). Subsequent sections of this paper apply this SES lens to identify and reflect upon the feedback loops resulting from non-native fish management in Aotearoa.

Realised and potential ecological impacts of non-native freshwater fish species in Aotearoa

To understand why freshwater fish legislation has evolved as it has, it is first important to appreciate the impacts of past and current invasions documented in Aotearoa, and second, to consider the risk profile of future potential invaders, which have had impacts in similar bioclimatic regions, albeit with different faunal assemblages (Kumschick et al. 2015; Torres et al. 2018). From the mid-19th century onwards, a range of freshwater fish were deliberately introduced to the new British colony of New Zealand via Victorian 'acclimatization societies'. These fish were part of a diverse array of what was then presumed 'innoxious' animal and plant introductions, for commercial and recreational benefit (McDowall 1990, 2006). This included species such as the brown trout (*Salmo trutta*), which from the 1860s onwards were repeatedly introduced from Britain for over a century (McDowall 2006; Jones and Closs 2018).

Such fish introductions can drive positive socioeconomic feedback loops that increase donor fish transfer out of source regions over time. This is because the perceived success and value of previous fish introductions in recipient areas increases demand, 'trade volume' and the number of potential species within these trades (Sinclair et al. 2020). Sinclair et al. (2020) has pointed out that the purely ecological 'invasional meltdown' hypothesis, where one INNS and the disturbance it causes in the recipient environment can facilitate further invasions (Simberloff and Von Holle 1999), can be mirrored by a socio-economic invasion meltdown, whereby economic and social benefits drive further introductions (Fig. 3). Such a feedback loop is evident in Aotearoa, whereby the perceived economic success of early brown trout introductions led to further introductions of not only brown trout but other salmonids including the Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*), with the aim of creating similarly successful fisheries (McDowall 1990,1994). Several coarse fish were also introduced to freshwaters deemed unsuitable for salmonids (de Winton et al. 2003) and other species were introduced as ornamental garden pond or aquarium specimens (NIWA 2020).

The active 'acclimatisation' movement continued up until as recently as the late 1960s in Aotearoa (Champion 2018). At the time of the original introductions, little thought was given by acclimatisation societies to the impact on native ecosystems or the people dependent upon them (McDowall 2006). For instance, writing in an 1880 newspaper, an anonymous commentator welcomed Societies' plans to continue repeated stocking of rivers with salmonids, noting that once established, native fish would provide an 'inexhaustible' food supply for the introduced fish (Anonymous 1880).

However, even in Victorian times there was some questioning of the general lack of appreciation of the impacts of introduced species on the receiving environment. The author of a letter to *The Colonist* newspaper in 1873 calling themselves a 'Disbeliever in Too Much Acclimatisation', jokingly suggested that if leopards were introduced, they could be sustained on local school children (Anonymous 1873). Another author, writing from the viewpoint of a native bird, the shag, complained about the replacement of native fish with introduced trout, which were too big for it to eat (Alic 1890). During all of this, the views of Māori, whose culture had been intimately connected with native freshwater fish, were marginalised or absent. It was not until the end of the 1960s that the ecological and economic risks of such introductions were highlighted by the scientific community (Champion 2018). Recognition of

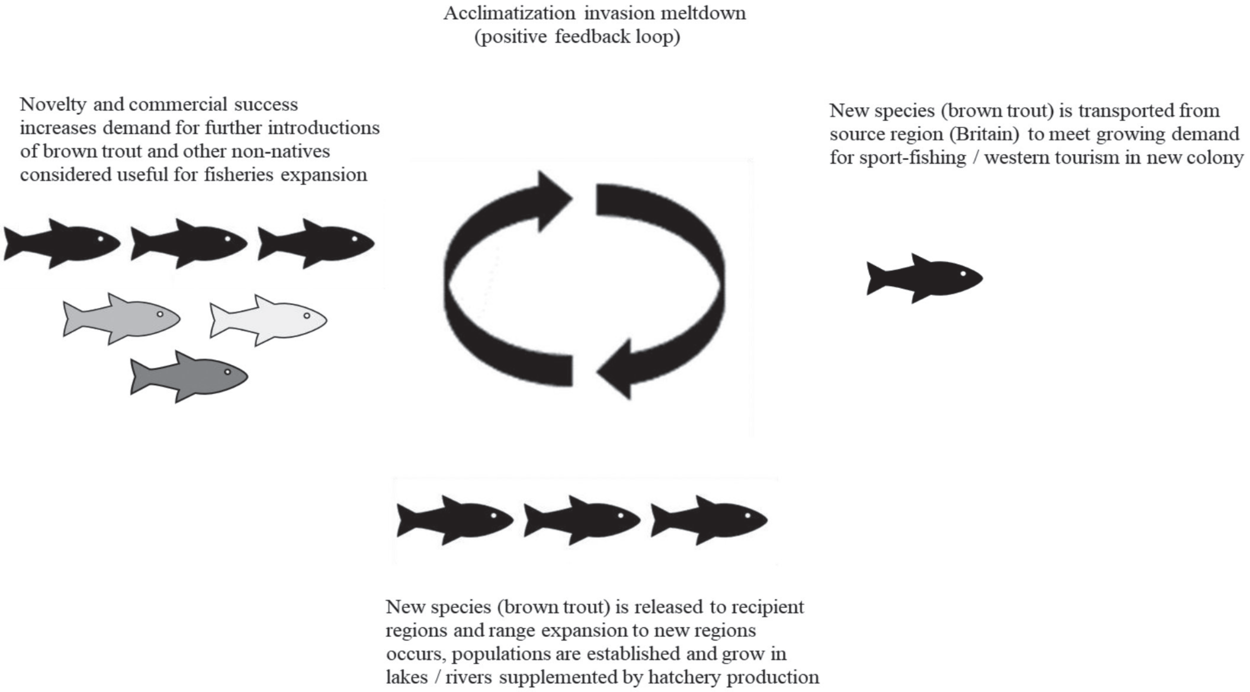


Figure 3. Socio-ecological invasion meltdown (positive, reinforcing feedback loop) for brown trout introductions into the British colony of New Zealand by Victorian acclimatisation societies. Intentional introduction and establishment in recipient regions is done without consideration of any potential negative impacts on native fish species and the cultural / resource values that the indigenous human population attribute to these.

the cultural impacts and the need to involve Māori in decision-making and management of freshwater fish is only now gradually being reflected in policy.

Introduced freshwater fish can have a wide variety of direct and indirect ecological impacts on native fish and ecosystems (for examples of major reviews see Gozlan et al. 2010; Cucherousset and Olden 2011; Bernery et al. 2022; Britton 2022). Direct impacts include competition with native fish for resources, such as food and habitat, predation on native species and alteration of ecosystem dynamics, including the structure and function of food-webs with cascading effects on ecosystem processes (Gozlan et al. 2010; Cucherousset and Olden 2011; Bernery et al. 2022; Britton 2022). Indirect impacts include alteration of physical and chemical water quality through bioturbation, changes in nutrient cycling and the introduction of novel diseases and parasites (Collier and Granger 2015; Bernery et al. 2022; Britton 2022). In the case of Aotearoa, many native freshwater fish species are small, benthic and cryptic and have evolved without large pelagic predatory species, while the majority of freshwater fish introduced for aquaculture, such as salmonids, are much larger (McDowall 2006; Joy and Death 2013). Consequently, introduced fish are at or near the top of food chains in many of the country's rivers and lakes and negatively impact on smaller native fish via predation and / or competition (McDowall 1990, 1995, 2006). Indeed, in the early years of the 'acclimatisation' of brown trout to Aotearoa, Spackman (1892) noted that a diet of small native fish accounted for the trout's high growth rates. Since then, the wide-ranging predatory impacts of brown trout on freshwater fish and food webs in Aotearoa have been well documented (McIntosh and Townsend 1996; McDowall 2006; McIntosh et al. 2010; Jellyman et al. 2018).

Although the brown trout is by far the best studied non-native fish in terms of ecological impacts, it is just one of many freshwater fish introductions that have impacted waterways in Aotearoa. Previous global reviews of the ecological impacts of freshwater non-native fish species have found it helpful to consider impacts at different levels or scales of biological organization, ranging from genetic to individual to population to community and finally ecosystem level (Cucherousset and Olden 2011; Britton 2022; Bernery et al. 2022). In Table 1 we adopt this useful organisational framework to consider ecological impacts of a selection of INNS in the freshwaters of Aotearoa. Our review identified that many of the potential ecological impacts of freshwater INNS already present in Aotearoa are assumed from documented impacts of the same INNS overseas. This is despite the uniqueness of Aotearoa's freshwater ecosystems and fish assemblages. We have therefore attempted to summarise the ongoing ecological impacts of INNS using only Aotearoa-based studies where possible. We would argue this is the optimum approach to ensure future policies and management are based on accurate and robust data from Aotearoa. It must also be appreciated that Table 1 is not exhaustive, in terms of fish species now present in Aotearoa and not all introduced NNS became invasive, for instance Mackinaw trout (*Salvelinus namaycush*) was introduced from North America for sport fishing in the early 1900s but never expanded beyond its introduction lakes or significantly increased in abundance over time (McDowall 1990).

It must also be appreciated that any consideration of ecological impacts needs to acknowledge the complicated and multifaceted mechanisms at play across different spatial and temporal scales. Thus, the various impacts on native species and ecosystems described in Table 1, such as food-web modification and alteration of physical habitat, seldom happen in isolation from one another and can be both additive and synergistic (Rowe 2007; Kumschick et al. 2015). In addition, the impacts of a single introduced fish species can span multiple scales of organization, ranging from impacts on individual animal behaviour, to population dynamics, to the structure and function of communities and ecosystems (Townsend 2003, Table 1). This can be readily witnessed for introduced cyprinids (carp) and Ictaluridae (catfish), which are archetypal 'ecosystem engineers', radically transforming their invaded physical environment with ramifications for multiple trophic levels (Field-Dodgson 1987; Jellyman et al. 2018; Britton 2022, Table 1). For instance, they disturb sediments, increasing turbidity and reducing light availability to aquatic plants that stabilise lakebeds (de Winton et al. 2003). At sufficiently high densities they can even actively uproot plants further destabilising lakebeds and increasing turbidity due to wave action on newly exposed sediments, which can in turn further increase nutrient availability (de Winton et al. 2003).

Nine non-native freshwater fish species have been identified as the most serious 'pests' for natural heritage managers in Aotearoa in terms of their ecological impact and spread (NIWA 2020). These are the brown bullhead catfish, goldfish, koi carp, mosquito fish, gudgeon, orfe, perch, rudd, and tench (NIWA 2020). This list purposely does not include the commercially important salmonids, which government agencies do not label as pests (see 'gaps and labels' section), despite their well-documented negative impacts on ecosystems (Table 1). All nine NNS seldom occur in isolation from one another (Collier et al. 2015), which in many invasion scenarios further complicates attempts to ascribe impacts to any one NNS in particular. For instance, an investigation on the impact of fish introductions on water clarity in 49 lakes in Aotearoa found 83% contained two or more NNS, with some containing as many as six NNS, including rudd, tench, perch, catfish, koi and goldfish (Rowe 2007). This meant the specific role of each individual NNS in causing identified ecological impacts could not be distinguished (Rowe 2007).

Table 1. Examples of ecological impacts of non-native freshwater fish species in Aotearoa. (Note only Aotearoa based studies are included and potential effects ascribed to overseas studies are purposely excluded).

Scale of biological organization	Impact and examples of mechanisms	Description
Genetic	Altering genetic resources via hybridization.	Tench may have the potential to hybridize with other introduced cyprinid fish such as goldfish, rudd and orfe (Rowe 2004). This is relevant if this increases the resilience, physiological tolerance and spread of hybrids.
Individual	Animal health and growth via altered behaviour, disease and parasite transmission.	Diel rhythm of habitat (water column and stream substratum) use by mayfly nymphs was affected by presence of brown trout, which exerted a different selection pressure on invertebrate drift behaviour than native galaxiids (McIntosh and Townsend 1996; Townsend 2003). Tench have a potentially high infestation rate with the gut parasite *Ligula intestinalis*, which is already present in North Island lakes. Tench populations could act as a reservoir for this parasite which also infects native fish such as common bullies (Rowe 2004). Interactions between mosquitofish and native mudfish (Galaxiidae) can negatively impact mudfish foraging behaviour and prey capture rates (Barrier and Hicks 1994)
Population	Population size decline via predation, competition, disease and parasite transmission.	Brown trout have replaced nonmigratory galaxiid fish in some streams and diminished population sizes in others and have altered the distribution / range of large invertebrates such as crayfish (Townsend 2003; Townsend and Simon 2006; McIntosh et al. 2010; Jellyman et al. 2018). Predation of native mudfish (*Neochanna* Galaxiidae) has potential to eliminate populations of this threatened 'nationally critical' species (Eldon 1979). Brown trout and rainbow trout feed on a range of macroinvertebrate species in a South Island lake (McCarter 1986). Predation by rainbow trout has caused significant declines in koaro (a native galaxiid fish) in North Island lakes (McDowall 1990). Small brown bullhead catfish feed on chironomids, Cladocera, gastropods, caddisfly larvae, plant material and detritus. and large catfish prey on native crayfish, fish and terrestrial invertebrates in Lake Taupo (Barnes and Hicks 2003). Catfish can also feed on other NNS including goldfish and brown trout (Dedual 2019). Perch include smelt, common bullies and macroinvertebrates such as mysids and damselflies in their diets in a South Island river (Griffiths 1976), while Ludgate and Closs (2003) found perch suppressed populations of common bullies in experimental ponds through a combination of predation and competition. Mosquitofish predate fry of native black mudfish and may exclude them from some habitats (Ling and Willis 2005). In lake field trials and tank experiments rudd selectively eat different macrophytes, potentially influencing composition of macrophyte communities and this selective feeding may prevent re-establishment of these species in restoration programmes (Lake et al. 2002). An intensive removal of koi carp from a North Island lake led to significant reductions in the koi carp population, which coincided with an increase in native eel abundance, suggesting dietary overlap and competition for food between carp and eels may have been suppressing eel population size (Tempero et al. 2019). Tench populations could act as a reservoir for a gut parasite and widespread transmission could reduce populations of native fish hosts such as common bullies (Rowe 2004).
Community	Species extinction and reduction in native biodiversity	Presence of brown trout is a driver for elimination of non-migratory galaxiids from some streams (Townsend 2003; Townsend and Simon 2006; McIntosh et al. 2010; Jellyman et al. 2018).
	Changes in composition of native species assemblages.	Brown trout have suppressed grazing pressure from macroinvertebrates on algae biomass and thus can enhance algal biomass and alter algal species composition and potentially macroinvertebrate community (Townsend 2003; Jellyman et al. 2018).
	Alteration / modification of food webs.	Annual production of macroinvertebrates is consumed by brown trout and not galaxiids where the native fish have been replaced and algal primary productivity can be six times higher in a 'brown trout stream' than a 'galaxiid stream' (Townsend 2003; Jellyman et al. 2018). Mosquito fish (*Gambusia affinis*) can induce changes in zooplankton community which diminish diets of native mudfish (Barrier and Hicks 1994).

Scale of biological organization	Impact and examples of mechanisms	Description
Ecosystem	Modification of nutrient cycles.	Greater algal primary productivity in streams where brown trout have replaced galaxiids leads to an increased nutrient flux from the water to benthic community (Townsend 2003; Jellyman et al. 2018). A range of NNS (rudd, tench, perch, brown bullhead catfish, goldfish, and koi carp) alters nutrient levels, affecting lake trophic processes, with excretion and bioturbation increasing nutrient levels (Hanchet 1990; Rowe 2007).
	Loss / modification of habitat, native species refuges.	Salmon redd construction by Chinook salmon in two salmon spawning streams decreased the abundance of mosses, algae, macrophytes and sediment and detritus, causing a geomorphic modification of pool-riffle sequences and this was associated with a decrease in the abundance of benthic macroinvertebrates (Field-Dodgson 1987).

Collier et al. (2015) summarised the main impacts of such multiple species assemblages acting in unison in Aotearoa lakes. These are: feeding, which influences bioturbation and water clarity; excretion, which influences nutrient levels; and predation or grazing which impact native food-webs, biodiversity and habitats. At a global scale, multiple NNS presence and their impacts are also a world-wide driver of increasing biotic homogenization of species, with more countries having an increasing number of species in common (Rahel 2000; Dudgeon et al. 2006; Joy and Death 2013; Bernery et al. 2022).

Table 1 purposely does not cover economic or cultural / social impacts of NNS introductions but as these are invariably interrelated with ecological impacts, they should also be acknowledged. Economic impacts can include negative impacts on local commercial and native fisheries (through competition / predation with focal species) and the local economy (i.e. adverse effects on tourism). Conversely, positive impacts can include new export opportunities, new fisheries / tourist opportunities (for a valued sports fish), increased employment and food resources (Dedual 2019). Economic damage and management costs of all biological invasions in Aotearoa have been estimated as US$120 million per year, and freshwater NNS would represent a significant proportion of this (Bodey et al. 2022). The cultural and social impacts of NNS tend to be largely negative for Indigenous people both globally and in Aotearoa (Rypel et al. 2021). Impacts include decline of native fish species relied on for food, with an associated loss of cultural practices and knowledge surrounding their use, and secondly the decline of aesthetics and 'contamination' by NNS of culturally important lake and river habitats (Dedual 2019). Approximately 83% of freshwater and marine taonga (treasured by Māori) native fish are classified as at risk of extinction (StatsNZ 2023) and there remains a lack of recognition of the cultural significance of native fish for Māori in policy and management. This 'blind-spot' is not unique to Aotearoa and arguably is still evident in the majority of European and North American fisheries management contexts (Rypel et al. 2021). Given that Indigenous communities have inter-generational experience in confronting NNS introductions, their knowledge and expertise would likely enhance NNS policy and management significantly (Harmsworth et al. 2016; Wheeler and Root-Bernstein 2020).

Dealing with risk – biosecurity policy and public messaging

At its simplest level, biosecurity consists of a number of feedback loops (positive and negative) aiming to influence different aspects of the biological invasion process (Fig. 1). Some governments and regions define biosecurity in purely scientific terms, such as in European Union (EU) guidelines (zu Ermgassen et al. 2020), whereas risks to cultural and social values are at the forefront of biosecurity policy in Aotearoa (MPI 2016a).

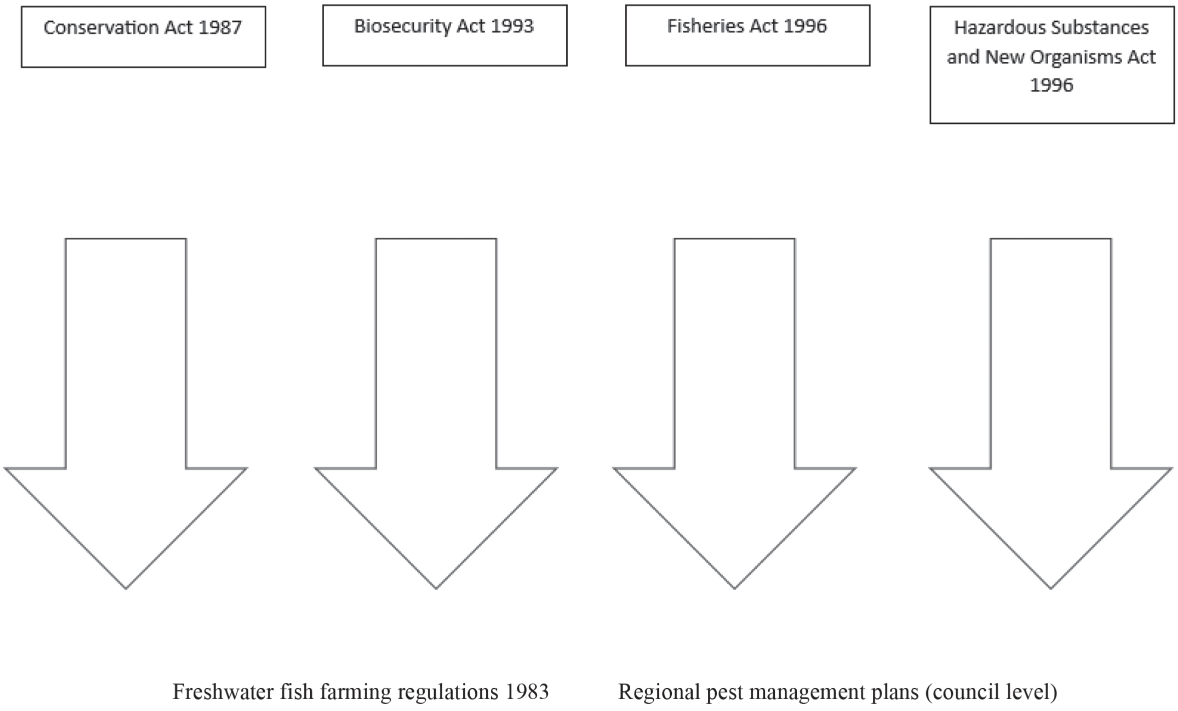
If we focus on fisheries management in countries such as Aotearoa, government resources and legislation are invariably applied to a limited suite of species deemed 'important', whether these are 'desirable' natives or non-natives which policies favour, or 'undesirable' non-natives which policies work against (see Davis et al. 2011). A complex and arguably fragmented governance and legal framework for biosecurity is currently driving very different socio-ecological feedbacks for 'important' freshwater fish species in Aotearoa.

Biosecurity, specifically as it relates to non-native freshwater fish in Aotearoa, is regulated in the context of four principal pieces of legislation: the Conservation Act 1987, the Biosecurity Act 1993, the Fisheries Act 1996 and the Hazardous Substances and New Organisms Act 1996, working alongside the Freshwater fish farming regulations 1983 and council administered regional pest management plans (see Fig. 4). These legislative components often operate in parallel but are not fully integrated, resulting in duplications and inconsistencies in both terminology and application of existing legislation. For example, under the Freshwater Fisheries Regulations 1983, it is illegal to have 'Noxious Fish' 'under control, or rear, catch, hatch or consign', whereas under the Biosecurity Act 1993, 'Unwanted Organisms' can have 'restricted sale, distribution and propagation' (Fisheries New Zealand 2021).

Prevention is always better than cure when it comes to biological invasion and the Hazardous Substances and New Organisms Act 1996 (Ministry for the Environment 1996) is another legislative tool that aims to regulate species introductions at the border (Fig. 4). This requires a rapid risk assessment and only permits entry if the species is unlikely to form a self-sustaining population, displace a valued or native species and / or affect native genetic diversity, cause habitat deterioration or a disease problem, or adversely affect human health and safety (Rowe and Wilding 2012). Despite this, a major flaw in the system is that there is no specific guidance on how the risk of these impacts should be assessed for taxa such as freshwater fish (Rowe and Wilding 2012). It has been suggested that non-native fish risk assessment models for Aotearoa, could be based on species traits associated with 'invasiveness', such as r-selected traits including rapid growth, early maturity and high fecundity, as well as tolerance of a wide range of physicochemical conditions, a large native range or a documented history of invasion success in other countries or regions (Ricciardi and Rasmussen 1998; Rowe and Wilding 2012).

Gaps and labels – how some species don't legally exist, and some can simultaneously be a 'problem species' and the 'right species' depending on where they are

A core aspect of any biosecurity programme requiring public support is the language used to describe species and biosecurity objectives. 'Branding' certain species as 'invasive aliens' or as 'pests', sends out an unambiguous message, that such species are undesirable (Nesbit 2020; NIWA 2020; Tadaki et al. 2023). The Department of Conservation (DOC) defines aquatic pests as 'aquatic organisms that may be problematic to aquaculture and ecosystems' (https://www.doc.govt.nz/nature/pests-and-threats/freshwater-pests/), but this general definition has no legal basis.



Sports Fish	Noxious Fish	Unwanted Organism	Prohibited Organism	Restricted Species
Brown trout	Walking catfish	Koi carp	Venomous fish	Grass carp
Rainbow trout	Rudd	Mosquitofish	Stickleback	Silver carp
Chinook salmon	Koi carp			
Tench				
Perch				
Rudd (Auckland -Waikato only)				

Figure 4. Main government legislation in Aotearoa dealing with the biosecurity, presence / possession and culture of non-native freshwater fish species and the classifications of selected fish species that have resulted from application of such legislation (table adapted from Dean 2003).

It could be argued that certain species emerge as 'pests' in the policymaker's mind due to ecological, economic and cultural damage associated with this species or its analogues, witnessed in other regions or countries. However, it has also been increasingly argued that deliberately prejudiced language has confounded reasoned, evidence-based ecological decision-making on NNS, and that these negative labels are being applied to animals which are just highly adaptable and successful generalists (Nesbit 2020).

Pest, alien, invasive, unwanted, undesirable and noxious are all terms that government agencies have purposely used to prompt the public to actively discriminate against non-native species labelled as such (Inglis 2020). Prevention, detection and elimination are all components of a negative feedback loop that seek to manage such 'problem species', because they are not the 'right species' such as a salmonid 'sports fish' (Abbate and Fischer 2019; Fig. 1). Fig. 4 shows non-native freshwater fish species classifications within the current legislative and regulatory framework (adapted from Dean 2001). These classifications represent the culmination of government risk assessments and management recommendations and are part of an ever-evolving legislative landscape. The resulting legislative landscape is so contradictory, that the 'right species' can be a 'valued introduced species' in one region of the country, while being a 'problem species' in another region. For instance, tench, perch and rudd are termed 'pest fish' in most regional council pest management plans but are also defined as 'sports fish' in the Auckland / Waikato Fish & Game region of the country (Freshwater Fisheries Regulations 1983 – Fish & Game being a non-government public entity representing a large hunting and fishing fraternity). Rudd, in fact, has three labels as it also bears the additional label of a 'noxious' fish, except where it is regarded as a 'sports fish'.

While this multiple labelling could be regarded as a pragmatic fisheries management approach, we suggest that it also reflects problems with policy (such as duplication), which are exacerbated by a current lack of 'joined up' centralised and holistic management of freshwater fisheries. Currently, different sectors of government, as well as public entities, have different responsibilities and accountabilities for different freshwater fisheries. To complicate matters further, a core piece of freshwater environmental legislation, the National Policy Statement for Freshwater Management (NPSFM-2020) only explicitly protects the habitats of two freshwater fish species and these are both NNS, namely trout and salmon. Perversely, only one native freshwater fish has full legal protection in Aotearoa legislation (the Freshwater Fisheries Regulations 1983), and that is the grayling (genus *Prototroctes*), a fish extinct since the 1930s (Mitchell 2018).

Members of the Carp family (Cyprinidae) are also subject to multiple labels in Aotearoa, depending on the species involved and their perceived 'usefulness' or threat. The koi carp is classified as both a noxious fish and an unwanted organism, depending on the legislation applied (Freshwater Fisheries Regulations 1983 for the former and the Biosecurity Act 1993 for the latter). McDowell (1990) also highlighted problems with the management of another NNS, that of rudd (*Scardinius erythophthalmus*), and pointed out that if an angler catches rudd, it is then illegal to keep it but also to release it, so an offence cannot be avoided. Despite this, Hicks (2001) also noted that the noxious status of rudd has failed to prevent its spread. Conversely, grass carp (*Ctenopharyngodon Idella*) and silver carp (*Hypophythalmichthys molitrix*) share a unique status in Aotearoa law as 'Restricted Fish' (section 26ZQA of the Conservation Act 1987), permitting both species to be farmed and released as biocontrol agents for aquatic weeds (http://legislation.govt.nz/act/public/1987/0065/latest/DLM106031.html).

While legal approval must be sought to possess a range of named freshwater animal and plant pests, including non-native *Gambusia* mosquito fish, no approvals at all are needed for a species such as the goldfish (*Carassius auratus*). Indeed, there are currently many freshwater NNS that are not covered by existing legislation and therefore effectively have no legal status in the context of current legislation. Apart from goldfish, these NNS 'gaps' with no legal status, include other aquarium / ornamental fish, brown bullhead catfish (*Ameiurus nebulosus*) and invertebrates. Although catfish and goldfish have no strict legal status, fishing regulations for recreational and commercial fishers require all captured catfish to be killed, and eradication of goldfish may be covered under regional council pest management plans such as in the Waikato Region of Aotearoa. Despite these latter attempts to 'cover these gaps', such omissions are worrying, especially given that the freshwater ornamental aquarium trade is arguably the greatest current biosecurity threat to freshwater ecosystems in Australasia (Ebner et al. 2020). This threat may only be exacerbated by climate change, with gradual increases in median water temperature favouring physiologically tolerant freshwater fish species (Dedual 2019).

The continuing tension between different legislation, governing organisations, and management objectives has been at least partially acknowledged. A recent proposal sought to create a new special permit to enable all species that can be defined as pest fish in areas where a specific problem has been identified, to be managed or eradicated under a single purpose, regardless of any other legislation (Fisheries New Zealand 2021).

Freshwater fish aquaculture – confused and confusing legislation

The sustainability of the freshwater aquaculture sector depends on minimising the environmental impact generated by freshwater farms (Mavraginis et al. 2017), and anything which inadvertently complicates this process or fails to engage the farmer in their statutory duties is problematic. Commercial farming of introduced fish is an inherently risky business in respect of the recipient ecosystem, as it introduces the threat of the possibility of escape or accidental release of NNS to a naïve ecosystem. To some extent a rudimentary negative feedback loop incorporating preventative biosecurity at the individual fish farm level, is the optimum approach to prevent ecosystem impact, with early detection and reporting the next 'best' approaches to minimise ecosystem impacts. Despite biosecurity regimes at fish farms, it should be acknowledged that fish farm escapes are relatively common and attempts to eradicate freshwater NNS have rarely been successful globally, so robust prevention in Aotearoa, as in other countries, remains as the critical safeguard against ecosystem impacts (MPI 2016b, c, d).

Fish farmer education and biosecurity initiatives do in their own way constitute negative socio-ecological feedback loops with respect to non-native fish (see Figs 1, 2 and associated definitions). These loops are working in the context of a species that policymakers have already decided is commercially valuable to the country and therefore 'worth the risk', as regards any potential negative impacts on native ecosystems from fish farm escapees, diseases and pathogens. Indeed, the New Zealand Government's Biosecurity 2025 Direction Statement refers to the protection of the environment, including 'valued exotic species' not just indigenous biodiversity (MPI 2016a). Only a limited number of selected non-native fish are legally licensable for aquaculture purposes (Freshwater Fish Farming Regulations 1983; MPI 2020). Despite the ecological impacts of fish introduction documented in Table 1, there have been notable 'near-misses' in terms of government-sanctioned species introductions for aquaculture purposes. We refer to these as 'near-misses' because if such species had escaped from farms or been deliberately released, they could have had far-reaching consequences on ecosystems in Aotearoa. Two such 'near-misses' occurred in the late 1980s.

In 1987, a proposal was made to introduce channel catfish (*Ictalurus punctatus*) from North America for aquaculture (Townsend and Winterbourn 1992). Despite information on the impacts of introducing this species in other countries being inadequate for a realistic assessment of potential effects in Aotearoa (Townsend and Winterbourn 1992), a government permit was granted to import fertilized catfish eggs. Eggs were hatched in quarantine, the need for environmental trials was dropped and the species was poised to be released for aquaculture, subject only to an independent two-person review team assessing whether the environmental risk was acceptable (Townsend and Winterbourn 1992). Because the available evidence from North America, albeit very limited, indicated that the catfish would probably eventually escape from an aquaculture facility and pose a major threat to one or more valued fish or invertebrate species, the review team recommended that the risk was unacceptable. This recommendation was accepted by the Fisheries Minister, and all catfish held in quarantine were subsequently destroyed.

In 1989, a fish farm in the Auckland region was initially granted permission to farm the Australian Marron Crayfish *Cherax tenuimanus* at one location (Rowe 1992). Shortly after this, approval to transfer the crayfish to other farm sites (new locations) was refused due to concerns over the potential impacts on native ecosystems, including displacement of native crayfish. This led to the 500,000 farmed Marron Crayfish already in Aotearoa being destroyed (Rowe 1992). A confused legal situation had been created in which introduced crayfish were being legally farmed and could be sold live throughout the country but could not be legally transferred to other farms (Rowe 1992). In 2005, two ponds in Auckland were subsequently found to contain 300 Marron crayfish, which then had to be destroyed by government agencies (Beston 2005; Champion 2018).

These cases demonstrate how the misalignment of legislation at the time allowed the legal importation of two INNS into Aotearoa despite their known histories of ecological damage in other jurisdictions, before subsequently legally preventing their release within the country. Both cases also demonstrate the continuing tension between economic interests and ecological protection as drivers in decision-making over the management of non-native fish (see Townsend and Winterbourn 1992; Gozlan et al. 2010).

Moving and stopping – translocation and fish barriers

Although many translocations of freshwater non-native fish in Aotearoa happened in the past, due to the activities of acclimatisation societies, legislation now restricts translocations to native fish only (section 26ZM ‘Transfer or release of live aquatic life’ of the 1987 Conservation Act (2019 amendment). The current government approval process has two broad pathways: First, releasing species where they don’t already occur, which includes stocking a freshwater species at a fish farm for the first time, and second, releasing a species where it already occurs. Because only indigenous species are legally allowed to be translocated, a positive (reinforcing) feedback loop can be established, where native freshwater species, valued for conservation purposes and native fish farm expansion to supplement native populations under pressure or decline, drive more translocations of selected native species (Fig. 1). If the public in the recipient environment value the translocated native species, this again reinforces the loop by driving increasing demand for further translocations (Raine et al. 2020).

As discussed earlier, there are two notable NNS exceptions to the ’native only’ translocation policy and these are the grass and silver carp which are legally termed ‘restricted species’, as opposed to ‘pests’. Their translocation and release are allowed under licence provided they are ‘under control’. However, this system is not foolproof and there have been instances of previously legally released carp escaping to water bodies, other than the ones allowed in the licence (Otago Daily Times 2024). In terms of unwanted NNS, the freshwater translocation of native species also carries risks, as NNS ‘hitchhikers’ can be inadvertently introduced as either parasites of translocated hosts or can exist free living in the water and/or on material used in the transport process (Duggan and Pullan 2017). This is currently a serious problem in parts of Aotearoa where the Gold-Asian clam has recently (2023) been detected and where fish translocations have been an established integral part of fisheries management (MPI 2023).

Fish passage in Aotearoa is managed to prevent the passage of 'undesirable' fish species in order to protect 'desirable' fish species, their life stages, or their habitats' (Ministry for the Environment 2024). Every regional council has to make or change its policies to identify 'desired fish species' for which instream structures must provide passage, while simultaneously identifying 'undesirable fish species' for which passage should be prevented. In effect, such management of fish passages in the recipient environment presents two basic simultaneous feedback loops, a negative (mitigating) feedback loop associated with the 'undesirable', usually non-native species, and a positive (reinforcing) feedback loop, associated with the 'desirable' native species (Fig. 2). The use of fish passages as a tool to both prevent non-native fish and enhance native fish movements is arguably creating tension in management strategies, especially where agencies are charged to deliver one without considering the other.

Past and present troubles with brown trout, as an exemplar of the persistent tensions surrounding deliberate introductions

As discussed previously, the acclimatization societies were dynamic drivers of fish introductions to bolster tourism and sport in the fledgling British colony of New Zealand (McDowall 1990). In these narrow aims, such introductions were largely successful. Nearly a century ago, Zane Grey, the famous American writer, declared 1920s New Zealand to be the 'angler's Eldorado' (Grey 1926), and a century later, in 2020, the New Zealand Federation of Freshwater Anglers (NZFFA) claimed to a government select committee, that the country's sports trout (brown and rainbow) fishery was worth over a billion New Zealand dollars annually (c. 700 million U.S.). Despite speculation on the economic benefits, the introduction, spread, management and continued presence of brown trout are becoming increasingly controversial issues. The current conservation status of brown trout in Aotearoa is 'introduced and naturalized'. These three words hide a myriad of complex, confusing, often contradictory and sometimes highly polarized views on the current 'status' of this iconic species. While brown trout is regarded as a 'pest' species by some as it out-competes and predates native species, to others it is the centre of their recreational lifestyle or culture (Tadaki et al. 2022). The management of such a fish remains an ongoing challenge for government agencies in Aotearoa (Chadderton 2001; Tadaki et al. 2022).

Being the only country in the world where trout farming and commercial sale of trout is banned, government documents with any reference to trout culture use the term 'trout production' to distinguish it from all other freshwater 'finfish aquaculture'. This is because although commercial farming is prohibited, hatcheries continue to produce trout for re-stocking purposes for recreational angling. The situation is culturally and politically complicated, with some iwi (Māori extended kinship group(s)) in some regions wanting the law changed to allow farming of trout (Kupenga 2019). This law change continues to be resisted by angling organisations, who see trout farming as a threat to the recreational fishery.

Non-native fish introductions can drive policy-based socioeconomic positive feedback loops that increase fish transport out of source regions over time, as the perceived success and value of the introduced fish in recipient areas induces a 'socio-economic invasion meltdown', whereby economics drive further introductions as part of a positive feedback loop (Fig. 3). Such a loop has been witnessed in the initial introductions of fish such as chinook salmon and brown trout by acclimatisation societies and although this historic feedback loop may no longer exist, its ramifications have arguably driven a host of more recent socio-ecological (negative mitigating) loops, as opposed to socio-economic (positive reinforcing) feedback loops. These more recent mitigating loops seek to manage the presence of introduced fish and their ecological or cultural impacts. Taking brown trout as an example, components of such loops include banning of trout fish farming, more restrictions on trout hatcheries, banning of trout translocation and establishment of instream structures to act as barriers to trout movement in and between watercourses. More controversially, such loops would also include elimination / removal of trout from water bodies, where native species are threatened and / or river restoration projects are underway. It could also be argued in general terms, that the extremely detailed and tightly policed biosecurity regulations at the borders of both Aotearoa New Zealand and Australia are strong negative feedback loops, which are just as much a response to a past history of ecological and economic damage involving INNS, as they are a current response to new INNS threats.

Conclusions

The debate over non-native freshwater fish management continues, and that is the 'new reality in New Zealand' (Jellyman et al. 2018). Legislation, policy, language and the social-ecological feedbacks generated, are a result of a series of value judgements on native and non-native fish. These value judgements have themselves evolved over time and will continue to change. Such change is inevitable, as the spread and impacts of INNS continues to accelerate, presenting policymakers and public with an issue that threatens to get worse and become more confrontational in the decades to come.

Currently, the prevailing legislation, despite its shortcomings, seeks to promote negative feedbacks mitigating against the establishment and spread of INNS, and positive feedbacks that reinforce the recovery, persistence and growth of culturally important native species and fisheries. Continued use of language and selective 'labelling' of some INNS as 'pests', 'noxious' and 'undesirable' is a powerful tool in policy terms to promote negative feedbacks seeking to detect, manage and eliminate INNS. However, it should be acknowledged that some non-native fish such as brown trout are now so widespread in Aotearoa and have already drastically changed the trophic ecology of freshwater systems, that it is probably impossible to eradicate them, even if they are increasingly regarded as pests that need to be removed so native biodiversity can be restored (Chadderton 2001; Jellyman et al. 2018). In contrast, it should also be acknowledged that there is increasing debate in the global scientific community, about all NNS being vilified as 'evil aliens' and natives being 'beloved', and it has been argued that conservationists should assess species on their impacts, rather than whether they are native or non-native (Davis et al. 2011). In the context of Aotearoa, where non-native fish introductions have largely reflected colonial values and interests, there is increasing recognition that the priorities and expectations of Māori communities need to be provided for, in weighing these questions.

In Aotearoa, arguments over ecosystem restoration from a western fisheries perspective, with a *status quo* of established non-native sport or aquaculture species desirable to some stakeholders, as opposed to 'reimagining' fishery management with indigenous knowledge and values as a focal point, continue. Māori values and perspectives concerning freshwaters, which were all too often ignored in the past (Stewart-Harawira 2020) are now increasingly informing freshwater management (Harmsworth et al. 2016), highlighting opportunities to incorporate more diverse values and impacts into legislation concerning NNS. While Aotearoa policy still has a long way to go in this regard, in recent years *mana whenua* [customary authority exercised by iwi or hapū (Māori descent groups) in an identified area] rights and obligations are increasingly manifest in legislative fisheries management frameworks, as well as in core freshwater environmental protection legislation (Ministry for the Environment 2024). In contrast, Fish and Game New Zealand in a 2023 manifesto document, have raised concerns among its angling members that recent legislative proposals focussing solely on protection of native fish biodiversity, are inevitably to the detriment of non-native sports fish valued by its members (Fish and Game New Zealand 2023). The debate over where non-native freshwater fish into future legislative and actual landscapes in Aotearoa continues (Jellyman et al. 2018). From a global perspective, some fish introductions judged beneficial for biodiversity and/or economy, may still be promoted in the future, alongside increasingly stringent measures against those species which have caused ecological damage in other jurisdictions (Gozlan 2008; Gozlan and Newton 2009). Biosecurity, fisheries and aquaculture policies in Aotearoa, as in other countries, will have no choice but to acknowledge and deal with this apparent paradox (Gozlan 2008; Gozlan et al. 2010).

We would argue the current legislative landscape in Aotearoa is a patchwork, 'make-do-and-mend' approach to freshwater fish management, rather than a coordinated, coherent pragmatic approach. We would advocate the latter, until a much wider national debate has taken place on what people need and want from their freshwater fisheries in Aotearoa and how this could be achieved. A coordinated approach would require government organisations, iwi entities, and different stakeholder groups working together in a more equitable, unified way than previously witnessed. Only by doing this, can modernised, coherent policies and legislation be produced, whereby gaps, inconsistencies, anomalies and duplication can be minimised and when identified, resolved quickly. Using a SES approach with a focus on causal-loop relationships and feedbacks will be extremely valuable to improve understanding of the dynamics of drivers and outcomes underlying current non-native freshwater fish polices and how these can be manipulated to achieve agreed outcomes.

Acknowledgements

This research was conducted through the Fish Futures MBIE Endeavour programme, contract CAWX2101. Thanks to Joanne Clapcott for reviewing this manuscript. We thank both the editor and reviewer for their very constructive comments.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

This study was funded by the Ministry of Buiness, Innovation and Employment, through the Fish Futures MBIE Endeavour programme.

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Data availability

All of the data that support the findings of this study are available in the main text.

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A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 127–143 (2024)
DOI: 10.3897/neobiota.94.120644

Research Article

Evidence of short-term response of rocky cliffs vegetation after removal of invasive alien *Carpobrotus* spp.

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Abstract

Invasive alien plant species are among the major drivers of change in natural ecosystems; therefore, their eradication or control is a common and effective conservation tool to reverse biodiversity loss. The LIFE LETSGO GIGLIO project was implemented with the objective of controlling the invasion of *Carpobrotus* spp., among the most threatening invasive alien species in Mediterranean ecosystems, on the Island of Giglio (Tuscan Archipelago, Italy). The management of *Carpobrotus* spp. was conducted across an area of approximately 33,000 m^2 of coastal habitats. The main intervention was conducted during the winter of 2021–2022, primarily through manual removal, with a limited use of mulching sheets. Subsequent years saw the continued removal of seedlings.

We monitored the habitats of vegetated sea cliffs and coastal garrigues (both protected under Directive 92/43/EEC), as these were the two habitats most affected by the control actions. A total of 24 permanent plots were sampled annually from 2020 to 2023 in a Before-After-Control-Impact (BACI) design. We analysed the variation pre- and post-removal of *Carpobrotus* spp. cover and litter and of native plant cover and diversity, as well as the changes in the composition of native plant communities.

Our results show that already two years after the main intervention of removal, thus in the short term, the community's composition shifted considerably towards the pre-invasion set of species. This recovery was also evident in terms of diversity indices, although the impact of *Carpobrotus* spp. on ecological parameters (mainly soil) favoured nitrophilous species. Furthermore, we highlight the need for yearly removal of *Carpobrotus* spp. seedlings for the next 5–10 years, in order to continue promoting the recovery of native communities.

Key words: Ecological restoration, island ecosystem, Mediterranean, N2000 habitats, plant community, plant management

Academic editor: Joana Vicente
Received: 29 February 2024
Accepted: 1 July 2024
Published: 26 July 2024

Citation: Misuri A, Siccardi E, Mugnai M, Benesperi R, Giannini F, Giunti M, Lazzaro L (2024) Evidence of short-term response of rocky cliffs vegetation after removal of invasive alien *Carpobrotus* spp. NeoBiota 94: 127–143. https://doi.org/10.3897/neobiota.94.120644

Introduction

Invasive alien plants (IAPs) are one of the major drivers of change in natural ecosystems (IPBES 2019) and represent one of the most severe threats to biodiversity (CBD 2018). There is strong evidence of the negative impacts of IAPs on islands, which are more vulnerable to biological invasions than the mainland (IPBES

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[*] The two authors contributed equally to this work (shared first authorship).

2023), but there is also increasing evidence for the effectiveness of eradication and maintenance management of invasive species on islands (Simberloff et al. 2018). Despite the persistence of certain challenges, such as the persistent seed bank (Munné-Bosch 2023) or the unaffordable costs of long-term management (Lorenzo and Morais 2023), islands represent the optimal context for successful control outcomes (Simberloff et al. 2018). For these reasons, eradicating or controlling introduced plants and animals from island contexts is a common and effective conservation tool to reverse biodiversity loss (Ruffino et al. 2015).

Carpobrotus acinaciformis (L.) L.Bolus, *C. edulis* (L.) N.E.Br., and their hybrids (hereafter collectively referred to as *Carpobrotus*) are among the most threatening invasive alien species in Mediterranean ecosystems (Acosta et al. 2006; Carranza et al. 2010; Santoro et al. 2011; Celesti-Grapow et al. 2016). These species are native to South Africa and were introduced to Europe for ornamental purposes at the end of the 17th century (Campoy et al. 2018). The invasion by *Carpobrotus* causes significant ecosystem changes at different scales, leading to a decrease in native plant richness and diversity (Santoro et al. 2011; Fried et al. 2014; Badalamenti et al. 2016; Mugnai et al. 2022; Lazzaro et al. 2023). The impacts include alterations in soil pH, salinity, moisture level, nutrient content, and microbial activity (Santoro et al. 2011; Novoa et al. 2013; Badalamenti et al. 2016; Vieites-Blanco and González-Prieto 2018). These may result in inhibiting native plants' germination, survival, growth, and reproduction (Vilà et al. 2006; Conser and Connor 2009; Affre et al. 2010; Novoa et al. 2013) and in the facilitation of nitrophilous species (Fried et al. 2014; Badalamenti et al. 2016; Lazzaro et al. 2023). Due to these peculiarities, the genus *Carpobrotus* has the highest number of records of control actions (Brunel et al. 2013). Eradication and control methods used on *Carpobrotus* range from chemical to mechanical methods, such as mulching and manual removal (Lazzaro et al. 2020a; Fos et al. 2021, 2022). Manual removal is considered an effective and cost-efficient method to control *Carpobrotus* invasion both in the short and long term (Munné-Bosch 2023). However, it also generates disadvantages, such as the formation of large quantities of waste material, resulting in additional management time and labour (Campoy et al. 2018; Chenot et al. 2018; Lazzaro et al. 2023). Integrating manual removal with mulching sheets can enhance effectiveness in suppressing *Carpobrotus* and facilitate the recovery of native vegetation (Lazzaro et al. 2023; Nascimento et al. 2023).

A focal point in the experiences of eradication or control of these IAPs is represented by the recovery or restoration of native communities. An increasing body of knowledge in *Carpobrotus* removal projects conducted on significant invaded surfaces shows that the recolonizing by native species occurs after the species removal, both on sand dunes (Andreu et al. 2010; Lazzaro et al. 2020), as well in low matorral (Buisson et al. 2021) or rocky cliffs vegetation (Lazzaro et al. 2023). These experiences seem to confirm that in the absence of other invasive species and with low post-removal disturbance, active revegetation through sowing or transplanting is not necessary to achieve diverse native plant communities, although the speed of development may vary. While there are some published experiences on these interventions, more are needed, and the availability of data in both the long and short term is essential to plan effective actions on these IAPs.

Within this work, we focus on the short-term effects of *Carpobrotus* control interventions conducted within the EU LIFE project LIFE18 NAT/IT/000828 "Less alien species in the Tuscan Archipelago: new actions to protect Giglio island habitats", on Giglio island (Tuscan Archipelago, Italy). In particular, our study builds upon the work of Mugnai et al. (2022), who highlighted the important impacts of these species on rocky cliff habitats, which caused a decrease in species richness, community diversity, and abundance, as well as a compositional shift in invaded communities.

In line with the above, the present study aimed to i) verify the short-term effectiveness of the intervention on *Carpobrotus*, evaluating the temporal changes in its cover and litter, as well as the recovery of native plant communities in terms of ii) plant abundance, species richness and diversity after the intervention, iii) species composition and iv) increase of nitrophilous species. Toward these aims, we monitored a series of vegetation plots within two coastal habitats invaded by *Carpobrotus* on Giglio Island.

Methods

Study area

Giglio Island (WGS84: 42.35527°N, 10.90134°E) is the second largest island of the Tuscan Archipelago (Italy) with a surface area of 21.2 km^2 and a perimeter of 28 km (Baldini 1998). Almost all Giglio Island's land surface, approximately 21 km^2, falls within the Natura 2000 Special Area of Conservation (SAC/SPA IT51A0023), with a portion of it, covering 8.9 km^2, being part of the Tuscan Archipelago National Park.

The island is mainly mountainous, with steep and rocky slopes up to the coastline. The climate of Giglio Island is Mediterranean, with mild, rainy winters and hot-arid summers, peaking in July and August (Baldini 1998; Foggi and Pancioli 2008). The vegetation is typically Mediterranean, with forests dominated by*Quercus ilex*, evolved scrubs of *Erica arborea* and *Arbutus unedo*, and other typical Mediterranean *Cistus* and *Helichrysum* garrigues (Foggi and Pancioli 2008). The coastal vegetation, of particular interest for this study, being the one invaded by *Carpobrotus* (see Fig. 1A, B), hosts a mosaic of habitats of conservation interest according to Directive 92/43/EEC "Habitat", including the habitat of Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp. (habitat code 1240 according to Dir. 92/43/EEC, hereafter referred to as Vegetated sea cliffs), and the habitat of Low formations of *Euphorbia* close to cliffs (hab. code 5320, hereafter referred to as Coastal garrigues). Vegetated sea cliffs are characterized by the presence of alophylous chasmophytes such as the endemic *Limonium sommierianum* Fiori and *Crithmum maritimum* L, with few other species very sporadic (f.e. *Lotus cytisoides* L., *Catapodium pauciflorum* (Merino) Brullo, Giusso, Miniss. & Spamp. or *Polypogon subspathaceus* Req.). This paucispecific habitat hosts species fully adapted to grow on rocky coasts and occupy the first colonizable zone in contact with the sea, forming a discontinuous belt along the entire perimeter of the island. Indeed, Vegetated sea cliffs are generally characterised by an almost total absence of soil and direct contact with seawater and marine aerosol, therefore requiring highly specialised species (Perrino et al. 2013). Coastal garrigues settle in the upper parts of rocky coasts, in a variable-width zone between the typical aeroalophilous vegetation of the Vegetated sea cliffs and the first elements of Mediterranean low and high maquis. These plant communities are characterized by a low, single-layer structure dominated by dwarf and small shrubs like *Helichrysum litoreum* Guss. and *Jacobaea maritima* (L.) Pelser & Meijden., prostrate *Pistacia lentiscus* L., and host several other Mediterranean annual species.

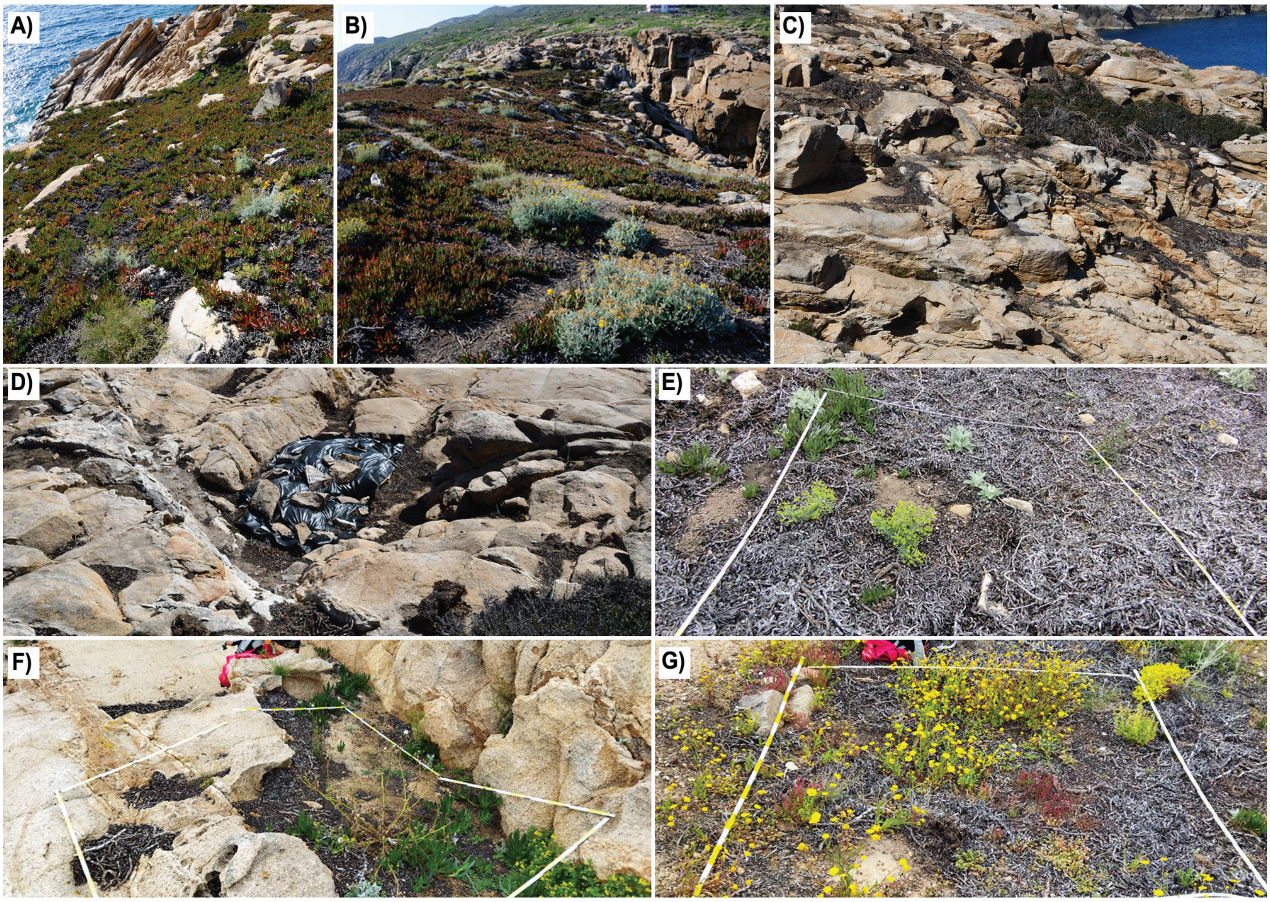

Figure 1. Some images of the interventions of *Carpobrotus* removal at Giglio Island **A**, **B** rocky cliffs invaded by *Carpobrotus*. In 2020 **C**, **D** images of the cliffs after the main intervention in early 2022 **E, F** monitoring plots after one year (2022) with the visible presence of *Carpobrotus* seedlings and **G** in 2023 with native vegetation.

The control of *Carpobrotus* at Giglio Island

The present study analysed the changes in *Carpobrotus* and native species presence and abundance during the actions of control of this invasive alien species, on the island of Giglio conducted within the EU LIFE project LIFE LETSGO GIGLIO "Less alien species in the Tuscan Archipelago: new actions to protect Giglio island habitats" (LIFE18 NAT/IT/000828). In the spring and summer of 2020, we conducted a preliminary phase of detailed mapping of the spread of Carpobrotus on the island. This involved the interpretation of aerial photos and surveys on the island. The initial distribution of this species on the island was recorded as approximately 61,000 m^2, with the majority occurring on cliffs or rocky areas. Approximately 50,000 m^2 of this area was found to be strictly invaded (Lazzaro et al. 2016; Mugnai et al. 2022). The intervention area extended over 33000 m^2 of coastal habitats, with an estimated net *Carpobrotus* surface of approximately 22000 m^2.

The main intervention for the removal began in the winter of 2021–2022 integrating two techniques: manual removal and covering with mulch sheets (landscape fabric, 105 g/m^2). It should be noted that approximately 90% of the *Carpobrotus* on the island were removed manually, while mulching sheets were used only in a limited number of areas, mostly used to contain and isolate the waste material (Fig. 1C, D). The manual removal involved the entire plant, including roots, with the objective of eliminating all visible *Carpobrotus* live plants within the intervention areas. As expected, regrowth of seedlings occurred in all the areas subjected to treatment, particularly in areas where the seed bank was present in the litter remaining on the ground (Fig. 1E, F). Hence, the project foresees continuous monitoring for three years following the main intervention and several rounds of follow-up interventions for the removal of new seedlings. Indeed, already in the first two years after the main intervention (from 2022 to 2023), any seedlings and resprouts were removed manually in April/May and, at the time of writing, continue to be removed annually (Fig. 1G). Furthermore, monitoring and the removal of seedlings will continue for at least five years after the main intervention.

Further technical details on the methods adopted are available in the executive project for the eradication (https://www.lifegogiglio.eu/wp-content/uploads/WEB_Relazione-illustrativa-generale_Carpobrotus-1.pdf).

Sampling design and data collection

The sampling was conducted at the promontory of “Punta Capel Rosso”, south of the island, largely invaded by *Carpobrotus*. In particular, the monitoring was carried out on both habitats, Vegetated sea cliffs and Coastal garrigues, as they were the two most invaded habitats of major conservation importance within the study area.

The monitoring began in 2020, and the impact assessment was carried out using the Before-After-Control-Impact (BACI), a suitable evaluation scheme consisting of pre- and post-intervention sampling of the restoration sites and control sites (Christie et al. 2019). The experimental sampling design has been implemented and maintained through a floristic survey of 2 × 2 m squared plots in two treatments: invaded (plots subjected to the removal) and control (plots in native vegetation). The sampling was stratified according to a random sampling design based on the surface of the habitats mapped concordant to the HaSCITu (Habitat in the Sites of Conservation Interest in Tuscany) program, and a detailed mapping of the distribution of *Carpobrotus* confirmed after several visits to the island before the commencement of the monitoring. Plots were paired, thus for each invaded plot a control plot was identified as close as possible. The sampling included a total of 24 permanent plots, 6 × 2 plots for Vegetated sea cliffs, and 6 × 2 plots for Coastal garrigues, evenly distributed between invaded and control.

Vegetation sampling was carried out during the vegetative season in May when most species were identifiable. Each plot was georeferenced and marked with a peg and a numbered nameplate. Data on native plant species occurrence and abundance was collected using a percentage scale, considering the overlapping of different species. Furthermore, the percentage of fresh *Carpobrotus* as well as its dead litter, was recorded. We present the data collected up to 2023, including thus 4 years of monitoring, resulting in the survey of 96 plots. A full list of species observed during the sampling within the two habitats between 2020 and 2023, is included in Suppl. material 1.

Statistical analysis

To verify the effect of *Carpobrotus* removal intervention on its cover and litter, and on the alpha diversity of native vegetation, we used a Repeated Measurement ANOVA-type modelling. We fitted a series of linear mixed models, with a random effect factor on plot identity to account for the autocorrelation linked to the repetition of the samplings across the four years of surveying and including a covariance structure based on a Gaussian spatial autocorrelation of the observations accounting for the paired structure of the sampling design. For each of the two habitats separately, we assessed whether the cover and litter of *Carpobrotus* varied according to treatment and time. Similarly, we tested whether native species richness (SR), native diversity expressed as H' index, and native species abundance, expressed as the sum percentage cover of each species, changed before, during, and after the interventions using time and treatment (Invaded vs. Control) as fixed effect explanatory variables. When required, the variables were log or asin-transformed to achieve the normality of residuals.

We assessed the changes in the species composition of plots using multivariate analysis for the two habitat types. The analyses included only plots with at least one species (a total of 92 plots; 4 plots had no species in 2022, the year of main interventions). Plot species composition differences were analysed using a non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis dissimilarities calculated on abundance data (expressed as percentages). Nitrophilous species were defined as those with Ellemberg's ecological indicator value for eutrophication above 6 (Pignatti 2005; Ellemberg 2009), the relative abundance and frequency of nitrophilous species were calculated and, due to the differences in the two types of plant communities, we conducted all the analyses separately for the two habitats. Moreover, we evaluated the extent and the divergence of species composition variations according to time and treatment using a Principal Response Curve (PRC) analysis (ter Braak and Smilauer 2012). In this analysis, time was treated as a categorical variable and was used as a covariate. The significance of the effect of the treatment on the species composition was assessed with a permutation analysis using 9999 permutations due to the hierarchical structure of the data and allowing freely exchangeable permutation on the whole plot level, and no permutation at the split-plot level. Given the differences in the two types of plant communities within the two habitats, we conducted the PRC analysis separately for the two habitats.

All analyses were conducted in the R environment (R version 2023.06.2): the LME models were fitted using the 'nmle' package version 3.1-162 (Pinhero and Bates 2006); the NMDS was produced using the 'vegan' package version 2.6-4 (Oksanen et al. 2020); PRC analysis was performed using 'prc' function in the 'vegan' package version 2.6-4, (Oksanen et al. 2020). All plots were drawn using 'ggplot2' package version 3.4.2. (Wickham 2016).

Results

The sampling resulted in the identification of 65 species in 96 plots. Within the invaded plots for both habitats, we observed a significant effect of time for both habitats (interaction terms Treatment: Year, see Table 1) with a decrease in *Carpobrotus* cover after the year of intervention, but a reappearance of *Carpobrotus* seedlings was recorded after two years (Fig. 2A). *Carpobrotus* litter increased in both Vegetated sea cliffs and Coastal garrigues treated plots after the intervention, and declined the following year. Within the Vegetated sea cliffs' control plots, some *Carpobrotus* litter was recorded after the intervention year (Fig. 2B). In the control plots of both habitats, no *Carpobrotus* cover was present in 2020, whereas *Carpobrotus* seedlings were found in the years after the intervention, with cover never exceeding 0.5%, except in one plot in Vegetated sea cliffs, which contained 6% *Carpobrotus* cover in 2023 (Fig. 2A, B).

Table 1. Repeated Measurement ANOVA table for the effect of Invasion Status (Control plots vs. Invaded plots) and Year (sampling year 2020, 2021, 2022, 2023) on *Carpobrotus* cover (%) and *Carpobrotus* litter cover (%), provided for Vegetated sea cliffs (Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp., habitat code 1240 according to Dir. 92/43/EEC) and Coastal garrigues (Low formations of *Euphorbia* close to cliffs, hab. code 5320). numDF: numerator degree of freedom; denDF: denominator degree of freedom. Significance codes: *p*-value <0.001 '***': *p*-value>0.01 '**'; *p*-value <0.05 '*'.

Response	Habitat	Variable	numDF	denDF	F value	*p* value
Carpobrotus Cover (%)	Vegetated sea cliffs	Invasive Status	1	10	25.94	<0.001***
		Year	3	28	12.39	<0.001***
		Invasive Status: Year	3	28	18.35	<0.001***
	Coastal garrigues	Invasive Status	1	10	248.04	<0.001***
		Year	3	28	93.39	<0.001***
		Invasive Status: Year	3	28	107.17	<0.001***
Carpobrotus Litter Cover (%)	Vegetated sea cliffs	Invasive Status	1	10	16.12	0.002**
		Year	3	28	4.99	0.007**
		Invasive Status: Year	3	28	2.42	0.087*
	Coastal garrigues	Invasive Status	1	10	112.51	<0.001***
		Year	3	28	4.46	0.011**
		Invasive Status: Year	3	28	5.75	0.003**

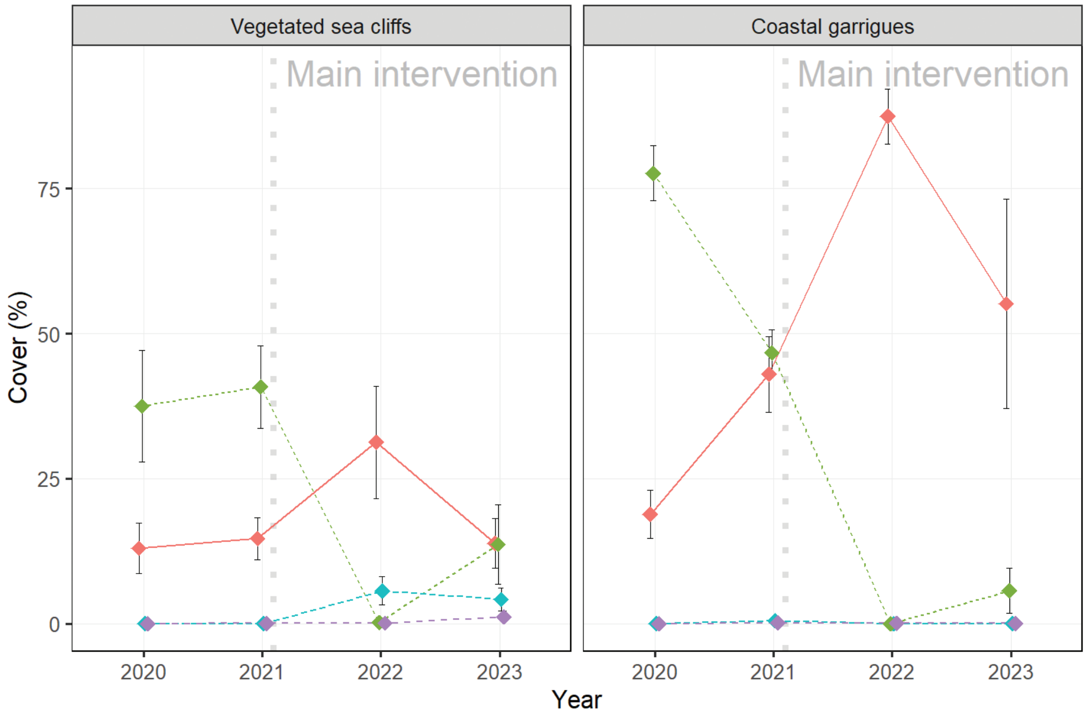

Figure 2. Litter cover of *Carpobrotus* and live *Carpobrotus* plants during 4 years of monitoring in the invaded and control plots of Vegetated sea cliffs (Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp., habitat code 1240 according to Dir. 92/43/EEC) and Coastal garrigues (Low formations of *Euphorbia* close to cliffs, hab. code 5320). The green dotted line represents live *Carpobrotus* plants in invaded plots, the red solid line represents the litter cover of *Carpobrotus* in invaded plots, the blue dashed line represents the litter cover of *Carpobrotus* in control plots and the violet long dashed line represents live *Carpobrotus* plants in control plots.

The native species cover was found to be significantly changed by time for Vegetated sea cliffs and by the interaction of time and invasive status for Coastal garrigues (respectively a p-value of <0.001 and 0.001, see Table 2). Moreover, the anal-

Table 2. Repeated Measurement ANOVA table for the effect of Invasion Status (Control plots vs. Invaded plots) and Year (sampling year 2020, 2021, 2022, 2023) on Native species cover (%), Species richness and Species diversity (Shannon Index), provided for Vegetated sea cliffs (Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp., habitat code 1240 according to Dir. 92/43/EEC) and Coastal garrigues (Low formations of *Euphorbia* close to cliffs, hab. code 5320). numDF: numerator degree of freedom; denDF: denominator degree of freedom. Significance codes: *p*-value <0.001 '***': *p*-value>0.01 '**'; *p*-value <0.05 '*'.

Response	Habitat	Variable	numDF	denDF	F value	*p* value
Native Species Cover	Vegetated sea cliffs	Invasive Status	1	10	7.89	0.018**
		Year	3	28	8.21	<0.001***
		Invasive Status: Year	3	28	1.51	0.234
	Coastal garrigues	Invasive Status	1	10	22.46	0.001**
		Year	3	28	0.98	0.418
		Invasive Status: Year	3	28	7.68	0.001***
Species Richness	Vegetated sea cliffs	Invasive Status	1	10	0.01	0.921
		Year	3	28	22.71	<0.001***
		Invasive Status: Year	3	28	10.43	<0.001***
	Coastal garrigues	Invasive Status	1	10	48.92	<0.001***
		Year	3	28	10.76	<0.001***
		Invasive Status: Year	3	28	2.47	0.083*
Species Diversity (Shannon Index)	Vegetated sea cliffs	Invasive Status	1	10	0.15	0.709
		Year	3	28	25.66	<0.001***
		Invasive Status: Year	3	28	7.43	0.001**
	Coastal garrigues	Invasive Status	1	10	35.98	<0.001***
		Year	3	28	11.79	<0.001***
		Invasive Status: Year	3	28	8.43	<0.001***

ysis of species richness and diversity index showed significantly changes by time and by the interaction of time and invasive status for both Vegetated sea cliffs and Coastal garrigues (Table 2). The analysis of native species richness, diversity index, and native species abundance shows that the index values are higher in the Coastal garrigues control plots than in the invaded one (Fig. 3A–C). Still, after the year of intervention, the values in the invaded plots increased. Interestingly, after the year of intervention, the values of the Shannon index and native species richness values for Vegetated sea cliffs-invaded plots, exceed those of the controls.

The NMDS analysis (stress = 0.1441, non-metric fit R^2 = 0.979, linear fit R^2 = 0.9, see Fig. 4) showed a well-defined differentiation between the composition of the two habitats in control plots across the time, highlighting strong and short-term changes in the species composition after the removal of *Carpobrotus* within the invaded plot. It is interesting to note that in the top-left corner of Fig. 4, Vegetated sea cliffs and Coastal garrigues invaded plots appear in proximity and therefore like each other in composition, both are characterised by the abundant presence of *Carpobrotus* However, after the *Carpobrotus* removal intervention, the differentiation between the invaded communities becomes more pronounced and the lines for invaded plots diverge (moving on both NMDS1 and NMDS2), getting closer in composition to their respective controls.

The PRC analysis of the composition of survey plots during the years is consistent with the mentioned trend of the fast-paced recovery of the invaded communities towards their habitat-related communities of the control plots. The analysis highlighted significant effects of treatment over time in both Vegetated sea cliffs (pseudo-F = 8.2, *p* value = 0.002) and Coastal garrigues (pseudo-F = 4.7, *p* value =

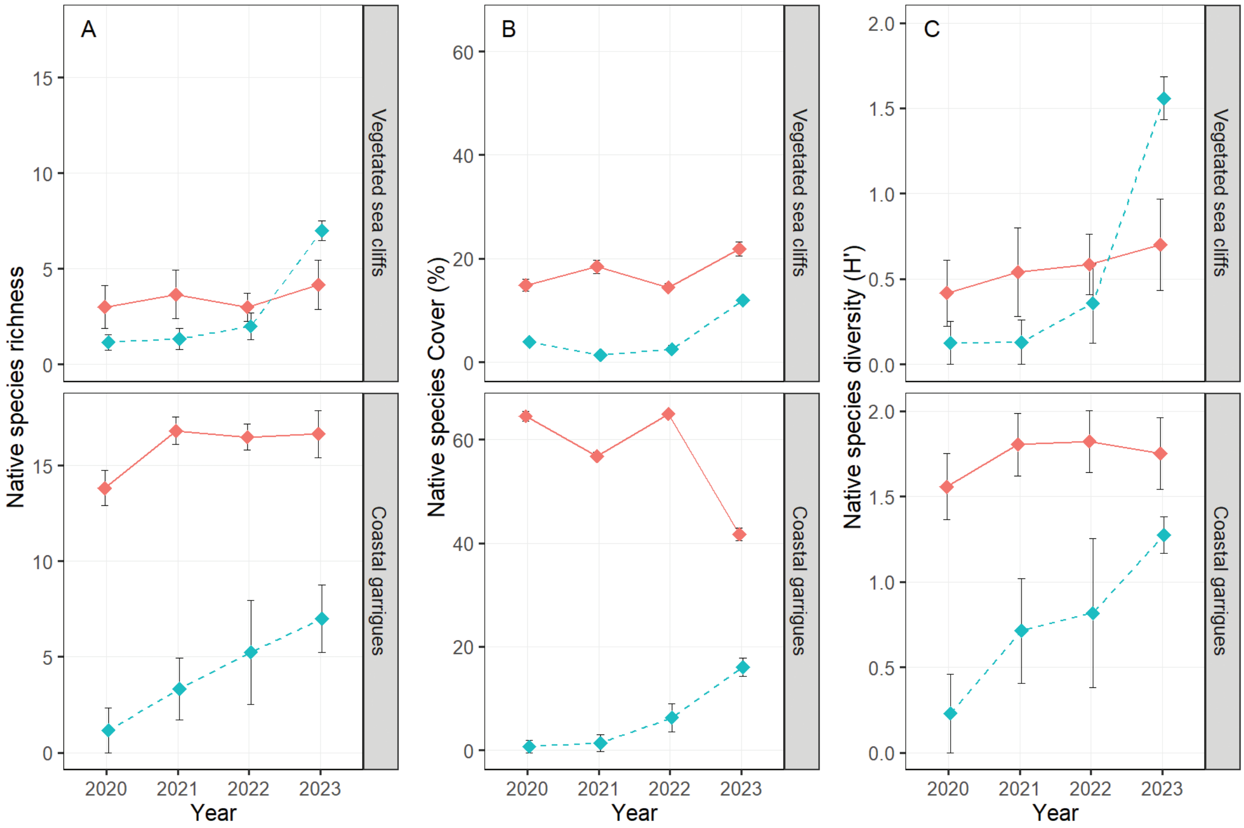

Figure 3. Native species richness, cover (%) and diversity (H') during 4 years of monitoring in the invaded and control plots of Vegetated sea cliffs (Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp., habitat code 1240 according to Dir. 92/43/EEC) and Coastal garrigues (Low formations of *Euphorbia* close to cliffs, hab. code 5320). **A** Native species richness **B** Native species cover (%) and **C** Native species diversity (H') in invaded (blue dashed line) and control plots (red solid line).

0.002). In both cases, a trend of convergence of the treated plots towards the control ones is visible (Suppl. material 2: fig. S1).

The cover of nitrophilous species was found to be significantly changed by time and by the interaction of time and treatment for Vegetated sea cliffs (respectively a *p*-value of 0.018 and 0.029, see Table 3), where the contribution of nitrophilous species in local communities increases drastically one year after *Carpobrotus* removal. Regarding Coastal garrigues, instead, we did not obtain significant values, even if there is a trend towards an increase that peaked in 2022, to then decline the following year, still maintaining values above that pre-intervention (see Fig. 5).

Discussion

Short-term effects after *Carpobrotus* control intervention

Our results build on the effects of *Carpobrotus* removal on coastal reef plant communities, based on a four-year survey period, focusing on describing and analysing the short-term response of native vegetation. Our results markedly indicated that the changes in community composition through the years correspond to a prompt recovery of the native plant communities following the removal of *Carpobrotus*. Prior to removal, both Vegetated sea cliffs and Coastal garrigues invaded communities were similar in composition, due to the very high impact of *Carpobrotus*

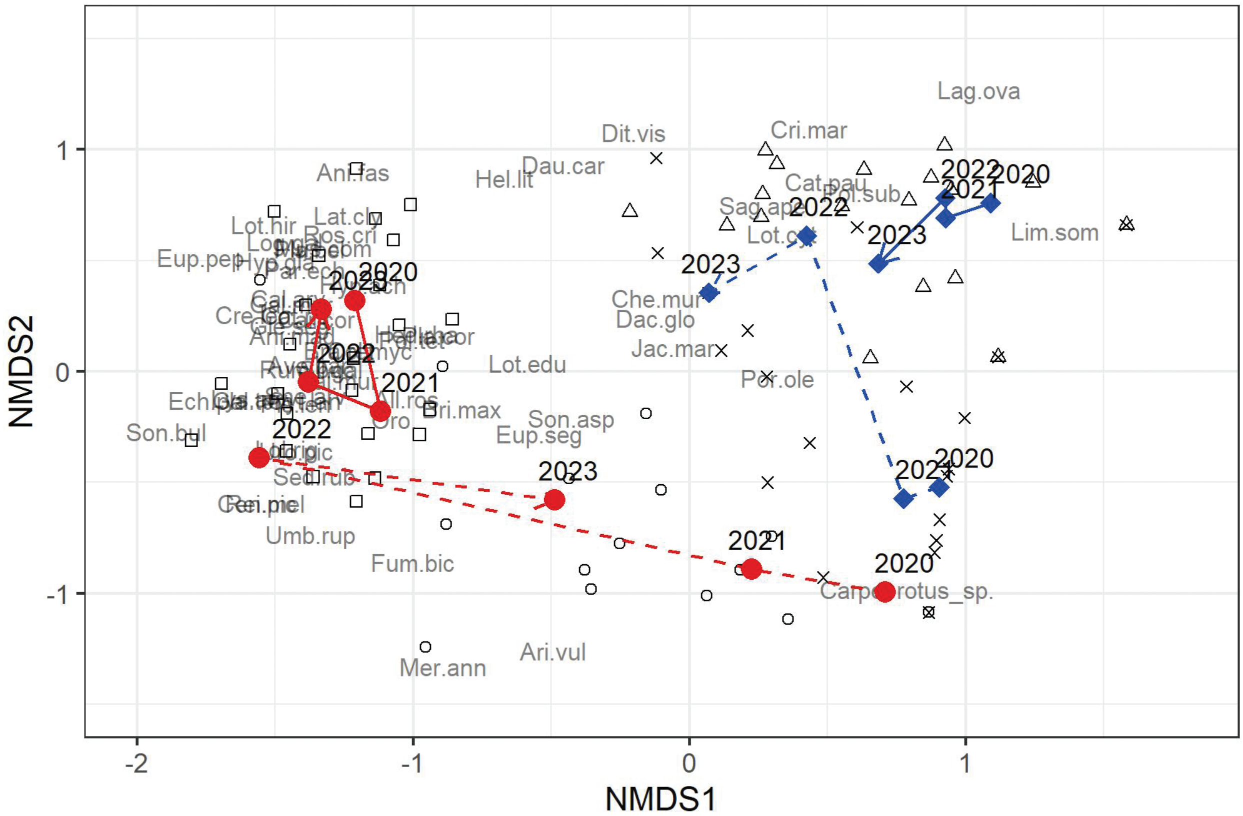

Figure 4. Non-metric multidimensional scaling (NMDS) ordination plot based on Bray–Curtis dissimilarities of the 96 sampled plots. Plots are grouped according to N2000 habitats and invasion status showing the years of monitoring. Solid lines represent control plots and dashed lines represent invaded plots. Blue lines and square symbols represent the Vegetated sea cliffs (Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp., habitat code 1240 according to Dir. 92/43/EEC) and red lines and round symbols represent the Coastal garrigues (Low formations of *Euphorbia* close to cliffs, hab. code 5320). Each symbol along lines indicates a year of monitoring. Codes of plant species are indicated in Suppl. material 1: table S3.

Table 3. Repeated Measurement ANOVA table for the effect of Invasion Status (Control plots vs. Invaded plots) and Year (sampling year 2020, 2021, 2022, 2023) on the relative abundance of nitrophilous species, provided for Vegetated sea cliffs (Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp., habitat code 1240 according to Dir. 92/43/EEC) and Coastal garrigues (Low formations of *Euphorbia* close to cliffs, hab. code 5320). numDF: numerator degree of freedom; denDF: denominator degree of freedom. Significance codes: *p*-value <0.001 '***': *p*-value>0.01 '**'; *p*-value <0.05 '*'.

Response	Habitat	Variable	numDF	denDF	F value	*p* value
Relative abundance of nitrophilous species	Vegetated sea cliffs	Invasive Status	1	10	4.43	0.062
		Year	3	28	3.93	0.018*
		Invasive Status: Year	3	28	3.47	0.029*
	Coastal garrigues	Invasive Status	1	10	0.68	0.430
		Year	3	28	2.23	0.107
		Invasive Status: Year	3	28	0.64	0.594

at the alpha diversity level replacing and outcompeting characteristic native species as already shown in several similar contexts (Fried et al. 2014; Badalamenti et al. 2016; Mugnai et al. 2022). However, invaded plots of both types tended to converge towards their respective control plots during the following growing season after the removal of *Carpobrotus*. Following the removal, the Vegetated sea cliffs communities, were primarily colonised by *Jacobaea maritima* subsp. *maritima*, *Lotus cytisoides* and *Limonium sommierianum*, whereas those of Coastal garrigues

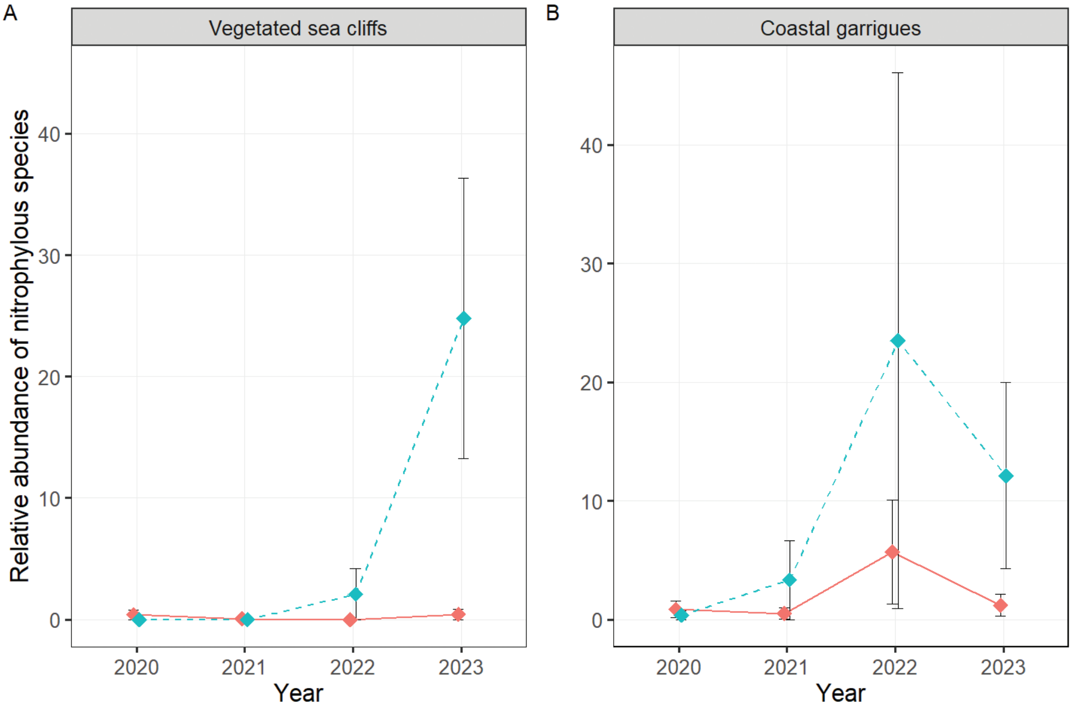

Figure 5. Relative abundance of nitrophilous species (%) for Vegetated sea cliffs (Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp., habitat code 1240 according to Dir. 92/43/EEC) and Coastal garrigues (Low formations of *Euphorbia* close to cliffs, hab. code 5320) during 4 years of monitoring in the invaded (blue dashed line) and control plots (red solid line).

were characterised by *Helichrysum litoreum*, and *Pistacia lentiscus*, which are indicator species of the typical coastal vegetation (Foggi and Pancioli 2008). Moreover, some of these species have a high conservation and naturalistic value, such as *Limonium sommierianum*, as it is endemic to the Tuscan Archipelago (Baldini 1998). Similarly, on Bagaud Island (Var, France), following the removal of *Carpobrotus*, the post-eradication plant communities of rocky coastal cliffs exposed to sea spray tended to converge toward baseline communities characterised by species such as *Jacobaea maritima*, *Spergularia* spp. and *Polycarpon tetraphyllum* (Krebs et al. 2015). Hence, our data confirm once more the effectiveness of control actions in the case of *Carpobrotus* invasion. Indeed, the effectiveness of manual removal and mulching sheet on the removal of *Carpobrotus* has been already shown (see Lazzaro et al. 2023; Nascimento et al. 2023), but while other experiences have shown that the recovery of native communities can be achieved on a medium/long term (7 and 5 years after the interventions, respectively in Buisson et al. 2021 and Lazzaro et al. 2023), we show that already in two years native plants can colonize the areas where *Carpobrotus* had been manually removed.

Although the removal of *Carpobrous* leads to an inevitable decrease in *Carpobrotus* coverage and a slow increase in the recolonization of native plants, the persistence of litter *in situ* can lead to a higher potential for reinvasion due to its large seed bank (Chenot et al. 2014). Indeed, as expected, in two growing seasons after removal, the emergence of seedlings of *Carpobrotus* has been observed within the survey plots and in all the study areas and appeared greater in the coastal garrigues and where litter was thicker and persistent after the interventions. Novoa et al. (2012, 2013) have shown that litter accumulation can repress the growth of native plants even after the removal of live plants, limiting the suitable area for the recolonisation of the natives.

However, removing the litter, other than adding a significant cost to the removal operation, may leave the soil subject to erosion (Chenot et al. 2018). On coastal reefs as per their exposure to atmospheric elements, the persistence of litter decreases quickly: we noted that litter cover diminished in 2023, probably due to soil runoff caused by wind and heavy autumn rains. Interestingly, with an opposite trend, litter was found in some control plots in Vegetated sea cliffs, with no live *Carpobrotus* present, probably transported from the invaded plots to the control plots during bad weather.

However, in one case, it has been shown that leaving the litter improves the germination of new *Carpobrotus* seedlings (Chenot et al. 2018) while, in another, the removal of both the living parts and the litter guarantees a more effective restoration of the native vegetation (Novoa et al. 2013), our results showed a good recovery of the native vegetation a few years after removal with a trend that increasingly approaches that of the control plots. In line with our results, Buisson et al. (2021) pointed out that coastal vegetation plant communities can recover and become relatively similar to the reference within a few years, while shrub communities may need more time to recover due to the competition from native herbaceous species. Buisson et al. (2021) and Campoy et al. (2018) suggested that *Carpobrotus* may have a persistent seed bank (> 5 years), but there are no exhaustive studies over a longer period. Gioria et al. (2012) placed *Carpobrotus edulis* in the category of short-term persistent seed banks, and Ruffino et al. (2015) reported that seeds of *Carpobrotus* can persist for 5 years after eradication. Numerous studies (Novoa et al. 2013; Ruffino et al. 2015; Buisson et al. 2021; Lazzaro et al. 2023) emphasised that it is, therefore, necessary to carry out regular monitoring of treated areas for 10 years before being able to assess the success of the eradication.

Effects on diversity indices and habitats composition

There are strong differences between Vegetated sea cliffs and Coastal garrigues habitats in terms of species richness and diversity (Foggi and Pancioli 2008; Mugnai et al. 2022), native species cover, and the response of their native communities to the removal of *Carpobrotus*. Moreover, the rapid response of the native vegetation follows the trend described by Buisson et al. (2021), Lazzaro et al. (2020a), and Andreu et al. (2010), confirming that, in the absence of post-removal disturbance, invaded and then treated areas can quickly and naturally revegetate, reaching levels of species richness and species diversity close to those of the corresponding control areas. Indeed, in the year following the removal of *Carpobrotus*, as shown in Fig. 2, species richness and diversity values in treated plots for Vegetated sea cliffs exceeded those of the control. This is likely due to the accumulation of *Carpobrotus* litter in areas that normally would not experience such soil buildup, which has allowed for the establishment of richer communities than those typically found in Vegetated sea cliffs. In 2023 there is also a slight divergence at the beta-diversity level, probably due to the entry of some ruderal and nitrophilous species, that are not typically found in either of these habitats, but which can be favoured by soil nutrient enrichment caused by *Carpobrotus* (Novoa et al. 2013; Fried et al. 2014; Malavasi et al. 2016; Lazzaro et al. 2023). The nitrophilous species with the highest cover values in both 2022 and 2023 are *Sonchus asper* (L.) Hill, *Polycarpon tetraphyllum* (L.) L., *Polypogon subspathaceus*, *Mercurialis annua* L. and *Dactylis glomerata* L. all with Ellemberg's ecological indicator values fluctuating between 6 and 8. Similarly, also for Bagaud Island (Var, France), following the removal of *Carpobrotus*, there was a shift of the post-eradication plant communities of the inner part of the island to the reference alonitrophilous ones. Although this inland area already had alonitrophilous herbaceous communities due to the presence of *Larus michahellis* (Naumann, 1840), there was no post-eradication community shift to low matorral communities, which are also present in this area (Krebs et al. 2015). Indeed, both *Carpobrotus* and *Larus michahellis* have been demonstrated to facilitate soil enrichment and entry of nitrophilous species (Novoa et al. 2013; Krebs et al. 2015).

Given the specialised flora that usually characterize the Vegetated sea cliffs, the occurrence of nitrophilous species in this habitat could probably be due to an unusual accumulation of *Carpobrotus* litter, which may have allowed the expansion of some non-characteristic species. In contrast, the relative abundance of nitrophilous species is reduced in habitat 5320 due to the greater complexity of this habitat, which develops on soils between the cliffs exposed to the action of the sea and the shrub communities of the more internal thermo-Mediterranean scrub (Perrino et al. 2013).

Conclusion

In conclusion, our results demonstrated that two years after the *Carpobrotus* removal, the habitat composition had shifted considerably towards a pre-invasion set of species. Furthermore, the recovery occurred also in terms of diversity indices, despite the initial impact of *Carpobrotus* on ecological parameters (mainly soil) favouring nitrophilous species in the early stages. We obtained significant and positive results in terms of native species re-establishment, in an optimistic short time, starting from the year following the removal. However, as the study focused on short-term patterns of regeneration following *Carpobrotus* management, the continuation of vegetation monitoring is pivotal to assess fully the recovery of native communities in the long term. Furthermore, the emergence of seedlings on the site due to the presence of litter and the persistence of the seed bank for many years, emphasises the importance of continued monitoring of the whole area for a long period (five to ten years from the main intervention), to ensure the seasonally repeated removal of new *Carpobrotus* spp. seedlings.

Acknowledgements

The authors acknowledge the support of NBFC to the University of Florence, funded by the Italian Ministry of University and Research, PNRR, Missione 4 Componente 2, “Dalla ricerca all’impresa”, Investimento 1.4, Project CN00000033.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

The research was funded by the LIFE Nature and Biodiversity project LIFE LETSGO GIGLIO “Less alien species in the Tuscan Archipelago: new actions to protect Giglio island habitats” (LIFE18 NAT/

IT/000828), and by the Italian Ministry of University and Research, PNRR, Missione 4 Componente 2, “Dalla ricerca all’impresa”, Investimento 1.4, Project CN00000033.

Author contributions

Alice Misuri: Methodology; Investigation; Visualization; Writing – Original Draft. Eugenia Siccardi: Methodology; Investigation; Visualization; Writing – Original Draft; Formal Analysis; Data curation. Michele Mugnai: Conceptualization; Methodology; Investigation; Visualization; Writing – review & editing. Renato Benesperi: Project administration; Validation; Writing – review & editing. Francesca Giannini: Project administration; Validation; Writing – review & editing. Michele Giunti: Project administration; Methodology; Validation; Writing – review & editing. Lorenzo Lazzaro: Project administration; Conceptualization; Methodology; Investigation; Writing – review & editing; Funding acquisition; Validation.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Data file used for analysis

Authors: Alice Misuri, Eugenia Siccardi, Michele Mugnai, Renato Benesperi, Francesca Giannini, Michele Giunti, Lorenzo Lazzaro

Data type: xlsx

Explanation note: **table S1.** Environmental variables for each plot; **table S2.** Species occurences per plot; **table S3.** Species list and names abbreviation.

Link: https://doi.org/10.3897/neobiota.94.120644.suppl1

Supplementary material 2

Principal response curves (PRC) showing the effect of the *Carpobrotus* removal on the plant species composition of the plots (only the 20 best fitting species are shown)

Authors: Alice Misuri, Eugenia Siccardi, Michele Mugnai, Renato Benesperi, Francesca Giannini, Michele Giunti, Lorenzo Lazzaro

Data type: pdf

Link: https://doi.org/10.3897/neobiota.94.120644.suppl2

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 145–158 (2024)
DOI: 10.3897/neobiota.94.121287

Research Article

Diet comparison suggests limited competition between invasive black rats (*Rattus rattus*) and sympatric endangered rodents

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Abstract

Black rats (*Rattus rattus*) are one of the most widespread invasive animals and have been implicated in the decline of species representing several wildlife taxa, particularly on islands. However, their impact on more closely related species, i.e. rodents, via competition is less well-understood. Using diet similarity as a metric of competition for food resources, we used stable isotopes to compare diets of two populations of black rats to diets of two endangered populations of rice rats (*Oryzomys palustris natator* and *Oryzomys palustris sanibeli*) in southern Florida, USA. Specifically, we analysed hair samples from 32 rice rats and 35 black rats for carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes. In addition, we analysed samples of 129 potential food items to characterise rodent diets using stable isotope mixing models. Despite considerable overlap in isotope-space, we observed differences between rice rats and black rats in the relative composition of plant and animal foods. Specifically, the diets of both populations of rice rats consisted of mostly animal foods, whereas the diets of black rats consisted mostly of plants. In combination with previous work revealing temporal niche partitioning, our results suggest competition between invasive black rats and endangered native rodents may be limited. As such, expensive and logistically complicated efforts to control black rats may have limited success for conserving endangered rodents.

Key words: Florida, island, mangrove, rodent, stable Isotope, Wetland

Academic editor: Sven Bacher
Received: 20 February 2024
Accepted: 7 June 2024
Published: 29 July 2024

Citation: Taillie PJ, Boone IV WW, Wilson-Seelig AL, McCleery R (2024) Diet comparison suggests limited competition between invasive black rats (*Rattus rattus*) and sympatric endangered rodents. NeoBiota 94: 145–158. https://doi.org/10.3897/neobiota.94.121287

Introduction

Invasive species represent a growing threat to global biodiversity (Vitousek et al. 1997; Clavero and Garcia-Berthou 2005; Doherty et al. 2016; Spatz et al. 2017). One of the most widespread, prolific and detrimental invasive species on the planet is the black rat (*Rattus rattus*; Drake and Hunt 2009; Spatz et al. 2017). This globally distributed generalist has contributed to declines in native populations, biodiversity and ecosystem function (Courchamp et al. 2003; Harris 2009; St Clair 2011; Harper and Bunbury 2015). Their broad diet, high fecundity and tolerance to environmental conditions have allowed them to become established in diverse environments ranging from tropical islands to polar regions (Ruffino et al. 2011), where they have contributed to declines in populations of native birds, mammals, lizards, invertebrates and plants via direct predation (Jones et al. 2008; St Clair 2011; Riofrío-Lazo and Páez-Rosas 2015).

In addition to direct predation, black rats can affect native fauna through other interspecific interactions, such as competition (Smith and Banks 2014). For example, invasive black rats are often implicated as a threat to native rodents with similar life histories (Holmes et al. 2019). However, direct evidence of competition can be challenging to quantify (Harris 2009) and is often conflated with other factors. As such, some have questioned whether the effects of black rats on native rodents may be overstated (Norman 1975; Towns et al. 2006). For example, black rats are closely associated with humans (Harper and Bunbury 2015) and, thus, native rodents' responses to black rats may actually be a function of other anthropogenic factors, such as habitat fragmentation, pollution and altered disturbance regimes, rather than competition from black rats (Harris 2009). In addition, it is often difficult to isolate the effects of a single invasive species in such contexts, as other invasive plants and animals are often present where black rats have become established (Towns et al. 2006). Given these challenges, investigating the degree of spatial or temporal overlap in habitat use or habitat characteristics, i.e. Grinnellian niche (Grinnell 1917), may provide limited insight into the degree of competition between black rats and native rodents.

In contrast to the Grinnellian conceptualisation of a species' niche, where the focus is on the environmental characteristics of a species' range, the Eltonian conceptualisation of niche focuses on functional traits and interspecific trophic interactions (Elton 1927; Sales et al. 2021), such as resource consumption (Soberón 2007). Thus, investigating the degree of overlap in Eltonian niche-space more directly corresponds to competition for resources than simply comparing spatial/temporal overlap in environmental conditions. Furthermore, considering competition in this way allows for better understanding processes like resource limitation, foraging dynamics and survival, which directly provide information for effective conservation strategies (Manlick et al. 2021).

Though previously understudied because of lack of data (Rosado et al. 2016), investigations of Eltonian niches have expanded in recent years as stable isotopes have become a well-established tool for quantifying animal diets (Bearhop et al. 2004; Manlick et al. 2019). Specifically, the values of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes can reveal important differences in the diets of individuals and populations (Kelly 2000), as well as variation over time and space (Ben-David et al. 1997). For example, carbon isotope ratios have been shown to correspond to the photosynthetic pathway of plants consumed (i.e. C_3, C_4 and CAM) and the proportions of marine, terrestrial and anthropogenic foods (Kelly 2000; Newsome et al. 2015). Similarly, nitrogen isotopes reflect trophic level, where $\delta^{15}N$ increases by 2–4‰ per trophic level (Crawford et al. 2008). Thus, these isotope ratios can reveal multiple ecologically meaningful aspects of animal diets.

In southern Florida, USA, diverse native wildlife communities are being transformed by multiple invasions by introduced species, making this region ideal for examining the role of competition between invasive and native species within taxonomic groups (e.g. Rodentia). Specifically, black rats have been implicated as a threat to multiple native and endangered rodents in southern Florida (Goodyear 1992; Frank et al. 1997; Boone and McCleery 2023). Empirical support for competition with black rats amongst native rodents in the region is limited to spatial and temporal overlap in occurrence and activity (McCleery et al. 2005; Taillie et al. 2020), though diet comparisons in a lab setting have suggested some differences in diet between native and invasive rats in the Florida Keys (Goodyear 1992).

We used stable isotopes to investigate the potential for competition between black rats and native rodents. Specifically, we compared the isotopic niches of invasive black rats and native rice rats (*Oryzomys* spp.) on two island groups in southern Florida, USA, which we used as a proxy for their Eltonian niches. In addition, we collected potential food items and used stable isotope mixing models to compare the diet composition amongst populations. Due to their more specialised, carnivorous diet (Sharp 1967; Goodyear 1992), we expected native rice rats to have narrower Eltonian niches (i.e. smaller ellipses in isotope space) than black rats, which are characterised by a more generalist diet. Furthermore, we expected the diets of both black rat populations to consist of mostly plants (Riofrío-Lazo and Páez-Rosas 2015; Shiels et al. 2017), compared to rice rats which specialise in consuming wetland macroinvertebrates (Sharp 1967; Goodyear 1992).

Methods

Study areas and focal species

We quantified trophic niches of four rodent populations on two island groups (hereafter: “islands”) in southern Florida, USA. Each of these islands supports a small-ranging subspecies of the marsh rice rat (*O. palustris* spp.). The Sanibel island rice rat (*Oryzomys palustris sanibeli*) is endemic to the Sanibel-Captiva barrier island complex on the south-western coast of Florida (Fig. 1). It is classified as threatened in the State of Florida and is currently under review for listing as endangered/threatened under the US Endangered Species Act. Though genetically distinct from the broadly distributed marsh rice rat, Sanibel Island rice rats appear to occur in similar environments, namely freshwater herbaceous wetlands (Indorf and Gaines 2013). The silver rice rat (*Oryzomys palustris natator*) is federally endangered and endemic to the Lower Florida Keys (USFWS 2021), an island group approximately 40 km southwest of mainland Florida (Fig. 1). Unlike other subspecies of *Oryzomys palustris* spp., which tend to occur in herbaceous wetlands, silver rice rats primarily occur in tidal dwarf mangrove communities (Taillie et al. 2020). Black rats co-occur with both rice rat populations and have been implicated as a potential threat to their conservation (Taillie et al 2020; Boone and McCleery 2023). As such, we compared the isotopic signature and diet composition between silver rice rats and black rats on the Lower Keys, as well as between Sanibel island rice rats and black rats on Sanibel Island.

Sample collection and processing

On both Sanibel and the Lower Keys, we trapped areas known to support the focal subspecies of *Oryzomys palustris* spp. (i.e. Sanibel Island rice rats and silver rice rats, respectively). All rodent isotope samples were collected between 1 October and 31 December 2021. At a given site, we deployed a grid of 25 Sherman traps for four consecutive nights. Each night, traps were opened within 2 hours of sunset and closed within 3 hours of sunrise the following morning. Upon closing traps, we collected all captured rodents and recorded the species, weight, length, and sex of each. In addition, we used small scissors to collect a ~ 2 mg sample of dorsal guard hairs to be analysed for stable isotopes. Each sample was stored in a sealable plastic bag and was frozen within 12 hours. All trapping and handling methods were approved by the University of Florida Animal Use and Care Committee (#202110390).

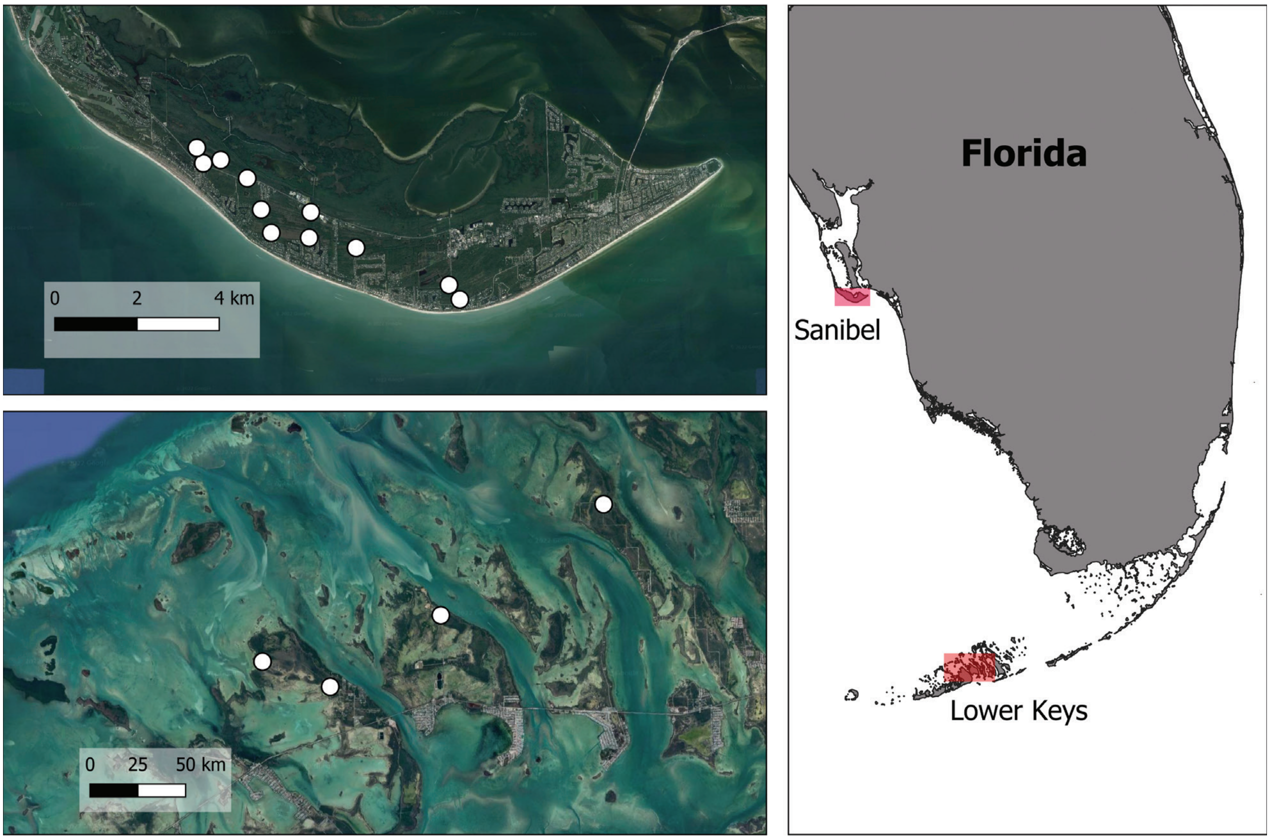

Figure 1. The location of rodent trapping locations (white circles) on each of Sanibel-Captiva Island and the Lower Florida Keys, USA (2017–2021). The right panel shows the location of these islands with respect to mainland Florida.

At each site, while traps were deployed, we opportunistically collected potential food items, based on previous studies of the diets of *Oryzomys palustris* spp. (Suppl. material 1: table S1). To represent the various potential food items of each rodent population, we aimed to collect at least two samples from each of five functional groups: plant, crustacean, fish, mollusc and other invertebrates from each island (i.e. Sanibel and Lower Keys). Based on previous literature, plant samples consisted of tissues most likely to be consumed by rodents, such as fruits, seeds and flowers (Suppl. material 1: table S1). The specific tissues collected from a specific plant were opportunistic, based on availability, but those previously documented in rodent diets were prioritised. As these samples were opportunistic, samples of the same plant species could represent different tissues. To supplement the potential food item samples collected in 2021 during the acquisition of rodent hair samples, we included 51 additional food item samples from Sanibel Island collected in a similar manner during 2017. All samples were stored in a cooler with ice upon collection and stored in a freezer within 12 hours. Prior to analysis, samples were thawed, rinsed with deionised water, dried in oven for 48 hr at 60 °C and then homogenised using a mortar and pestle. Samples were analysed at University of Florida's Light Stable Isotope Mass Spectrometry Lab for isotopic values of both carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$). Two reference materials, $USGS_{40}$ (L-glutamic acid) and $USGS_{41b}$ (L-glutamic acid enriched in ^{13}C and ^{15}N) were used to calibrate the system at the beginning, end, and at regular intervals. The precision for $USGS_{40}$ was 0.07 and 0.11 for ^{13}C and ^{15}N, respectively.

Analysis

We first quantified each population's Eltonian niche by plotting the values of $\delta^{13}C$ and $\delta^{15}N$ in isotope-space. We made qualitative comparisons amongst the four populations by comparing the relative positions of individuals in isotope-space. In addition, we used an ANOVA to test for statistically significant ($p < 0.05$) differences of each isotope between rice rats and black rats on each island. We then considered the area of isotope-space occupied by a population as a metric of niche breadth. Specifically, we used the standard ellipse area corrected for small sample sizes (SEAc) to quantify and compare the isotopic niche breadth of rice rats and black rats on each island. To account for variation in sample size amongst populations and to formally account for uncertainty (Jackson et al. 2011), we used a Bayesian approach to calculate 95% prediction ellipses for each population using the R package *SIBER* v.2.6.1 (R Development Core Team 2018; Jackson and Parnell 2021). We sampled the posterior distribution over 10,000 iterations and discarded the first 1,000.

To compare the relative position of the ellipses in isotope-space, we used two metrics of ellipse overlap. First, we calculated the proportion of the overlapping area to the total area of the ellipses being compared (hereafter: "total area proportion"). This total area proportion could range from 0 (no overlap) to 1 (total overlap). As black rats are generalists (Cox et al. 2000; Ruffino et al. 2011; Shiels et al. 2013), we expected their ellipses to be larger than those of rice rats, which would influence the total area proportion. Thus, we also calculated the proportion of the area of overlap to the area of the native rodent's ellipse (hereafter "native proportion"), to serve as a complementary overlap metric that did not depend on the size of the black rat ellipse. As with the total area proportion, the native proportion could range from 0 (no overlap) to 1 (completely contained within black rat ellipse). To acknowledge uncertainty in these overlap metrics, we reported the 10th and 90th quantiles of the posterior sample of each metric.

To link isotope signatures from rodent tissues to food items and make inferences about rodent diets, we used stable isotope mixing models using the R package *simmr* (Parnell 2021). We first grouped the food items according to three broad taxa: animals, C_3 plants and C_4 plants. We based these categories not only on consumer diet (e.g. herbivore, omnivore), but also on clustering of isotope values within groups. For example, C_4 plants were enriched in $\delta^{13}C$ and isotopically distinct relative to C_3 plants and the strong differentiation in $\delta^{13}C$ we observed corresponded closely to the reported ranges for plants using C_3 photosynthesis (range = –35 to –21‰) and C_4 photosynthesis (range = –14 to –10‰; (Kelly 2000)). To accommodate variable rates of isotope discrimination during assimilation and excretion (Olive et al. 2003), we adjusted isotope values using diet-tissue discrimination factors (TDF; Phillips et al. 2014). In addition to the consumer tissue being analysed, the consumer's diet can also influence the TDF (Stephens et al. 2022). Thus, we used previously reported TDF's for hair samples from omnivorous mammals (1.5 and 2.8 for $\delta^{13}C$ and $\delta^{15}N$, respectively; (Stephens et al. 2022)), which were consistent with those used previously in southern Florida (Cove et al. 2018). We fitted the mixing models using the simmr_mcmc function with 100,000 iterations of four chains thinned by every 10th iteration and a burn-in of 10,000 iterations. We checked for model convergence by ensuring the Rhat statistic was less than 1.05 for all estimated parameters (Parnell 2021). We used the compare_groups function to determine the probability that the diet proportion for a given food was different between two populations. We considered probabilities greater than 0.9 to have strong support.

Results

Our rodent trapping efforts resulted in hair samples from 23 silver rice rats and 18 black rats on the Lower Keys, as well as 9 Sanibel Island rice rats and 17 black rats on Sanibel. Though we trapped 11 sites on Sanibel Island where Sanibel Island rice rats were captured previously, we only detected them at three of these 11 sites. Meanwhile, we collected 78 potential food items, which we supplemented with 51 additional items collected from Sanibel in 2017 (Suppl. material 1: table S2). Isotope values for all rodent hair samples (including 30 Sanibel Island rice rat samples not included in this analysis) and food item samples are provided in the supplemental material (Suppl. material 1: table S3).

Broadly, we observed greater values of both $\delta^{13}C$ and $\delta^{15}N$ in rice rats compared to black rats on their respective islands (Table 1). The means of both isotopes were significantly (ANOVA $p < 0.05$) greater for rice rats compared to black rats on both islands. When considering both isotopes simultaneously, the standard ellipse areas were smaller for rice rats compared to their respective populations of black rats, but the 95% credible intervals overlapped for these comparisons. The ellipses for all four populations of rodents overlapped in isotope-space (Fig. 2). The 95% credible intervals for the total area proportion were 0.05–0.35 and 0.17–0.38, on Sanibel and the Lower Keys, respectively. The native area proportion was more variable, with the 95% credible intervals ranging from 0.09–0.59 and 0.27–0.72, respectively.

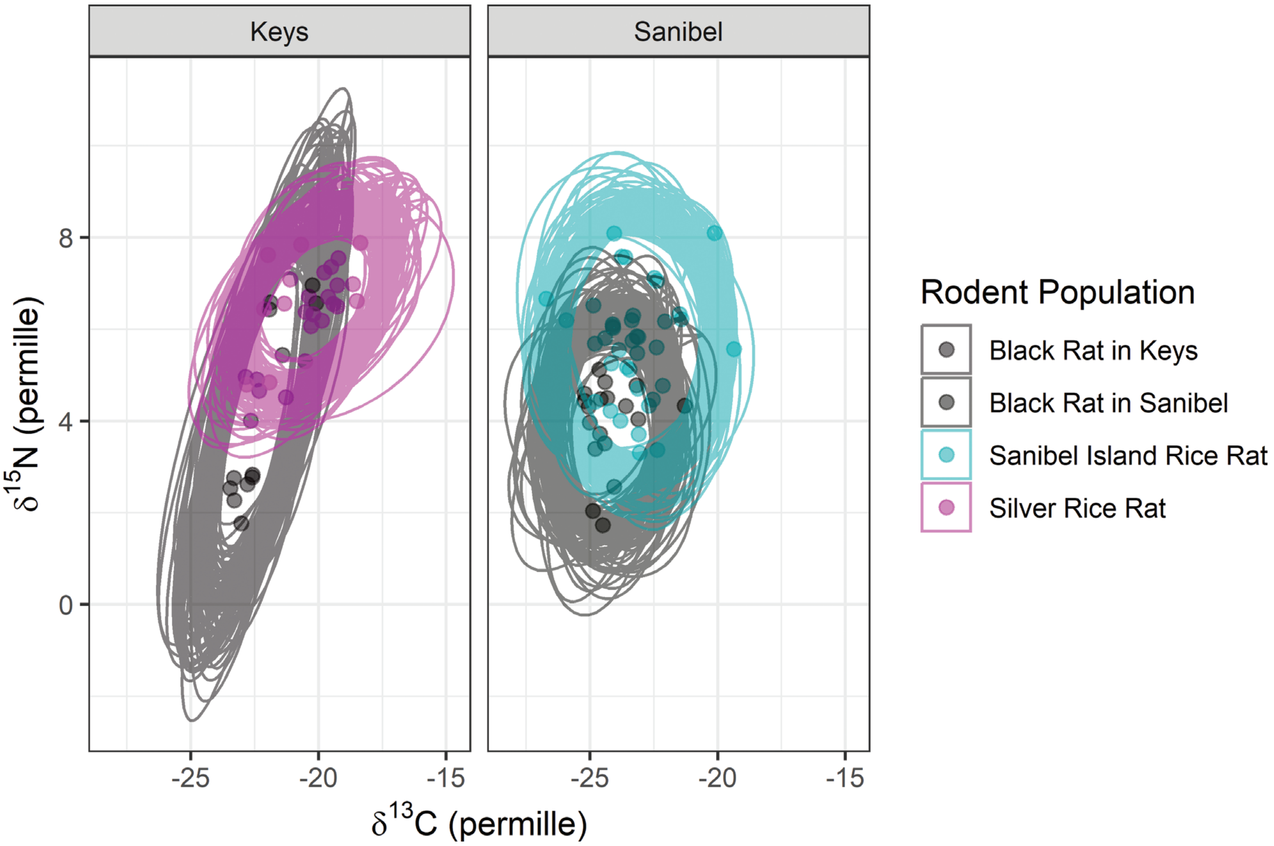

Figure 2. The nitrogen and carbon stable isotope values for four populations of rodents on two islands (Lower Keys and Sanibel-Captiva) in southern Florida, USA. The corresponding ellipses represent the posterior distributions of estimated bi-variate ellipses used to compare isotopic overlap. Shown also are the means (black dots) and standard deviations (coloured crosses) of the stable isotope values of the three taxa of potential food items (i.e. sources).

Table 1. Minimum and maximum isotopic values and Standard Ellipse Area with small sample correction for four populations of rodents in southern Florida, USA.

Species	Island	Range $\delta^{13}C$	Range $\delta^{15}N$	Mean $\delta^{13}C$	Mean $\delta^{15}N$	SEA_c
Silver Rice Rat	Keys	-22.8, -18.4	4.8, 7.9	-20.2	6.6	2.9
Black Rat	Keys	-23.5, -20.1	1.8, 7.0	-22.2	4.3	3.4
Sanibel Island Rice Rat	Sanibel	-23.6, -20.1	5.5, 8.1	-22.7	6.6	2.7
Black Rat	Sanibel	-25.2, -21.3	1.7, 5.1	-24.3	3.9	3.1

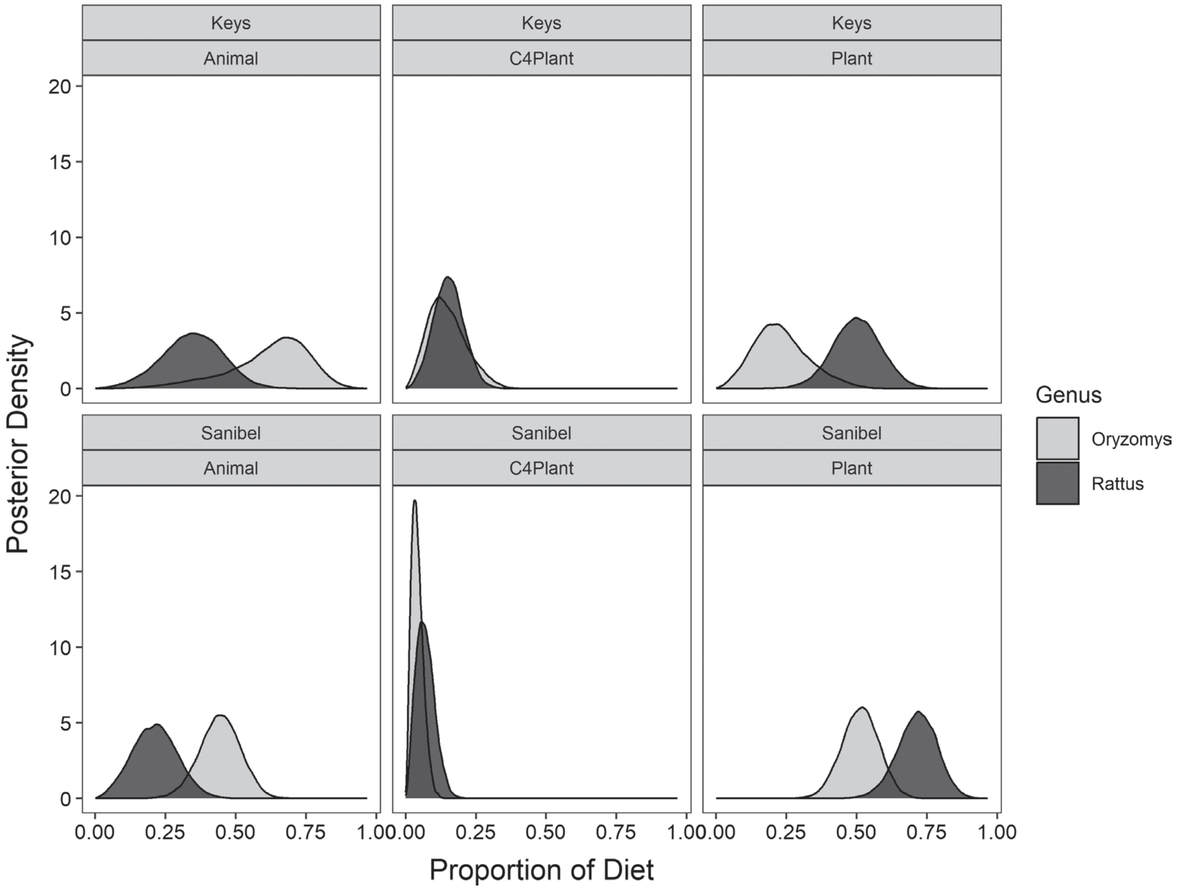

Figure 3. Posterior distributions and boxplots comparing the relative proportion of three food categories (animal, C3 plant and C4 plant) between two genera of rodents (rice rats [*Oryzomys*] and black rats [*Rattus*]) in each of two islands in southern Florida, USA (the Lower Keys and Sanibel-Captiva; 2021). Included in each panel is the probability (P) that the food item's proportional composition of diet is greater for one genus when compared to the other.

Despite this overlap, stable isotope mixing models suggested the diets of native rice rats differed from those of black rats on their respective islands. On the Keys, there was a high probability ($P > 0.9$) that rice rats consumed more animals and fewer plants compared to black rats, which consumed more plants (Fig. 3). We observed similar differences on Sanibel; however, the low sample size of Sanibel Island rice rats resulted in greater uncertainty in diet proportions. On the Lower Keys, the 95% credible intervals of the diet proportions were wider and, thus, the differences were less significant (Suppl. material 1: fig. S1). However, both these data, as well as unpublished data from Sanibel Island rice rat samples collected in 2017 (Suppl. material 1: table S3) reflect a similar diet of approximately equal parts plant and animal, compared to black rats which ate mostly plants (Fig. 3).

When we compared between islands, we observed strong evidence that the diet of black rats on Sanibel consisted of more plants than black rats on the Keys. Otherwise, comparisons within species between islands did not exceed the 0.9 probability threshold.

Discussion

Consistent with our expectation, the isotopic niche of black rats was larger than that of rice rats, reflecting a more generalist diet. Both rice rat populations appeared to be more carnivorous than black rats, evidenced by greater δ^{15}N than black rats on their respective islands. However, there was considerable overlap in isotope-space between rice rats and black rats on both islands. Despite this overlap, we observed two important differences in diet. First, rice rats consumed comparable amounts of animal and plant foods, compared to both populations of black rats, which ate mostly plants. On the Keys specifically, rice rats consumed mostly animal foods. These differences in diet suggest competition between native rice rats and exotic black rats may be limited. Second, the diet composition and stable isotope signatures of closely related rodents, as well as the food they consume, differed between the islands we investigated suggesting that the relative impact of black rats on native rodents may vary considerably.

Broadly, we observed that rice rats specialised in animal foods, whereas black rats consumed mostly plants, which is consistent with other studies of rodent diets (Goodyear 1992; Shiels et al. 2013; Riofrío-Lazo and Páez-Rosas 2015). Specifically, for silver rice rats on the Lower Keys, these differences in diet are further supported by temporal niche partitioning between silver rice rats and black rats, where the former are most active at low tide when tidal macroinvertebrates are more available (Taillie et al. 2020). Given that black rats have co-occurred with native rodents on Caribbean islands for several centuries (Harper and Bunbury 2015), some degree of partitioning is requisite for the continued co-existence of native and invasive rodents (Hardin 1960; MacArthur and Levins 1967).

Importantly, we observed some notable differences between the Lower Keys and Sanibel. Though rice rats on both island groups consumed primarily animal foods, plants represented a larger proportion of rice rat diets on Sanibel. Similarly, black rats on Sanibel consumed proportionally more plants compared to black rats on the Lower Keys. These between-island differences could result from differences in marine subsidies that have been shown to be important to mammalian diets (Stapp and Polis 2003; Manlick et al. 2019; Davidson et al. 2021). Specifically, silver rice rats in the Lower Keys may rely more on tidal macroinvertebrates compared to Sanibel where water levels are less variable and the vegetation community is less salt-tolerant (Indorf and Gaines 2013). More generally, previous research has suggested that Eltonian niches are driven by environmental factors such as landscape composition and prey availability (Manlick et al. 2019) and even species considered to be specialists can exhibit Eltonian niche plasticity (Terry et al. 2017). As such, the degree of competition between native rodents and invasive black rats likely varies as a function of environmental context and geography. Other studies have suggested that competition from black rats may limit populations of rodents on other islands (Harris and Macdonald 2007; Harper and Cabrera 2010; Russell et al. 2015), which could result from differences in resource availability, as well as traits of competing species.

On both islands, isotope signatures of rice rats and black rats differed more in the values of $\delta^{15}N$ than $\delta^{13}C$, suggesting that these differences in diets were driven more by trophic levels than primary producer photosynthetic pathways. The minimal differentiation in $\delta^{13}C$ was surprising given that we expected differential consumption of native C4 plants (e.g. grasses), C4 plants in anthropogenic foods (e.g. corn) and marine foods between rodent populations, all of which have been shown previously to influence $\delta^{13}C$ (Ben-David et al. 1997; Kelly 2000; Newsome et al. 2015). For example, previous work has suggested that anthropogenic food subsidies likely drove $\delta^{13}C$ enrichment in feral cats (*Felis catus*) on the Florida Keys (Cove et al. 2018). However, the individuals in our study may consume fewer anthropogenic foods, given the wetland environments away from human development in which our study took place.

One important limitation of our study is that we only sampled rodent diets during a single season (late autumn/early winter). Previous studies have shown that stable isotopes can vary seasonally as a function of diet, as well as other factors (Ben-David et al. 1997; Willson et al. 2010). Though temperature variation in the subtropical climate of southern Florida are minimal, seasonal variation in precipitation has been shown to be an important driver of animal phenology and demographics (Henry et al. 2022). As such, resource abundance and, as a result, diet and competition of rice rats and black rats are likely to change seasonally. Future work should focus specifically on times of the year when resources are limited to better understand how those limited resources are partitioned between sympatric species.

Our results suggest that the degree of competition between black rats and native rodents may vary as a function of environmental factors, such as prey availability and geographical context. Consequently, responses amongst native rodents to invasive black rats and the need for control or eradication efforts may also vary. Therefore, previously documented conservation successes for taxa such as seabirds resulting from invasive rat eradication efforts (Jones et al. 2016) may not translate to similar benefits for the conservation of native rodents. In addition, eradication of invasive rats is expensive and can have unintended negative consequences for both native rodents and other taxa (Simberloff 2001; Howald et al. 2010). In contexts where competition from invasive rats is limited, conservation practitioners should work to identify and address more direct threats limiting the populations of threatened species, such as habitat loss and other anthropogenic stressors.

Acknowledgements

We thank our dedicated field technicians A. Veselka, K. Carey, and A. Merchlinsky who made the project possible. In addition, we thank United States Fish & Wildlife staff (K. Kalasz and Sanda Sneckenberger, among others) for providing assistance with accommodations, access, and logistics. Lastly, we thank the University of Florida Stable Isotope Lab for processing isotope samples.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

No funding was reported.

Author contributions

Conceptualization: RM, WWBI, PT. Data curation: WWBI, ALWS, PT. Formal analysis: PT. Funding acquisition: PT, RM. Investigation: ALWS. Methodology: ALWS, RM, WWBI. Project administration: RM, PT. Supervision: WWBI. Writing – original draft: PT. Writing – review and editing: RM.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Data and summary statistics

Authors: Paul J. Taillie, Robert McCleery

Data type: docx

Link: https://doi.org/10.3897/neobiota.94.121287.suppl1

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 159–177 (2024)
DOI: 10.3897/neobiota.94.128736

Research Article

Disentangling the effects of abiotic and biotic processes on non-indigenous species dominance

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Abstract

Relatively little attention has been paid to the underlying mechanisms determining the dominance of non-indigenous species (NIS) once established, despite being regarded as a proxy of invasion success and potential impacts in recipient communities. To bridge this knowledge gap, here we evaluate the potential direct and indirect effects of community filters on the dominance of two widespread NIS in the Baltic Sea: *Marenzelleria* spp. and the round goby (*Neogobius melanostomus*) within their corresponding communities. We applied a structural equation modelling approach to assess the direct and indirect effects amongst multiple abiotic and biotic variables on the relative biomass (as proxy of dominance) of NIS. The biotic variables represented the taxonomic- and functional diversity of the recipient communities, as well as the trait similarity between NIS and native species. We observed a comparable influence of abiotic and biotic drivers on the dominance of both NIS, with biotic variables having a somewhat stronger overall direct effect. Specifically, the dominance of both NIS was similarly affected negatively by the richness and positively by the evenness of the native communities. However, we also detected that both NIS might need different ecological strategies to become dominant in their recipient communities, which underwent similar assembly processes. Such strategies were partly highlighted by the different degrees of trait similarity between each NIS and their respective co-occurring native species. A better understanding of the underlying processes affecting NIS dominance is of high relevance to mitigate potential impacts of NIS once established. Furthermore, the provided approach could be further applied to unveil the potential strategies that NIS might follow in other regions and ecosystem types.

Key words: Benthos, biological invasions, coastal fish, community assembly rules, dominance, functional distinctiveness, functional ecology, SEM, traits

Academic editor: Anthony Ricciardi
Received: 31 May 2024
Accepted: 6 July 2024
Published: 30 July 2024

Citation: Vivó-Pons A, van Denderen PD, Flensborg L, Jaspers C, Lindegren M (2024) Disentangling the effects of abiotic and biotic processes on non-indigenous species dominance. NeoBiota 94: 159–177. https://doi.org/10.3897/neobiota.94.128736

Introduction

The spread of non-indigenous species (NIS) pose a major threat to biodiversity and the integrity of ecosystems worldwide (IPBES 2023). The global spread of NIS is mostly driven by human activities linked to increasing trade and transport (Seebens et al. 2021). While only a minimal fraction of NIS become naturalised and further invasive (Blackburn et al. 2011; IPBES 2023), the global impacts of NIS establishment in marine communities are predominantly negative (Anton et al. 2019) and often intensified by anthropogenic pressures (Geraldi et al. 2020). However, at more local scales, the effects of NIS on ecosystem structure and functioning can also be innocuous or even positive (Thomsen et al. 2014; Vivó-Pons et al. 2020). Hence, the effect and potential impacts of NIS on native communities is likely to be highly scale-, context- and species-dependent (Guy-Haim et al. 2018).

The establishment of NIS has been shown to be influenced by the same community assembly processes structuring the composition of native communities (Gallien et al. 2014; Pearson et al. 2018). Communities exposed to a strong environmental filtering (e.g. extreme temperatures) tend to harbour functionally similar species with a limited set of physiological, morphological or behavioural characteristics (i.e. traits), which NIS may also display in order to cope with the environmental conditions (Cleland 2011; El-Barougy et al. 2020). Conversely, in communities mainly shaped by biotic interactions (e.g. limiting similarity), NIS might need to display more dissimilar traits or strategies compared to natives in order to limit niche overlap and avoid competitive exclusion (Mathakutha et al. 2019; Xu et al. 2022). As evident from the abovementioned studies, considerable effort has been dedicated to understand NIS establishment (Richardson and Pyšek 2012; Gallien and Carboni 2017). However, relatively little attention has been paid to the underlying mechanisms determining the dominance of NIS once established (Lundholm and Larson 2004). This lack of knowledge is critical since NIS dominance is regarded as a key proxy of invasion success (Crall et al. 2006) and may provide information about the magnitude of potential impacts on recipient communities (Bradley et al. 2019; Cadotte 2023). Hence, a better understanding of the multiple processes affecting NIS dominance is essential to evaluate potential risks of already established NIS to become hazardous in the future (Richardson and Pyšek 2012).

Here, we aim to bridge this knowledge gap by providing one of the first comprehensive assessments of the main drivers of NIS dominance in recipient communities by integrating existing community assembly theory (Pearson et al. 2018) with the functional similarity between NIS and co-occurring species. As model organisms, we performed a comparative analysis on two widespread and well-known NIS in European waters and beyond: the benthic polychaete worm *Marenzelleria* spp. (Mesnil, 1896); (Blank et al. 2008) and the demersal fish round goby (*Neogobius melanostomus*; Pallas, 1814). We conducted the analysis in the Baltic Sea region, where both species are currently well established. The Baltic Sea provides an illustrative case study area as it is highly susceptible to the arrival and establishment of NIS from both marine and limnetic origin (Paavola et al. 2005) and currently contains > 100 recorded NIS, many of which form self-sustaining populations (Ojaveer et al. 2017).

The first report of the *Marenzelleria* species complex in the Baltic Sea was in 1985, probably introduced through ballast water from North America (Bastrop et al. 1997; Blank et al. 2008). Subsequently, it spread and established throughout the entire Baltic Sea (Blank et al. 2008; Maximov 2011), often reaching high densities (Delefosse et al. 2012). *Marenzelleria* is regarded as an ecosystem engineer species, as it has been documented to change sediment and nutrient dynamics of recipient areas together with other multifaceted impacts on native communities (Delefosse et al. 2012; Kauppi et al. 2018). The round goby is native to the Ponto-Caspian region and was first recorded in the Gulf of Gdansk in 1990 (Skóra and Stolarski 1993). This NIS has been spreading throughout several regions within the Baltic Sea, being established in many coastal areas (Puntila-Dodd et al. 2018). Round goby attains very high population densities partly due to a wide tolerance to environmental conditions and high reproductive turnover rate (Behrens et al.

2017; Kruze et al. 2023). To date, the overall impacts of round goby's establishment are assumed ecosystem- and context specific (Hirsch et al. 2016), yet it has been shown to decimate local invertebrate populations (van Deurs et al. 2021; Wallin-Kihlberg et al. 2023) and compete with native species due to overlapping dietary preferences (Karlson et al. 2007).

To evaluate the potential direct and indirect effects of both abiotic and biotic community filters on the dominance of both NIS, we applied a structural equation modelling (SEM) approach (Shipley 2009) using high-resolution monitoring data on species biomass and environmental conditions as input. SEM has previously been used to study the associated impacts of NIS (Britton-Simmons 2004) and the mechanisms behind their establishment success (Xu et al. 2022); but, to date, not to assess the main processes affecting their dominance once established. More specifically, we aimed to analyse how environmental conditions and biotic variables, reflected by key taxonomic and functional community metrics, as well as the degree of niche (trait) overlap between NIS and native species, determine the dominance of NIS. This overall aim was achieved by pursuing the following research questions and their associated hypotheses (Fig. 1):

Q1: What are the effects and relative importance of abiotic and biotic drivers on NIS dominance?
Q2: Are the responses to drivers similar or different between NIS?
Q3: To what extent does trait similarity and niche overlap with native species determine the dominance of NIS?

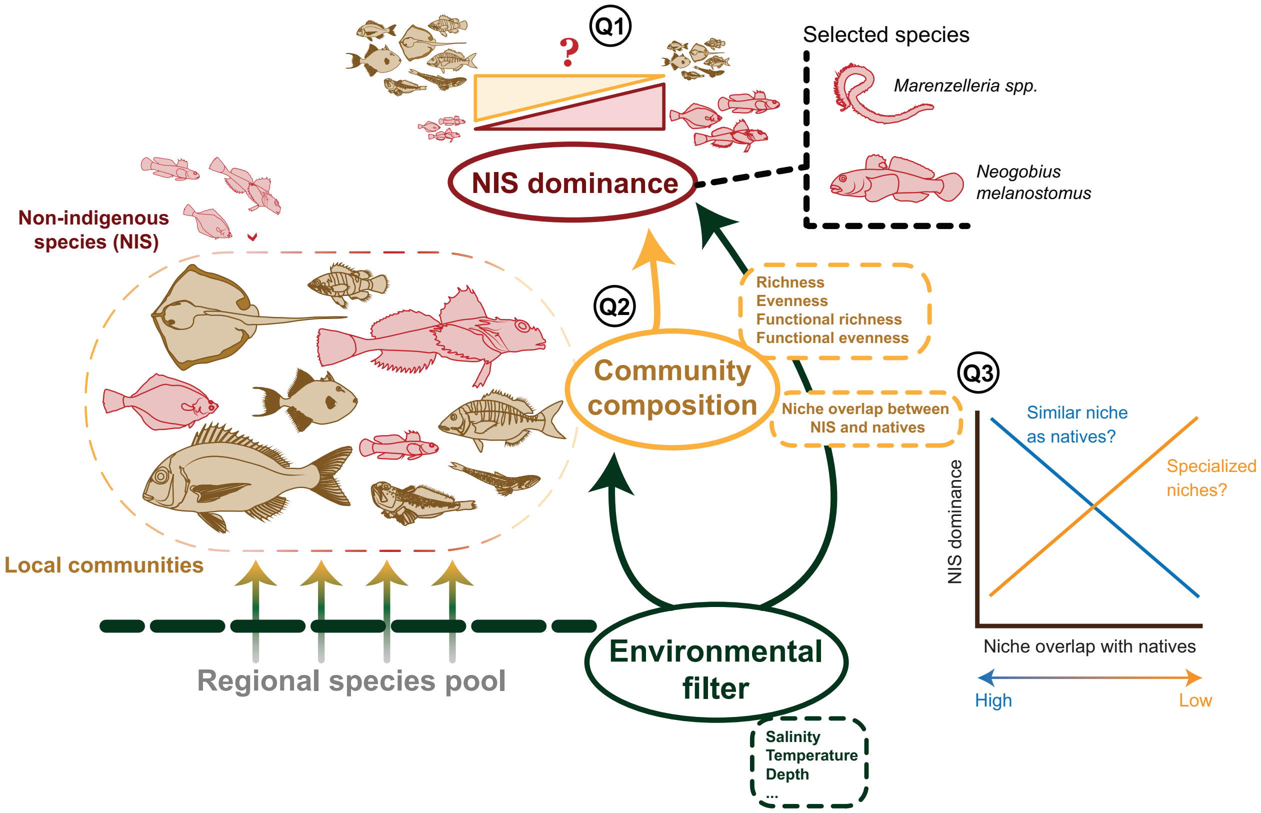

Figure 1. Conceptual figure summarising the key assembly processes acting on community composition and NIS dominance along with the three main questions and selected species.

Community assembly theory predicts that the environment is more important in shaping communities across larger spatial scales, while biotic interactions gain relevance at more local scales (Zobel et al. 1998; Pearson et al. 2018). Given the fine spatial resolution of the analysis (i.e. by sample unit), we therefore expect that the biotic drivers have a greater influence than the abiotic ones in determining NIS dominance (Q1). Furthermore, we assume that the benthic invertebrate and fish communities are subjected through analogous environmental filtering processes and that the dominance of both NIS will respond similarly to the community attributes (i.e. richness and evenness of species and traits; Q2). Finally, we expect that the NIS will adopt different strategies depending on the degree of niche overlap with their corresponding native species (Q3).

Materials and methods

Data collection

We collected available monitoring data on *Marenzelleria* and round goby, as well as the co-occurring native benthic invertebrates and fish species throughout the study area. For *Marenzelleria* and the native benthic invertebrates, wet weight was obtained from the Swedish Ocean Archive (https://sharkweb.smhi.se), containing a total of 3534 unique sampling events from 1993 to 2020 covering the Baltic Sea from the Bothnian Bay in the north-east, to the south-western Baltic Sea (Fig. 2A). Only grab samples were included with approximately 0.1 m^2 area sieved through a 1-mm meshed net. Wet weight was converted to ash-free dry weight (AFDW), with conversion factors obtained from Rumohr et al. (1987) and Gogina et al. (2022). For round goby and the co-occurring fish species, data on wet weight (g) per unit effort (WPUE) was obtained from the Swedish national and regional monitoring programme (https://www.slu.se/en/departments/aquatic-resources1/databases/database-for-coastal-fish-kul/). The data comprised 14 locations sampled from 2005 to 2021, covering most parts of the *Marenzelleria* programme, from the Bothnian Sea in the NE to the south-western Baltic Sea, with a total of 735 sampled communities (Fig. 2B). Two gear types, Nordic coastal multi-mesh gillnets or sets of nets, were used in the selected monitoring locations (see Suppl. material 1: appendix S1 for details). To avoid the inclusion of highly sporadic species, we retained all species representing 99.5% of the total species occurrences in each dataset. This led to a final selection of 173 benthic invertebrates and 27 coastal fish species.

In addition to the monitoring data, we collected available trait information for all species representing the fundamental ecological processes of feeding, growth, reproduction, survival and behaviour following existing trait-based descriptions of marine organisms (Törnroos and Bonsdorff 2012; Litchman et al. 2013). For benthic invertebrates, 10 traits with 56 different trait categories or modalities were selected: size, adult life span, reproductive type, developmental mechanism, environmental position, living habits, feeding habits, mobility, movement method and bioturbation ability (Suppl. material 1: table S1, appendix S1). For coastal fishes, 11 traits with 37 different modalities were selected: habitat switching, parental care, territorial behaviour, diet, temperature preference, development mode, pharyngeal bones, habitat, fin type, body type and length class (Suppl. material 1: table S1, appendix S1).

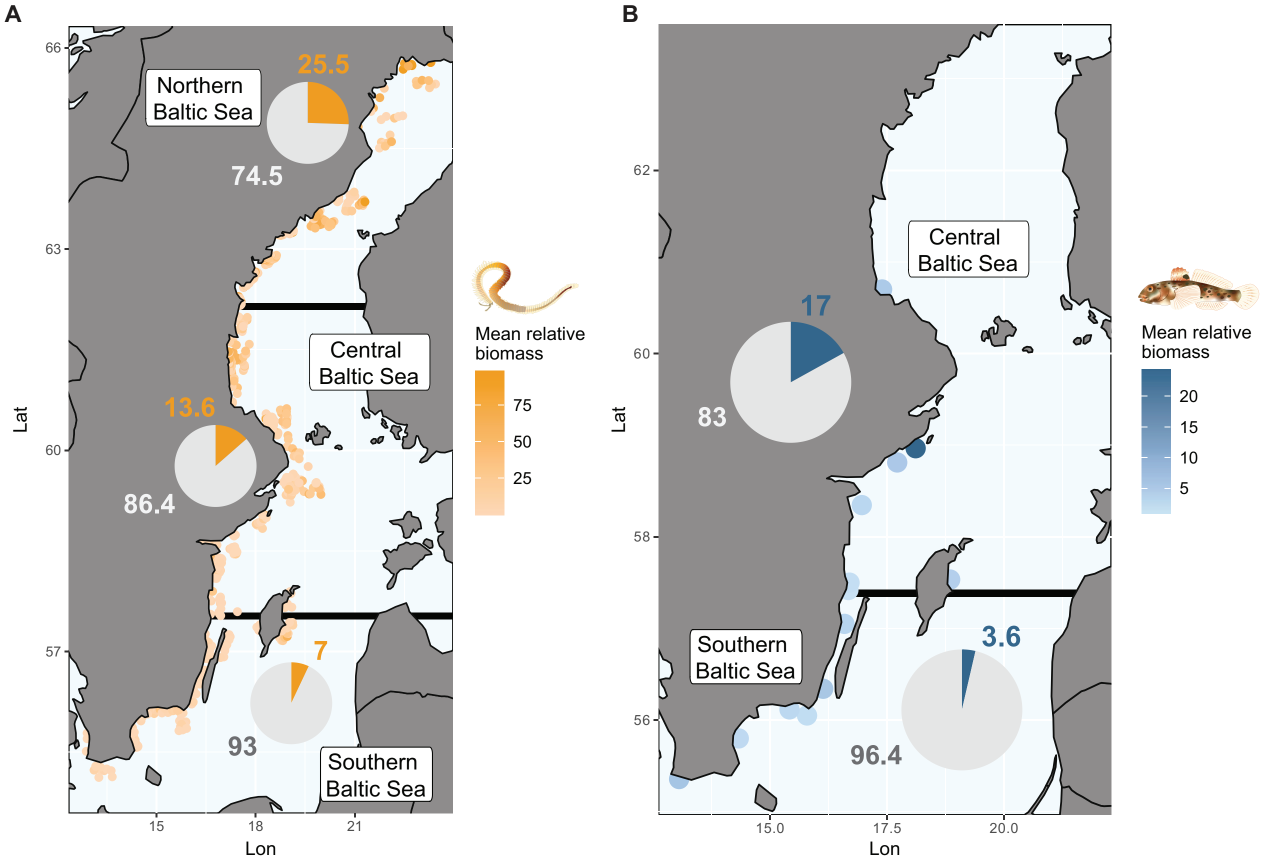

Figure 2. Mean relative biomass of *Marenzelleria* (**A**) and round goby (**B**) at each sampling location. The pie plots illustrate the percentage of total biomass corresponding to NIS (coloured) or native species (grey) in each region.

Biotic variables

On the basis of the data, the relative biomass of NIS compared to the native species at each sample unit was used to represent NIS dominance. Furthermore, to examine potential biotic factors affecting dominance of NIS, we computed several community metrics representing the taxonomic and functional richness and evenness in each sampled community. For species evenness, we used Pielou's Index (J), based on the specific measure of biomass of species at each unique sampling event. Functional richness (FRic) was measured as the minimum amount of functional space (convex hull) filled by all the species in a community (Villéger et al. 2008). Functional evenness (FEve) corresponds to how evenly the species biomass is distributed in the functional space (Villéger et al. 2008). Evenness was calculated with the function *diversity* from the *vegan* package (Dixon 2003) and FRic and FEve were calculated with the function *dbFD* from the *FD* package (Laliberte and Legendre 2010).

To assess the potential individual strategies of NIS in their recipient communities, we further included the functional distinctiveness metric as a predictor. This metric reflects the degree of niche differentiation between species given by their traits, measured as the mean functional distances from one species to all the others within the same community (Violle et al. 2017). If applied to biological invasions, functional distinctiveness provides information about the strategy that a certain NIS could be following in the recipient communities, in relation to how the individual trait expression might be favouring that NIS over the native community

(Vivó-Pons et al. 2023a). We estimated the functional distances between species needed to compute distinctiveness with the function *compute_dist_matrix* from the *funrar* package (Grenié et al. 2017). Further details about the distinctiveness calculation can be found in the Suppl. material 1: appendix S1.

Environmental variables

Spatial differences in salinity and bottom oxygen concentrations, as well as temperature and depth have been shown to influence the structure and composition of benthic invertebrates and fish communities in the Baltic Sea (Gogina et al. 2016; Pecuchet et al. 2016). Hence, we included these four environmental factors as potential predictors in the analysis of NIS dominance for both *Marenzelleria* and round goby. For round goby, we also included chlorophyll A and coastal exposure as environmental predictors since both variables have been regarded to be more relevant in communities from shallower areas in the Baltic Sea (Frelat et al. 2018; Kraufvelin et al. 2018). We did not include these factors for *Marenzelleria*, since this NIS was sampled over a pronounced depth gradient (0–240 m).

Additionally, we computed the standard deviation for the set of environmental predictors by year (*Marenzelleria*), month (round goby) and location to represent the variability and seasonality of environmental conditions. To test for potential multi-collinearity amongst predictor variables, we performed a variance inflation factor (VIF) analysis. Based on the VIF results, we removed bottom temperature variation from *Marenzelleria* (VIF > 5; Suppl. material 1: fig. S2) and species richness from the round goby analysis (VIF > 10; Suppl. material 1: fig. S2). More details about the sources and processing of the environmental data are found in the Suppl. material 1: appendix S1.

Statistical analysis

To assess multiple relationships between NIS dominance and the set of environmental and biotic variables, we used a structural equation modelling (SEM) framework, based on linear mixed models. We first developed a SEM with links considered only between NIS dominance and biotic variables and between biotic variables and environmental drivers separately. After evaluating model fits (Suppl. material 1: appendix 2), we developed a final model where we added all significant direct paths between environmental predictors and NIS dominance identified as missing in the initial setup. We fitted the final SEM for each NIS on the basis of the following setup:

$$\begin{gathered}
\textit{NIS relative biomass} = a + \beta_1\,(\textit{NIS functional distinctiveness}) + \beta_2\,(\textit{Richness}) + \beta_3 \\
(\textit{Evenness}) + \beta_4\,(\textit{Functional richness}) + \beta_5\,(\textit{Functional evenness}) + \beta_6 \\
(\textit{Environmental predictor 1}) + \ldots + \beta_N\,(\textit{Environmental predictor N}) + \\
d(\textit{Year}) + e \ldots n(\textit{Random effects}) + \varepsilon
\end{gathered}$$

where α and β reflect the intercept and regression coefficients for each predictor (N) on NIS relative biomass (as a response) and ε the residual error term. We also tested for non-linear relationships by adding a second term for each predictor variable x that reflected the quadratic effect: $(x - \text{mean}\,(x))^{2}$ (Maureaud et al. 2019). This term was only retained in the final SEM if both the quadratic and non-quadratic term were significant. Additionally, we transformed NIS relative biomass, richness and FRic using a natural logarithm. To account for potential temporal effects of repeated measures over time, all models contained a random effect *d* for year, as well specific random factors to account for other potential biases, such as the type of gear or the sampling location (Suppl. material 1: appendix 2). We tested each model for spatial autocorrelation and selected the best covariance structure (if applicable) using Akaike Information Criteria (AIC). Please note that the same considerations were taken when fitting the separate regression models for each biotic variable, with the environmental variables as predictors and with non-causal links between biotic variables that are correlated. Further details about the initial SEM tests, data exploration, the model setup and formulation can be found in the Suppl. material 1: appendix 2. The linear mixed models were performed using the *lme* function from the *nlme* package (Pinheiro et al. 2017). The SEMs were performed using the package *piecewiseSEM* (Lefcheck 2016). All analyses were conducted in R version 4.1.0.

After model fit and validation, we compared the strength and relative importance of environmental and biotic predictors by summarising the standardised coefficients of all the significant direct and indirect effects on NIS dominance. We estimated indirect effects of environmental variables by multiplying the path coefficients from any environmental variable by the path coefficient of any biotic variable that showed a significant link with NIS dominance. We also estimated the overall effect of environment and biotic variables on NIS dominance by obtaining the absolute sum of all the direct effects within each group.

Results

Overall, both SEMs demonstrate pronounced direct links between the environmental and biotic variables, including dominance (Fig. 3A, B) and indirect effects on NIS dominance channelled through the biotic variables (Fig. 4C, D). Taken together, both SEMs show a slightly higher relative importance of biotic versus environmental variables as direct drivers of NIS dominance, indicated by the higher absolute sum of standardised coefficients (i.e. direct joint effects) across variables within each group (Fig. 4E).

NIS dominance in both SEMs was positively related to species evenness (Fig. 3), but the effect was somewhat stronger for *Marenzelleria* (Fig. 4A). The significant non-linear effect indicates that *Marenzelleria*'s dominance decreases in highly evenly distributed communities. Furthermore, the dominance of *Marenzelleria* and round goby was negatively related to species richness and FRic, respectively (Fig. 3; Fig. 4B, C). Regarding distinctiveness, we found a negative relationship for round goby dominance, whereas a weak positive, but non-significant relationship for *Marenzelleria* (Fig. 3B, Suppl. material 1: table S2). This indicates that round goby is more dominant when it co-occurs with functionally similar species (Fig. 4D). No significant effects were detected for FEve on NIS dominance in the SEMs.

Amongst the set of environmental predictors, the dominance of *Marenzelleria* showed strong positive and negative (non-linear) links with depth and bottom salinity, respectively (Figs 3A, 5E, F). Weaker relationships were found with bottom temperature (negative) and bottom oxygen variation (positive) (Figs 3A, 5G, H). For round goby, we found strong and negative relationships between dominance, bottom temperature and bottom oxygen concentration (Figs 3B, 5G, I).

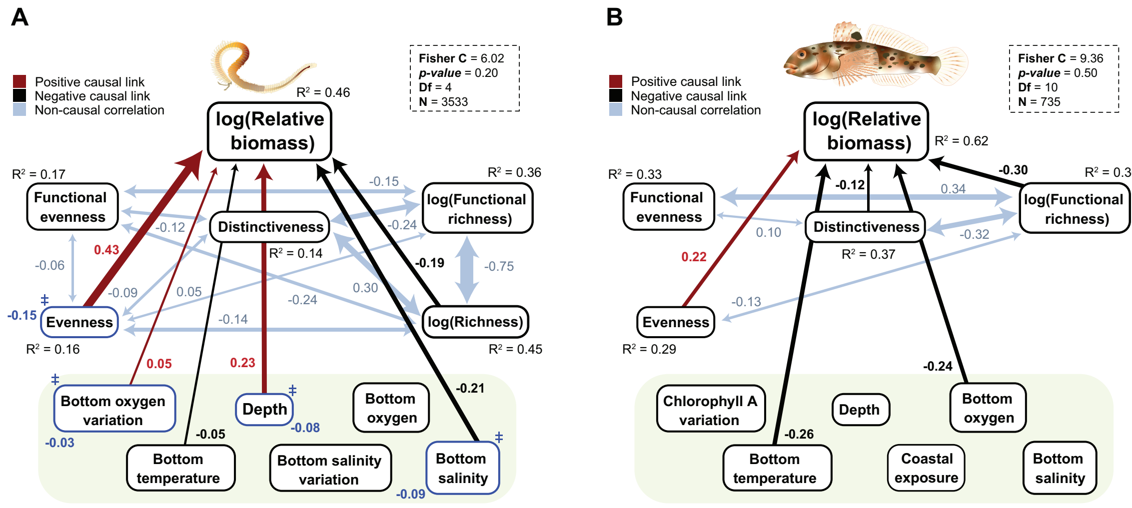

Figure 3. SEM structures for *Marenzelleria* (**A**) and round goby (**B**) dominance showing the direct and indirect links between abiotic and biotic variables. Blue boxes indicate a significant quadratic effect of the corresponding predictor. The values next to the arrows show the standardised coefficients. Non-causal correlations are expressed as light blue arrows. Fisher's C test parameters and corresponding p-value (i.e. goodness-of-fit) of each SEM structure are shown in the dashed box. The coefficient of determination (R^2) is shown for each biotic variable and NIS dominance. The direct links between abiotic and biotic variables are shown in Fig. 4A, B.

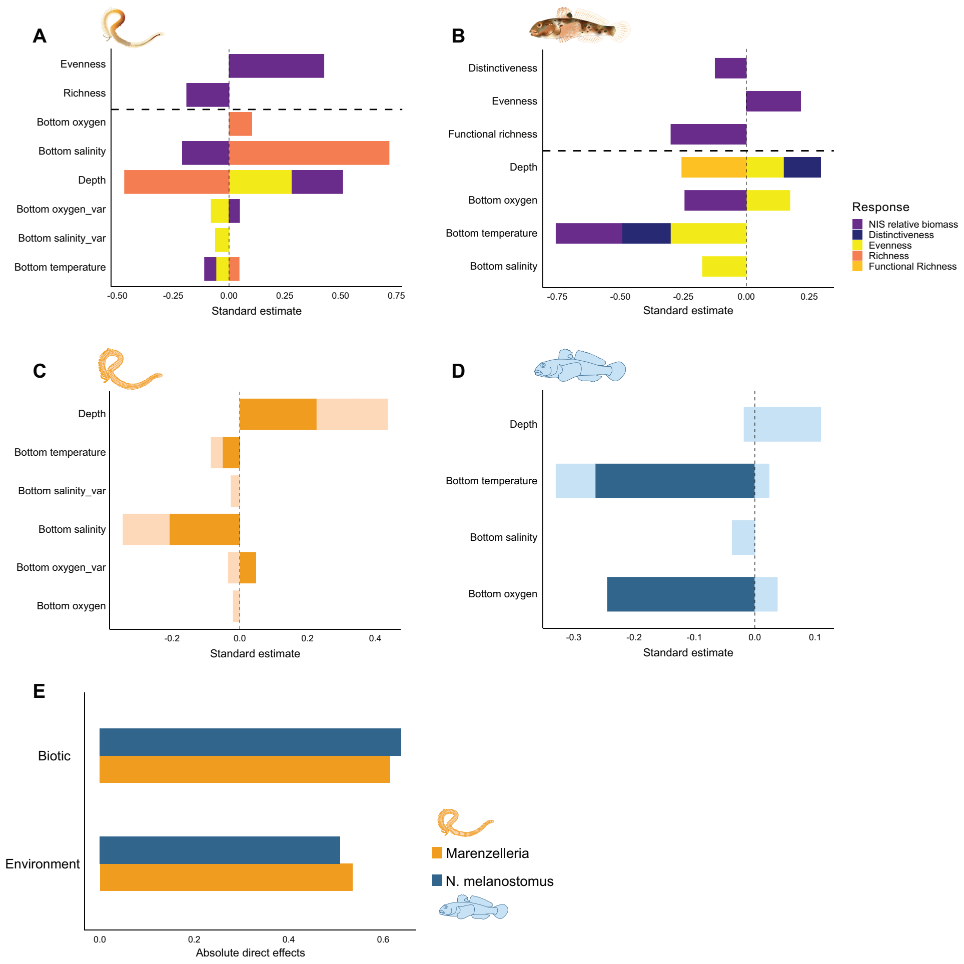

Figure 4. Direct significant effects of the environment on biotic variables and relative biomass on *Marenzelleria* (**A**) and round goby (**B**). Direct (darker colour) and indirect effects (lighter colour) from environmental variables on the relative biomass of *Marenzelleria* (**C**) and round goby (**D**). Cumulative absolute direct effects from both environmental and biotic variables on NIS relative biomass (**E**).

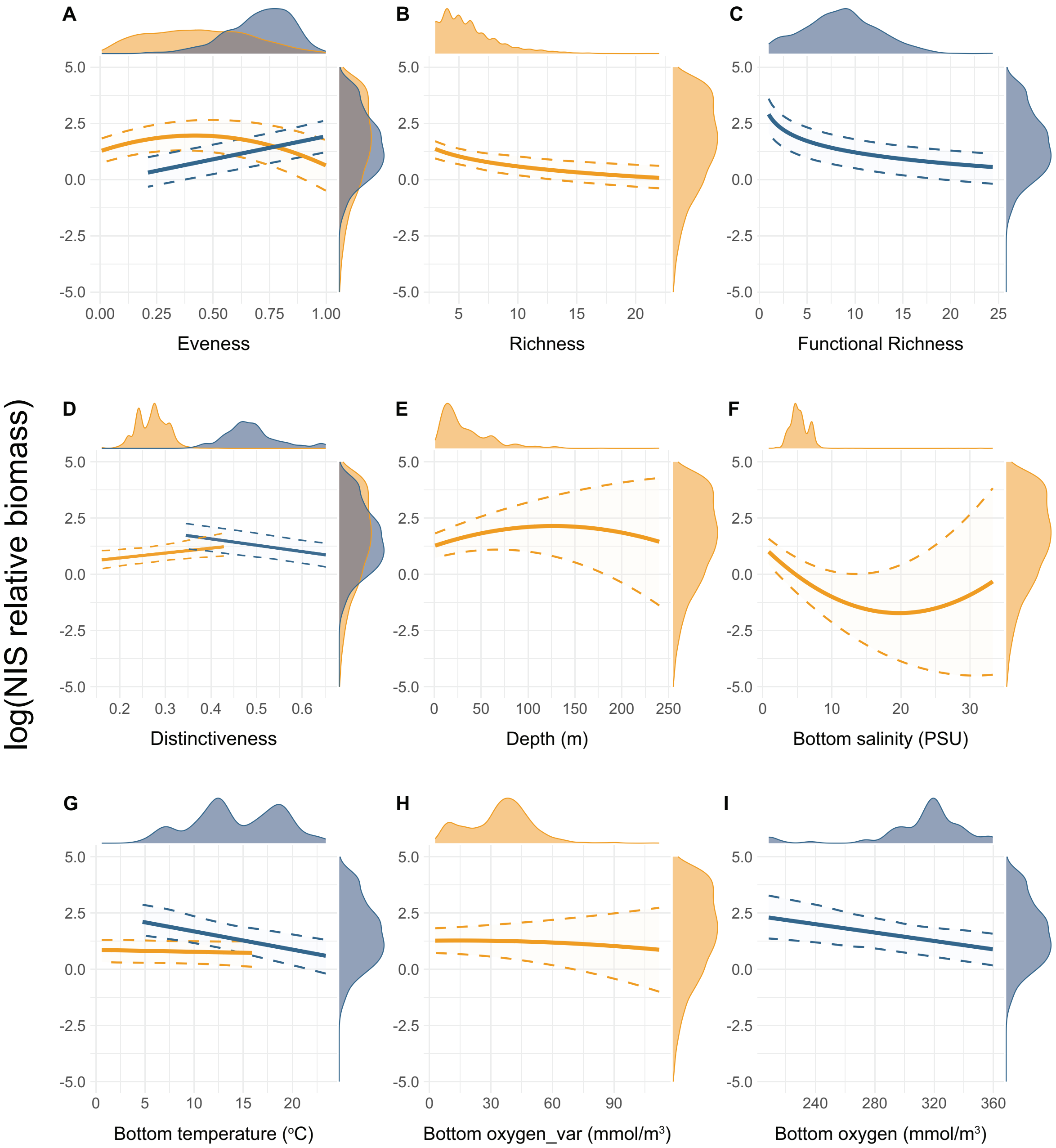

Figure 5. Partial effects plot from all significant variables (**A–I**) illustrating their effects on the relative biomass of *Marenzelleria* (orange) and round goby (blue). Panel D included the non-significant relationship between distinctiveness and NIS dominance for *Marenzelleria* to illustrate the opposite direction of both trends. The y-axis in each plot represents the change of NIS relative biomass values in function of each variable, with its entire range of values represented in the x-axis.

The most important direct predictors affecting richness and evenness in both communities were depth and salinity for *Marenzelleria* along with bottom temperature for round goby (Fig. 4A, B). Additionally, depth and salinity showed large indirect effects on *Marenzelleria's* dominance (Fig. 4C). For round goby, depth and bottom temperature had a pronounced indirect effect on dominance (Fig. 4D).

Marenzelleria's dominance was reasonably well explained by the environmental and biotic variables (r^2 = 46%) (Fig. 3A). Environmental variables also explain a considerable proportion of the variance in two of the biotic variables, FRic (r^2 = 36%) and richness (r^2 = 45%), while distinctiveness (r^2 = 14%), evenness

(r^2 = 16%) and FEve (r^2 = 17%) were less well explained (Fig. 3A). For round goby, the dominance was well explained by both the environment and biotic variables (r^2 = 63%). The biotic variables show a more moderate degree of explained variance; distinctiveness (r^2 = 37%), FRic (r^2 = 36%), FEve (r^2 = 33%) and evenness (r^2 = 29%) (Fig. 3B). The final SEM structures for both NIS showed a favourable goodness-of-fit (Fisher's test with $p > 0.1$) after including several links between NIS dominance and the environmental variables deemed missing in the initial runs (Suppl. material 1: appendix 2, table S2).

Discussion

Our findings indicate a comparable direct influence of environmental conditions and biotic factors on the dominance of NIS, with biotic variables exerting a slightly stronger overall effect. These outcomes emphasise the importance of biotic drivers (i.e. potential biotic interactions) as small-scale community assembly processes, although biotic interactions are also relevant beyond local extents (Wiens 2011; Wisz et al. 2013). Such results conform with previous works (Zobel et al. 1998; Paine et al. 2011; Gaüzère et al. 2023) and support our initial hypothesis. In addition, we observed that environmental filtering processes play a dual role in defining NIS dominance. The abiotic variables indirectly influenced the dominance of NIS by shaping the structure and composition of local communities, but also by determining suitable conditions for NIS to become dominant.

The biotic attributes from the host community showed a similar influence on NIS dominance in both SEMs, in line with our second hypothesis. More specifically, the observed negative relationship with richness suggests that a higher number of native species or functional groups, present at a given sampled location, may provide some sort of biotic resistance towards both NIS (Elton 1958). Indeed, previous studies have shown that richer communities in species, functions or both, tend to hinder the establishment of NIS, as well as their subsequent expansion and impacts (Kennedy et al. 2002; Santamaría et al. 2021; Delavaux et al. 2023). The underlying mechanisms are likely due to increased competition and predation from native species affecting population numbers of NIS through lower growth, reproduction and survival (Kimbro et al. 2013). In addition to richness, the positive effect of evenness, demonstrated in both SEMs, indicates that NIS may have a higher chance of becoming dominant if the biomasses are more equally distributed amongst native species, compared to a situation where the biomass is skewed towards one or a few taxa. Whether this implies that the success of NIS may be lower in the presence of a highly competitive or locally better adapted native species (i.e. highly dominant) is unclear and merit further study.

Amongst the environmental variables considered in the SEMs, only depth and bottom temperature had strong and similar effects on richness and evenness in both communities. This corroborates previous studies on the role of both depth and temperature as primary factors structuring marine communities in the Baltic Sea (Olenin 1997; Gogina et al. 2016) and in marine systems elsewhere (Zintzen et al. 2017; Beukhof et al. 2019). In addition to depth and temperature, salinity also impacted the richness and evenness, especially for benthic invertebrates, thus supporting previous findings on the role of salinity structuring the diversity and composition of marine organisms in the Baltic Sea (Törnroos et al. 2015; Gogina et al. 2016; Pecuchet et al. 2016). Although the dominance of both NIS and the attributes of their associated communities show generally similar responses to several biotic and abiotic drivers, we observed that both NIS can follow different strategies in order to become dominant, as shown by their corresponding relationships with distinctiveness. More specifically, our findings show a higher dominance of round goby when co-existing with functionally similar species, indicating that it might be a good competitor despite native species occupying a similar niche. In the Baltic Sea, round goby has been found to compete with flounder (*Platichthys flesus*) and juvenile cods (*Gadhus morhua*) due to overlapping dietary preferences (Karlson et al. 2007; Ericsson et al. 2021). The apparent success of round goby, even in the presence of competitors, could be due to its aggressive behaviour (Dubs and Corkum 1996; Balshine et al. 2005), coupled with a high degree of territoriality and offspring protection (Vivó-Pons et al. 2023b), which might provide an adaptive advantage for round goby compared to native species, thus limiting the inter-specific competition for resources. Likewise, round goby has also been shown to tolerate a wide range of environmental conditions, specifically for temperature, oxygen and salinity (Christensen et al. 2021; Puntila-Dodd et al. 2021). This broader environmental tolerance could help with increasing the dominance of round gobies under suboptimal conditions, such as low temperatures or hypoxia. For example, oxygen-poor waters may act as physiological refuges where round gobies would not overlap with predators such as Northern pike (*Esox lucius*) or Atlantic cod (Herlevi et al. 2023), as these species appear to largely avoid hypoxic areas (Neuenfeldt 2002; Yamanaka 2013).

While no significant effect of distinctiveness was found for *Marenzelleria*, the observed negative effect of species richness may provide additional insight. It has been observed that *Marenzelleria* has the potential to displace or strongly compete with other native species (Kotta and Ólafsson 2003; Delefosse et al. 2012). However, this NIS can be outperformed in some cases (Kotta et al. 2004) and several of *Marenzelleria's* colonisation events occurred only after a mass mortality event of a potential competitor, the native amphipod *Monoporeia affinis* (Maximov 2011). These results may suggest that *Marenzelleria* performs better in the absence of functionally similar species, likely benefitting from a unique or more specialised niche to become dominant. Such a specialised niche may be further evidenced by the positive direct effect of depth on *Marenzelleria*'s dominance. Deeper waters in the Baltic Sea are often characterised by hypoxic conditions (Jovanovic et al. 2014; Carstensen and Conley 2019). Although such conditions are not optimal for *Marenzelleria*, it can withstand fluctuating oxygen conditions or even anoxic events (Schiedek et al. 1997) thanks to its unique pumping behaviour ensuring access to oxygen more efficiently than native species (Jovanovic et al. 2014). This behaviour may help to explain the higher relative biomass of *Marenzelleria* in deeper areas, facing variable oxygen conditions with frequent hypoxia or anoxia. Similarly, the higher dominance of *Marenzelleria* in low-saline areas could be due to its remarkable tolerance to low salinities by both larvae and adult life-stages (Bochert 1997; Stigzelius et al. 1997).

In conclusion, our results show that local-scale biotic drivers together with the environment constitute key determinants of both NIS dominance in recipient communities. These findings highlight that biotic interactions may play a fundamental role in community assembly at small spatial scales (Wisz et al. 2013). However, biotic interactions are often overlooked in studies about the potential future impacts or distribution of NIS, as the influence of environmental variables and other anthropogenic impacts receive most attention (Geraldi et al. 2020; Bennett et al. 2021; Lindegren et al. 2022). Our findings demonstrate that the diversity and composition of native communities have the potential to control NIS populations (Levine et al. 2004; Santamaría et al. 2021; Delavaux et al. 2023), since the outcomes of interactions with natives (i.e. competition, predation, facilitation) may ultimately define NIS success (Richardson and Pyšek 2012). Finally, the approach used in this study provides a broadly applicable framework to address the potential drivers and community assembly processes influencing NIS dominance in other regions or ecosystem types. In that sense, improved knowledge on where, under what conditions and within which communities NIS can be more successful can enhance spatial management actions directed to address biological invasions (Lodge et al. 2016; Buchadas et al. 2017). Especially at an early stage, fostering preventive actions to keep NIS from becoming invasive and harmful to the overall structure and functioning of ecosystems (Richardson and Pyšek 2012).

Acknowledgements

We wish to thank the Swedish Agency for Marine and Water Management and OKG Aktiebolag for funding the regional and national monitoring programmes together with all the people involved who sorted, identified and measured the included species in the database, ensuring the access and availability to high-quality.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

A.V.P and M.L. acknowledge financial support from the European Union’s Horizon 2020 projects “Mission Atlantic” (ID: 862428), “B-USEFUL” (ID: 101059823) and “ACTNOW” (ID: 101060072). P.D.V.D. was funded by the European Union's Horizon 2020 Research and Innovation Programme under the Marie Sklodowska-Curie grant agreement No. 101024886. C.J was funded by Villum Fonden (agreement No. 25512).

Author contributions

A.V.P. and M.L. conceived the ideas and designed methodology; A.V.P., D.vD. and M.L. conducted the main research with contributions of C.J. and L.F.; A.V.P. analysed the data with contributions of M.L., D.vD. and L.F.; A.V.P. and M.L. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data availability

The data and R code used in this study are publicly available in Dryad (https://doi.org/10.5061/dryad.4f4qrfjkr) and GitHub (https://github.com/ToniVP/NIS_dominance).

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Supplementary material 1

Supplementary information

Authors: Antoni Vivó-Pons, Pieter Daniël van Denderen, Louise Flensborg, Cornelia Jaspers, Martin Lindegren

Data type: docx

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Link: https://doi.org/10.3897/neobiota.94.128736.suppl1

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 179–203 (2024)
DOI: 10.3897/neobiota.94.121987

Research Article

Exploring the efficacy of predacious diving beetles as potential nature-based solution for combatting the invasive mosquito *Aedes albopictus* (Skuse, 1894)

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Academic editor: Sabrina Kumschick
Received: 1 March 2024
Accepted: 3 July 2024
Published: 2 August 2024

Citation: Vanslembrouck A, Scheers K, Vermeersch X, Hendrickx R, Schneider A, De Witte J, Deblauwe I, Van Bortel W, Reuss F, Müller R (2024) Exploring the efficacy of predacious diving beetles as potential nature-based solution for combatting the invasive mosquito *Aedes albopictus* (Skuse, 1894). NeoBiota 94: 179–203. https://doi.org/10.3897/neobiota.94.121987

Abstract

The invasive mosquito species *Aedes albopictus* (Skuse, 1894) is rapidly spreading in Europe, posing an increasing threat because of its high vector competence for chikungunya and dengue virus. An integrative and eco-friendly control of these populations is required to prevent mosquito-borne disease outbreaks. Traditionally-used insecticides or other chemical control agents are often expensive, harmful to the environment, strictly controlled or completely banned in several countries. Additionally, insecticide resistance is a potential threat. One possibility for biological control agents is the use of native aquatic beetles as natural predators of mosquitoes to boost *Bacillus thuringiensis israelensis* (Bti) interventions. Thirty predatory aquatic beetle taxa were caught in Belgium and kept at the Institute of Tropical Medicine's insectary to test predation rate and prey choice on *Aedes albopictus* and *Culex pipiens* Linnaeus, 1758. Predation rates suggest at least four efficient dytiscid predators that are known to inhabit small, temporary habitats in Europe. Further experiments on prey choice reveal a clear preference for *Aedes albopictus* over alternative larval prey (*Culex pipiens*, *Daphnia* sp., Chaoboridae). We found a strong ecological overlap of the feeding niche of *A. albopictus* and the hunting zone of dytiscid predators in the benthic layer of small waterbodies. Our findings on the efficacy are very encouraging to further assess the potential of native predacious diving beetles as a biological control agent against the invasive *A. albopictus* in Europe.

Key words: Arbovirus, biodiversity, Dytiscidae, invasion, predation

Introduction

The Asian tiger mosquito *Aedes albopictus* (Skuse, 1894) is an invasive species that rapidly spreads throughout Europe (Sherpa et al. 2019). It poses an increasing threat because of its high vector competence for arboviruses such as chikungunya, dengue and zika (Martinet et al. 2019; Ryan et al. 2019). Since 2000, an increase in autochthonous infections of dengue and chikungunya virus was observed in Europe (Giunti et al. 2023), for example, 65 autochthonous cases of dengue were recorded in France in 2022 (Gossner et al. 2018; Cochet et al. 2022).

Aedes albopictus females search for blood meals during the day and prefer human hosts in urban areas (Bonizzoni et al. 2013), resulting in potential pathogen transmission and biting nuisance. The establishment of invasive mosquitoes in a yet unaffected area is mainly influenced by climate conditions (Schindler et al. 2015; Ryan et al. 2019), globalisation and urbanisation (Deblauwe et al. 2022b). In addition, these mosquito species are also adapting to climatic changes (Kramer et al. 2020, 2021). At the local scale, interspecific interactions and, foremost, the competition for ecological niches may shape the micro-distribution and abundance of a given *A. albopictus* population (Deblauwe et al. 2015; Müller et al. 2018).

To date, there are no effective vaccines or treatments widely available for dengue and chikungunya virus (Flandes et al. 2023; Thomas 2023; Al-Osaimi et al. 2024). Hence, the prevention of those arboviral infections is primarily based on the control of the mosquito vector (Vontas et al. 2012; Bonizzoni et al. 2013; Abdelnabi et al. 2017). The insecticides used for space spraying, indoor residual spraying or container treatment lead to insecticide resistance, which can rapidly develop in mosquitoes (Su et al. 2019; Pichler et al. 2022; Vereecken et al. 2022). In addition, insecticide-based control efforts over the past decades have not been successful in controlling *Aedes* populations (Achee et al. 2019). In Europe, a biological control method with *Bacillus thuringiensis israelensis* (Bti) is widely used to control mosquito populations (Giunti et al. 2023); however, its activity period is limited (Kroeger et al. 2013; Pauly et al. 2022). In addition, cryptic and domiciliary larval habitats may frequently go unnoticed (Achee et al. 2019). The enrichment of native aquatic biodiversity might be a promising nature-based solution to control discontinuously distributed *A. albopictus* populations. This form of biological control can synergise other control strategies, such as Bti, by including cryptic or domiciliary larval habitats, which often remain undetected or unidentified in other control strategies (Achee et al. 2019; Donald et al. 2020). Additionally, biological vector control generally receives more public acceptance if compared to chemical control (Reuss et al. 2020).

Alternative strategies are mainly focused on adult control and involve *Wolbachia* bacteria (Caputo et al. 2023), sterile insect technique (Balatsos et al. 2024), release of insects carrying a dominant lethal gene (Dobson 2021), attractive toxic sugar baits (Chiu et al. 2024), mass-trapping (Jaffal et al. 2023), spatial repellents, insecticide treated materials (Senapati et al. 2019), antipathogen genetic modifications and lethal ovitraps (Achee et al. 2019; Jones et al. 2021; Ogunlade et al. 2023). For juvenile stages, the use of a new entomopathogenic fungi (Cafarchia et al. 2022) or autodissemination (such as pyriproxyfen) (Pleydell and Bouyer 2019) are proposed alternative strategies (Achee et al. 2019). Biological mosquito control is so far mainly based on predatory copepods, *Toxorhynchites larvae* and fish; however, none of these control agents is currently used in Europe (Baldacchino et al. 2015; Achee et al. 2019).

Biological vector control increases the necessity to identify the most locally effective natural predators of Culicidae, which is especially true for areas recently invaded by *Aedes albopictus* (Younes 2008; Bofill and Yee 2019). The use of native cyclopoid copepods already proved to be a successful method to control first larval instars of *A. albopictus* (Pauly et al. 2022) and is applied in the Americas, Asia and Oceania (Baldacchino et al. 2015). *Toxorhynchites* species show positive results in the control of *Aedes* species, but they are native to the Tropics with some species found in Asia and North America (Donald et al. 2020; Malla et al. 2023). Moreover, fish such as mosquitofish (*Gambusia* sp.) showed no preference towards mosquito larvae, were introduced far outside its natural distribution range and some species are now considered invasive species (Alcaraz et al. 2008; Jourdan et al. 2021; von der Leyen 2022).

In contrast, many Dytiscidae or predacious diving beetles show a preference to feed on mosquito larvae (Culler and Lamp 2009; Bofill and Yee 2019). The presence of Dytiscidae caused Culicidae populations to decrease significantly (Lundkvist et al. 2003; Chandra et al. 2008; Culler and Lamp 2009; Bofill and Yee 2019). Both larvae and adults of Dytiscidae are considered ubiquitous top predators in lentic systems (Yee 2014), particularly in fishless waters (Larson et al. 2000; Bofill and Yee 2019). Adults of most dytiscid species are capable of active dispersal due to their ability to fly (Bofill and Yee 2019) and many are pioneers occupying freshly-formed waters (Lundkvist et al. 2003; Reyne et al. 2020). They are also found in urban areas (Lundkvist et al. 2002; Liao et al. 2020), which is of paramount importance since urbanisation decreases species diversity and favours *Aedes albopictus* population growth (Perrin et al. 2022).

Dytiscidae are known to migrate, entering a large variety of aquatic habitats and may even have seasonal habitat-shifts or winged migrations (Nilsson and Holmen 1995). Larson et al. (2000) mention that adults of many Dytiscidae are known to disperse readily and are frequently encountered across a wide variety of waterbodies. This also includes artificial habitats as observed by Bameul (1990), Shaverdo et al. (2013) and Nilsson (2024). Fransiscolo (1979) states that isolated individuals can be found anywhere and refers to specimens that were found near and in the sea. Many species reproduce in ephemeral waterbodies, such as small ponds, bogs or ditches with aquatic vegetation. Some even choose small ponds, pits or ditches with little vegetation (Galewski 1971).

Since mosquito larvae are an important prey item for Dytiscidae (Galewski 1971), predacious diving beetles are known to naturally colonise habitats with mosquito larvae, which has been observed by Lundkvist et al. (2003). Especially *Agabus* species seem to prefer smaller waterbodies (Davy-Bowker 2002; Lundkvist et al. 2003).

Onyeka (1983) found eight species of Dytiscidae in 107 litre artificial containers, with 46.3% of 432 specimens testing positive for *Culex pipiens*/*torrentium* antiserum. Dytiscidae also inhabit tree holes, phytotelmata and water-filled leaves (Kitching and Orr 1996; Miller and Bergsten 2016). Kehl and Dettner (2007) observed them in wells, cattle troughs, swimming pools and rain barrels. Balfour-Browne (1940), Yanoviak (2001) and Nilsson (2024) noted dytiscids in open tub aquaria, paddling pools and plastic cups and pans. Bameul (1990) identified 16 species in an urban pool. James (1965) and Bay (1974) found dytiscids in rock pools preying on *Aedes atropalpus*, which breeds in the same habitats as *A. albopictus* (Kesavaraju et al. 2011; Farajollahi and Price 2013). Young (1954) reported *Laccophilus* sp. in puddles, rain barrels, tin cans and water-filled tyres. Bashir et al. (2017, 2018) observed them with *A. albopictus* larvae in temporary pools and latex collection cups, while Sulaiman and Jeffery (1986) noted Dytiscidae preying on *A. albopictus* larvae.

Until now, research on predation by Dytiscidae focused mainly on their habitat characteristics (Ohba and Ushio 2015), type of prey (Culler and Lamp 2009), preferred larval stage of the prey (Chandra et al. 2008; Younes 2008) and dytiscid stages (larvae and adult) predating on mosquito prey (Bofill and Yee 2019). For example, larval *Acilius sulcatus* collected in India consumed 34 specimens of late instar *Culex quinquefasciatus* in 24 h (Chandra et al. 2008). Though literature supports the statement that predacious diving beetles are effective predators of mosquito larvae, it is unknown which species are the most effective and suitable predators, particularly in areas newly invaded by *Aedes albopictus* (Ohba and Takagi 2010). Some studies in India (Kumar et al. 2014) and Malaysia (Sulaiman and Jeffery 1986) showed positive results using Dytiscidae as a natural predator for *A. albopictus* in laboratory and field conditions. Bashir et al. (2018) proposed to use a dytiscid species as an efficient biological control agent against *A. albopictus* larvae. In general, literature on the topic is mainly focused on Asia and the United States of America. Only very little research has been conducted on whether and which dytiscid species in Europe would be suitable for integrated biological control of mosquitoes, especially for *A. albopictus*. Are all dytiscid species equally suitable as biological control agent against mosquito larvae? Additionally, would aquatic beetles prefer mosquito larvae over other aquatic invertebrates? We here provide research on predatory aquatic beetles for Europe.

We hypothesise that dytiscid species are potentially good biological control agents given that they: 1) show high feeding rate with preference towards Culicidae; 2) are common and widespread throughout Europe and 3) occur in the same region and habitat as *Aedes albopictus* larvae.

In this study, we aimed to assess the feeding preference of 30 predacious diving beetle taxa comparing: 1) mosquito larvae with other aquatic invertebrates and 2) *Aedes albopictus* to *Culex pipiens* larvae. Based on the experimental data, we evaluated whether predacious diving beetles have the potential as a biological control agent against *A. albopictus* and compared the overlap in the field distribution of the most efficient predator species with the points of entry (PoEs) of *A. albopictus* in Belgium.

Methods

Beetle material

A total of 29 species of Dytiscidae and one species of Noteridae were tested. They were collected in semi-permanent pools with a hydrobiological hand net with diameter of 30 cm and a mesh size of 1 mm. Sampling took place in Stekene (51°14'35.5"N, 4°04'11.4"E; Stropersbos), Verrebroek (51°14'44.0"N, 4°14'16.3"E; Haazop) and Kallo (51°15'18.7"N, 4°15'42.4"E; Steenlandpolder) in Belgium on 7 April and 15 November 2021. Species identification was done in the field and nomenclature follows Nilsson (2011) and Nilsson and Hájek (2024). All specimens were maintained in climate chambers (CPS-P530 Climatic Cabinet, RUMED Germany) at the insectary of the Institute of Tropical Medicine and placed at 10 °C with 80% relative humidity and a 16:8 hour light/dark cycle. Adults were maintained together in 200 ml soft water and provided with substrate to hang on to. Larvae were placed separately to avoid cannibalism. Adults and larvae were fed *ad libitum* with frozen chironomid larvae. All specimens were maintained under these conditions two weeks prior to the experiments to allow acclimatisation. The acclimatisation temperature in the insectary mirrored the mean water temperature during sampling of aquatic beetles. Six mixed water samples from the upper half metre of the water column were taken in Flanders (Belgium) on 13 April (10.5 ± 0.6 °C), 19 October (15.6 ± 0.6 °C) and 1 December 2021 (8.6 ± 0.5 °C) using a WTW Multi 3430 and acid electrode WTW IDS Sentix 940. For the experiments, only healthy, active specimens were retained. Beetles were starved 48 to 72 hours before conducting the experiments. Every specimen was placed in a 100 ml cup with 80 ml of soft water and a stone as substrate at 23 °C. Acclimatisation was allowed for a minimum of one hour prior to every experiment.

Mosquito material

Two mosquito species with different feeding strategies were selected as prey for prey-preference studies with aquatic beetles. *Aedes albopictus* (20AAlb.DE-HU.11) and *Culex pipiens* cf. *molestus* (20CPip.BE-ITMf.6) strains used for the experiments were reared in climate chambers (CPS-P530 Climatic Cabinet, RUMED Germany) at the insectary of the Institute of Tropical Medicine (ITM), Antwerp, Belgium. The *C. pipiens* colony originated from larvae collected in Hove, Belgium (51°09'05.2"N, 4°28'45.2"E) and was reared with overlapping generations for one year at 23.8 °C ± 0.7 °C with 80% relative humidity and a 16:8 hour light/dark cycle. The *A. albopictus* colony derived from a lab strain established at Heidelberg University in 2017 and reared at ITM for six months at 28 °C with 80% relative humidity and a 16:8 hour light/dark cycle. All larvae were fed TetraMin (Tetra, Germany) fish flakes *ad libitum* (Bock et al. 2015).

Feeding experiment

Only third and fourth instar *Aedes albopictus* larvae were used during the experiment and were kept at 20 °C with 80% relative humidity and a 16:8 hour light/dark cycle. To test which predacious diving beetles feed on *A. albopictus* during a feeding experiment, five *A. albopictus* larvae were added to a 100 ml cup hosting a single beetle when starting the experiment. After one hour, the surviving larvae were counted excluding moribund and non-moving larvae. Sometimes the predators started feeding on one larvae and stopped after injuring or killing it. Since this predation is also effective as biological control, we included these moribund and non-moving larvae as dead larvae. All data were obtained in triplicate, except for *Dytiscus marginalis* larvae, *Liopterus haemorrhoidalis*, *Bidessus unistriatus* and *Hydaticus seminiger* with one or two replicates. Feeding rate results of beetles collected in April and in November were compared to assess the effect of seasonality on the feeding behaviour.

Prey choice experiments

To test if predacious diving beetles prefer mosquito larvae over other aquatic invertebrates, a four-choice and a two-choice experiment was performed on a set of effective predators. Third and fourth instar mosquito larvae and freshly bought *Daphnia* sp. and Chaoboridae, kept at 20 °C with 80% relative humidity and a 16:8 hour light/dark cycle, were used during the experiments.

In the four-choice experiment, two larvae of *Aedes albopictus*, *Culex pipiens* and *Chaoborus* sp. and five specimens of *Daphnia* sp. were added to a 100 ml cup hosting one beetle when starting the experiment. After one hour, the surviving prey were counted, excluding moribund and non-moving prey. Five beetle species, that showed to be successful predators in the feeding experiment, were tested in one or two replicates.

A two-choice experiment was performed to test if predacious diving beetles prefer *Aedes albopictus* larvae over *Culex pipiens* larvae. Six dytiscid species that showed to be successful predators in the feeding experiment were tested on their preference for *A. albopictus* over *C. pipiens* larvae in duplicate. This limited number of species and replicates tested was depending on available specimens per dytiscid species. Five larvae of both *A. albopictus* and *C. pipiens* were allotted to a 100 ml cup hosting one beetle when starting the experiment. After one hour, the surviving larvae were counted excluding moribund and non-moving larvae.

Photometric assays

To evaluate the potential bias from seasonal sampling and, hence, probably seasonally varying ecophysiological status of aquatic beetles, that could potentially have an effect on their rate of predation, the content of the energy reserves glycogen and lipid of the collected beetles was quantified. Four dytiscid species (*Agabus bipustulatus*, *A. undulatus*, *Hyphydrus ovatus* and *Laccophilus minutus*) that were sampled in high numbers in both April and November, were analysed in triplicate. The length of elytra and wet weight per specimen was measured prior to the homogenisation in order to allow size normalisation. Per specimen, the total content of glycogen, lipids and proteins was analysed via photometric assays according to Van Handel (1985a), Van Handel (1985b) and Bradford (1976), respectively, as described by Bock et al. (2015). Lipid, glycogen and protein concentrations and weight were divided by elytra length to correct for individual size.

Habitat overlap

The habitat overlap between Dytiscidae and invasive mosquitoes such as *Aedes albopictus* is largely understudied. We observed an influence of separation of entomological disciplines and combined observations of Dytiscidae in invasive *Aedes* habitats in Table 3.

Statistical analysis

The analysis of experimental data and data visualisation was carried out with Prism® (version 9.3.1, GraphPad Software Inc., USA). Statistical significance was defined as $P < 0.05$. The Kolmogorov-Smirnov test and Shapiro-Wilk test were used to test for normality and residuals were plotted to test for homoscedasticity. The feeding rate obtained in triplicate in April and November of five dytiscid species (*Agabus bipustulatus*, *Graptodytes bilineatus*, *Hydroporus angustatus*, *Hygrotus impressopunctatus* and *Laccophilus minutus*) was tested for normality via the Kolmogorov-Smirnov test and verified for homoscedasticity via the homoscedasticity plot. Lipid data were log transformed and glycogen data were sine transformed to meet assumptions of normality. A repeated measures two-way ANOVA was conducted to verify differences in variation of the feeding rate between both experimental points of time with factors *Species* and *Seasonality*. To merge data from April and November and to compare lipid, protein and glycogen content, a two-way ANOVA was used to test significant differences in variation. Afterwards, the Šídák's multiple comparisons test was conducted to test the species separately.

To assess the overlap between the distribution of predacious diving beetles and the points of entry of *Aedes albopictus*, distribution and presence data were obtained from the *A. albopictus* surveillance programme in Belgium that has been conducted by ITM from 2007 to 2020 (Deblauwe et al. 2022a, 2022b). The Belgian Hydradephaga Database (managed by Scheers, Research Institute Nature and Forest) and Scheers et al. (in press) provided annual information since 1834 on the distribution (exact location or 1 km quadrant) of the dytiscid species. A distribution table with *A. albopictus* presence and presence/absence data of selected beetle species was created resulting in an overlapping distribution table of both Dytiscidae and PoE of *A. albopictus* in Belgium. Presence/absence data of Dytiscidae was given in following classifications: confirmed presence (already been observed), presumed presence (not been observed, but occurrence in the region and suitable habitat present), plausible presence (not been observed, no occurrence in the region, but suitable habitat present) and not present in the region.

A duplicated scoring with variation of expert judgement was performed to rank the top ten predatory beetle species according to our hypothesis that dytiscid species are potentially good biological control agents when they: 1) show high feeding rate with preference towards Culicidae; 2) are common and widespread throughout Europe and 3) occur in the same region as *A. albopictus* larvae. Scoring of the suitability of a given dysticid species as biological control tool against *A. albopictus* was given on ten, including categories such as habitat suitability (small, temporal, ephemeral waters), abundance, dispersal (ability to fly) and distribution, based on Kehl and Dettner (2007), Nilsson and Hájek (2024) and expert judgement.

Results

Rate of predation on *Aedes albopictus* by aquatic beetles

A total of 369 specimens representing 29 predacious diving beetle taxa (Dytiscidae) and one burrowing water beetle species (Noteridae) were collected (Suppl. material 1: table S1). During the feeding experiment, only *Agabus undulatus* consistently consumed all five *Aedes albopictus* larvae in one hour in April. However, the beetles collected in November showed no interest in feeding (Fig. 1). Other *Agabus* species, such as *A. bipustulatus* and *A. nebulosus*, also proved to be very effective predators, consuming on average 83% and 76% of the provided *Aedes albopictus* larvae, respectively. *Rhantus exsoletus* and *Hyphydrus ovatus* consumed 80% and 67%, respectively; however, no *H. ovatus* showed interest in feeding during experiments in November. The Agabinae larvae consumed on average 40% of the *Aedes albopictus* larvae. *Hygrotus impressopunctatus* and *H. parallellogrammus* consumed on average 37% and 53% of the larvae, respectively. *Hydroporus figuratus* fed on average on 47% and *Ilybius quadriguttatus* and *Hydroporus palustris* consumed 40% and 32% of the provided larvae, respectively. *Laccophilus minutus* consumed on average 43% of the larva. All other species (*Acilius sulcatus*, *Clemnius decoratus*, *Colymbetes fuscus*, *Dytiscus marginalis* adults, *Graptodytes bilineatus*,

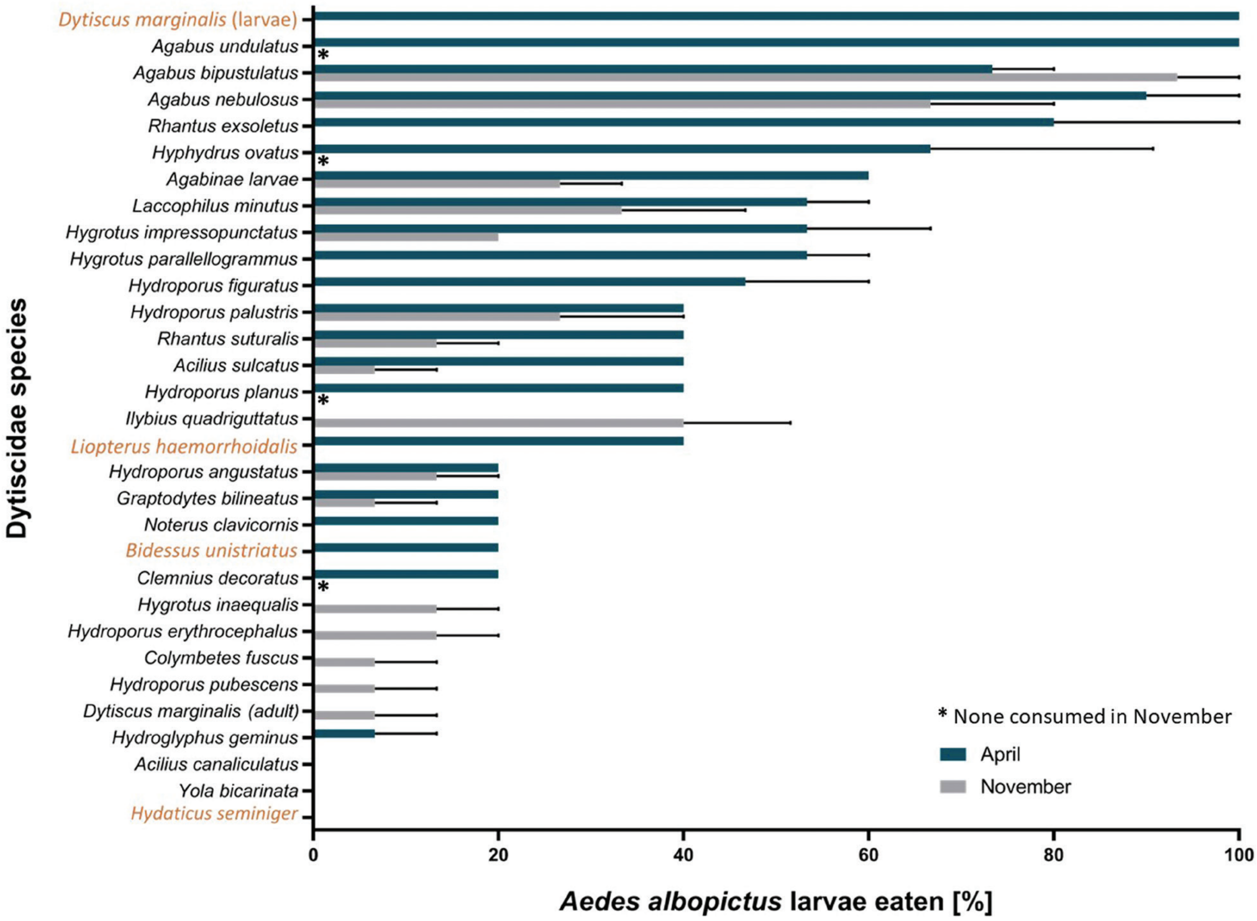

Figure 1. Rate of predation on *Aedes albopictus* larvae by different aquatic beetle species [% larvae eaten per hour]. The percentage of eaten mosquito larvae (n = 5) per dytiscid species during one hour is separately shown for dytiscid specimens collected in the field in either April or November (mean = 3, less replicates for species marked in orange font). * None consumed in November: no predation observed.

Hydroporus angustatus, *H. erythrocephalus*, *H. planus*, *H. pubescens*, *Hydroglyphus geminus*, *Hygrotus inaequalis*, *Noterus clavicornis* and *Rhantus suturalis*) consumed less than 20% of the provided larvae. No predation was observed by *Acilius canaliculatus* nor *Yola bicarinata*. *Dytiscus marginalis* larvae, *Liopterus haemorrhoidalis*, *Bidessus unistriatus* and *Hydaticus seminiger* consumed 100%, 40%, 20% and 0% of the larvae, respectively. These latter species were tested with less than three replicates per species. The following Belgian aquatic beetles were identified as effective predators on *Aedes albopictus*: *Agabus bipustulatus*, *A. nebulosus*, *A. undulatus*, *Hydroporus figuratus*, *Hygrotus impressopunctatus*, *H. parallellogrammus*, *Hyphydrus ovatus*, *Ilybius quadriguttatus*, *Laccophilus minutus* and *Rhantus exsoletus.* These species were included in further experiments.

Normality of the feeding rate data was assumed for both April (P > 0.1) and November (P > 0.1) datasets. The repeated measures two-way ANOVA indicated that the factor *Beetle species* (73.90%) accounted for most of the variation in the feeding rate and was highly significant (F = 20.82; P < 0.001). The interaction between the factors *Species x Seasonality* (9.19%) and *Seasonality* (3.34%) were significant (F = 1.89; P = 0.02 and F = 7.11; P = 0.02, respectively). Therefore, the seasonal rate of predation was separately shown for each of two sampling months (Fig. 1). Overall, the experimental block *Month* was not a significant factor (F = 1.89; P = 0.17), but it accounted for 8.87% of total variation. The seasonal predation efficiency of *Hydroporus angustatus* (P = 0.96), *Graptodytes bilineatus* (P = 0.60), *Laccophilus minutus* (P = 0.22) and *Agabus bipustulatus* (P = 0.22) was not significantly different between the months, but Šídák's multiple comparisons showed that the feeding rate of *Hygrotus impressopunctatus* was significantly different between April and November (P = 0.02), with a higher predation rate in April compared to November.

Prey preference of Dytiscidae

1. Dytiscid preferences towards *Aedes albopictus*, *Culex pipiens*, *Chaoborus* and *Daphnia*

From the five most predatory Dytiscidae, both *Agabus nebulosus* and *A. undulatus* preyed on all four prey choices (Fig. 2a). Four species - *A. nebulosus*, *A. undulatus*, *Laccophilus minutus* and *Rhantus exsoletus* - consumed all provided *Aedes albopictus* larvae. Only one species - *A. nebulosus* - consumed all *Culex pipiens* larvae. *Daphnia* sp. were consumed by *A. nebulosus*, *A. undulatus* and *R. exsoletus* and *Chaoborus* larvae were eaten by *A. nebulosus* and *A. undulatus*.

2. Dytiscid preferences towards *Aedes albopictus* and *Culex pipiens*

The six dytiscid species all preyed on *Aedes albopictus* larvae, for which *Agabus undulatus* ate all five larvae in one hour (Fig. 2b). In contrast, only four species, *A. bipustulatus*, *A. undulatus*, *Hygrotus parallellogrammus* and *Laccophilus minutus* also consumed *Culex pipiens* larvae. For most experiments, the dytiscid specimen started feeding on *Aedes albopictus* larvae and switched to *C. pipiens larvae* when *A. albopictus* larvae were depleted.

The effect of season on dytiscid ecophysiology exemplified by four abundant beetle species

Overall, the weight of the aquatic beetles *Agabus bipustulatus*, *A.undulatus*, *Hyphydrus ovatus* and *Laccophilus minutus* was not significantly different between specimens collected in April or November (Table 1). The energy reserves of aquatic

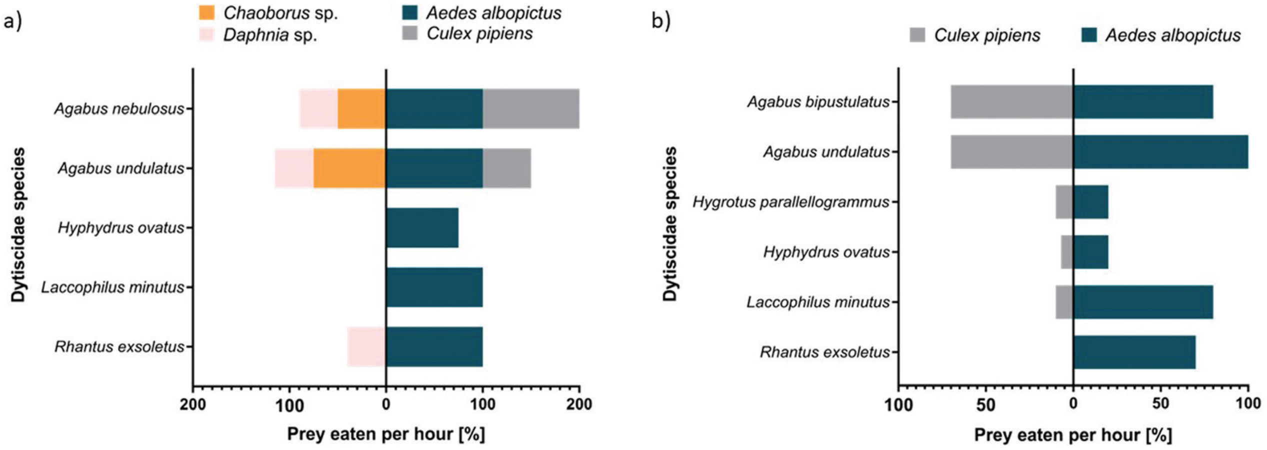

Figure 2. The preferred invertebrate prey of aquatic beetles [% prey eaten per hour] **a** four prey choice experiment offering two larvae of *Aedes albopictus*, *Culex pipiens* and *Chaoborus* sp. each and five *Daphnia* sp. in a volume of 80 ml of water for one hour **b** two prey choice experiment offering five larvae of *A. albopictus* and *C. pipiens* each in a volume of 80 ml of water for one hour.

Table 1. Ecophysiological status of aquatic beetles collected in April and November. The weight [mg], the content of energy reserves and the protein concentration of aquatic beetles [size-corrected lipid, glycogen and protein concentration in µg per adult] are shown for four top predator Dytiscidae.

Weight [mg]	**Mean ± SD April**	**Mean ± SD November**
Agabus undulatus	6.44 ± 0.49	5.75 ± 0.17
Agabus bipustulatus	12.41 ± 0.15	12.56 ± 0.01
Hyphydrus ovatus	4.39 ± 0.56	4.96 ± 0.51
Laccophilus minutus	2.15 ± 0.05	1.75 ± 0.08
Lipids [µg/pupae]	**Mean ± SD April**	**Mean ± SD November**
Agabus undulatus	111.38 ± 14.34	84.19 ± 4.60
Agabus bipustulatus	291.20 ± 49.31	142.35 ± 20.60
Hyphydrus ovatus	80.60 ± 22.58	75.28 ± 4.78
Laccophilus minutus	83.16 ± 11.99	92.38 ± 5.42
Proteins [µg/pupae]	**Mean ± SD April**	**Mean ± SD November**
Agabus undulatus	146.86 ± 7.12	145.33 ± 1.04
Agabus bipustulatus	124.19 ± 3.41	125.05 ± 1.28
Hyphydrus ovatus	25.41 ± 18.33	67.92 ± 19.57
Laccophilus minutus	27.02 ± 4.56	62.89 ± 10.68
Glycogen [µg/pupae]	**Mean ± SD April**	**Mean ± SD November**
Agabus undulatus	22.34 ± 5.70	22.40 ± 4.22
Agabus bipustulatus	31.99 ± 10.39	23.80 ± 6.31
Hyphydrus ovatus	37.86 ± 11.05	118.24 ± 10.11
Laccophilus minutus	3.56 ± 1.05	20.75 ± 2.26

beetles were likewise quite uniform in spring and autumn. However, the lipid content of *A. bipustulatus* was significantly higher in the specimens sampled in April compared to November (P = 0.003) and the glycogen content of *H. ovatus* was much lower if specimens have been sampled in April compared to November. Likewise, the protein content of *L. minutus* and *H. ovatus* was lower in specimens sampled in April compared to November.

Distribution overlap between Dytiscidae and *Aedes albopictus* in Belgium

The known point of entry of *Aedes albopictus* in Belgium were tyre companies, parking lots, a port and a Lucky Bamboo import company (Deblauwe et al. 2022a, 2022b). At least three of the topmost predatory species were expected to be present in the region where *A. albopictus* were already found (Table 2).

Habitat overlap between Dytiscidae and Culicidae

Aedes albopictus has been reported from artificial habitats such as pots, buckets, manhole/scupper and rain barrels with a typical water volume ranging from less than one litre up to 200 litres (Graziosi et al. 2020), which are similar habitats observed for Dytiscidae by Young (1954), Onyeka (1983), Sulaiman and Jeffery (1986) and Bashir et al. (2018). Since the habitat overlap between Dytiscidae and invasive mosquitoes is largely understudied, observations of Dytiscidae in

Table 2. Distribution overlap between Dytiscidae within 5 km from a points of entry (PoE) of *Aedes albopictus*.

Selected beetle species	**Points of Entry of *Aedes albopictus***										**Points of Entry of *Aedes albopictus***		
	AB	**AT**	**BA**	**E0**	**E12**	**E2**	**E5**	**E6**	**EB**	**PA1**			
Agabus bipustulatus	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	🟧	**Tire companies**	AB	Kallo
Agabus nebulosus	🟧	🟧	🟧	⬜	🩷	⬜	⬜	🟧	🟧	🟧		AT	Vrasene
Agabus undulatus	🟧	🟧	⬜	⬜	⬜	⬜	⬜	🩷	🟧	🟧		BA	Frameries
Hydroporus figuratus	🟧	🟧	⬜	⬜	⬜	⬜	⬜	🟦	🟧	🟧	**Parking lots**	E0	Sprimont
Hygrotus impressopunctatus	🟧	🟧	🟦	⬜	🩷	🩷	⬜	🟧	🟧	🟧		E12	Eghezée
Hygrotus parallellogrammus	🟧	🟧	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟧		E2	Messancy
Hyphydrus ovatus	🟧	🟧	🟧	🟦	🟧	🟦	🟦	🟧	🟧	🟧		E5	Wanlin
Ilybius quadriguttatus	🟧	🟧	🩷	⬜	⬜	🩷	⬜	🟧	🟧	🟧		E6	Kortrijk
Laccophilus minutus	🟧	🟧	🟧	🟦	🟧	🟦	🟧	🟧	🟧	🟧	**Lucky Bamboo**	EB	Lochristi
Rhantus exsoletus	🟧	🟧	🩷	⬜	⬜	⬜	⬜	⬜	⬜	🟧	**Port**	PA1	Antwerp

Orange: confirmed presence within 5 km (since 2010); blue: presence within 5 km not confirmed but presumed; rose: presence within 5 km not confirmed but plausible; gray: not present in the region.

artificial habitats that were made or verified by the authors were added to Table 3. These observations include plastic trays, plastic foil, buckets, bird baths, ornamental water features, cattle drinking troughs, rain barrels, shallow puddles, garden ponds and disused swimming pools, ranging from less than one litre up to 250 litres. These observations were made in small city gardens, urban parks, suburban gardens, agricultural areas, heathland, nature reserves and forests in Belgium.

Ecological portfolio of the topmost predatory dytiscid species

Agabus bipustulatus has the highest total scoring because of its wide habitat preference, abundance, excellent dispersal ability (Kehl and Dettner 2007) and wide distribution throughout Europe (Nilsson and Hájek 2024) (Table 4). *Agabus nebulosus*, *Laccophilus minutus* and *Hygrotus impressopunctatus* are also suitable species. *Agabus undulatus*, *Hyphydrus ovatus* and *Hydroporus figuratus* were excluded due to their reduced flight ability (Kehl and Dettner 2007). *Ilybius quadriguttatus* was excluded due to its preference for well-vegetated semi-permanent lentic habitats and *Hygrotus parallellogrammus* and *Rhantus exsoletus* were excluded, based on both habitat preference (coastal habitat and vegetated permanent waterbodies, respectively) and distribution (Nilsson and Holmen 1995; Nilsson and Hájek 2024).

Discussion

This study is a first step to understand the value of the use of native Dytiscidae and Noteridae species as a biological *Aedes albopictus* control tool. Firstly, a high feeding rate on *A. albopictus* has been observed in several dytiscid species. Based on Kehl and Dettner (2007), Nilsson and Hájek (2024) and expert judgement, these species resulted in a top ten of best predators for *A. albopictus* larvae. Two prey-choice experiments carried out with a selection of the top ten predators revealed a clear prey preference towards mosquito larvae and especially towards *A. albopictus* larvae. For Belgium, a significant distribution overlap was detected for three efficient

Table 3. Dytiscidae found or verified in small and artificial habitats by the authors in Belgium, overlapping with habitats of *Aedes albopictus*.

Water volume (L)	Larval habitat	Dytiscidae species	Number of individuals	Stage
<1	plastic tray	*Agabus bipustulatus*	1	Adult
4	puddle in piece of plastic foil	*Agabus bipustulatus*	1	Adult
5	bucket	*Hydroporus dorsalis*	1	Adult
		Hydroporus planus	4	
		Hydroporus tesselatus	2	
		Hydroporus pubescens	1	
		Rhantus suturalis	1	
10	small steel bird bath	*Agabus bipustulatus*	1	Adult
		Hydroglyphus geminus	5	
		Hydroporus planus	2	
15	display table for pond plants	*Hydroglyphus geminus*	1	Adult
	small concrete bird bath	*Hydroporus pubescens*	2	Adult
20	disused metal cattle drinking trough	*Agabus bipustulatus*	1	Adult
		Hygrotus inaequalis	4	
	small wooden ornamental water feature	*Hydroporus planus*	1	Adult
30	disused prefab plastic water feature	*Agabus bipustulatus*	9	Adult
		Hydroporus nigrita	3	
		Hydroporus tesselatus	1	
40	disused prefab plastic water feature	*Agabus bipustulatus*	10	Adult
		Hydroporus nigrita	10	
		Ilybius chalconatus	1	
50	cattle watering basin	*Agabus bipustulatus*	21	Adult
		Hydroglyphus geminus	2	
		Hydroporus planus	3	
		Hydroporus pubescens	3	
	plastic ornamental water feature	*Agabus bipustulatus*	>10	Adult
60	concrete water feature	*Hydroporus tesselatus*	1	Adult
90	rain barrel	*Agabus bipustulatus*	1	Adult
		Dytiscus marginalis	1	
	cattle drinking bucket	*Rhantus suturalis*	1	Adult
100	metal cattle drinking trough	*Agabus bipustulatus*	30	Larvae
		Hydroporus discretus	2	Adult
	plastic drinking container for cattle	*Agabus bipustulatus*	3	Adult
		Hydroporus planus	6	
		Rhantus suturalis	2	
	shallow puddle on concrete slab at construction site	*Agabus nebulosus*	1	Adult
		Hydroglyphus geminus	>10	
		Hydroporus planus	1	
		Rhantus suturalis	3	
150	plastic drinking container for cattle	*Agabus uliginosus*	1	Adult
	shallow puddle on concrete slab at construction site	*Agabus bipustulatus*	1	Adult
		Colymbetes fuscus	1	
		Hydroglyphus geminus	>10	
		Hydroporus palustris	1	
		Hydroporus planus	>10	
		Rhantus suturalis	2	
220	rain barrel	*Acilius sulcatus*	1	Larvae
250	metal cattle drinking trough	*Agabus bipustulatus*	1	Adult

Table 4. Ecological portfolio of the top ten dytiscid predators for *Aedes albopictus* based on Kehl and Dettner (2007), Nilsson and Hájek (2024) and expert judgement.

Habitat	To what extent does this species occur in small ephemeral waterbodies?
Common	Is this species common?
Dispersal	How is this species' ability to fly?
Distribution	To what extent does this species occur in Europe?

Selected beetle species	**Habitat**	**Common**	**Dispersal**	**Distribution**	**Total scoring [%]**
Agabus bipustulatus	7.5	10.0	10.0	10.0	93.75
Agabus nebulosus	6.0	8.0	9.5	9.5	82.50
Laccophilus minutus	3.5	9.5	10.0	10.0	82.50
Hygrotus impressopunctatus	3.5	8.0	8.5	8.5	71.25
Rhantus exsoletus	2.5	5.5	10.0	8.5	66.25
Ilybius quadriguttatus	1.0	7.5	10.0	6.5	62.50
Hyphydrus ovatus	1.0	9.5	1.0	8.5	50.00
Hygrotus parallellogrammus	1.0	1.5	8.0	7.5	45.00
Hydroporus figuratus	1.0	5.0	3.5	6.5	40.00
Agabus undulatus	1.0	2.0	1.0	4.5	21.25

predator species (*Agabus bipustulatus*, *A. nebulosus* and *Laccophilus minutus*) and ten points of entry where *Aedes albopictus* has been introduced in Belgium between 2007 and 2020 (Deblauwe et al. 2022b). The results are very encouraging to further explore the use of Dytiscidae as a potential biological control agent against *Aedes albopictus*.

The obtained results showed evidence for efficacy of predacious diving beetles to predate on *Aedes albopictus* larvae. Following our observations, of the topmost predatory dysticid species are *Agabus undulatus*, *A. bipustulatus*, *A. nebulosus*, *Rhantus exsoletus*, *Hyphydrus ovatus*, *Laccophilus minutus*, *Hygrotus impressopunctatus*, *H. parallellogrammus*, *Hydroporus figuratus* and *Ilybius quadriguttatus*. The tested beetle larvae proved to be good predators, which is in line with Chandra et al. (2008), Culler and Lamp (2009) and Bofill and Yee (2019). However, dytiscid larvae were not included in the topmost predator list, since the active dispersal of the predators is necessary for biological control. Adults are found in a larger variety of waterbodies, while dytiscid larvae are generally ecologically more specialised than adults (Galewski 1971). It is important to note that Dytiscidae show different types of feeding behaviour in their life stages. Larvae can inject digestive protease enzymes and feed on the prey's body contents (Yee 2014). In the majority of dytiscids, the first larval stage has shorter mandibles and feeds mostly on planktonic Crustacea, but older larval stages have longer mandibles and hunt insect larvae such as Culicidae (Galewski 1973). Especially larvae from the genera *Agabus*, *Colymbetes*, *Hydroporus* and *Rhantus* are known to feed mainly on mosquitoes (Galewski 1971). Adults have chewing mouthparts and are thus gape-limited (Yee 2014); however, they will attack any prey they can overtake (Galewski 1971).

The efficient predation on mosquito larvae by *Agabus* species are in line with Ohba and Takagi (2010), where they found a predation rate on fourth instar *Culex tritaeniorhynchus* of 100% and 55% for *Agabus conspicuous* and *A. japonicus*, respectively. The feeding rate for *Hyphydrus ovatus* is comparable to the observation in Ohba and Takagi (2010), with a 44% predation rate for *H. japonicus* on *Culex tritaeniorhynchus*. A similar predation rate was reported for *Laccophilus minutus* on

C. pipiens and *L. difficilis* on *C. tritaeniorhynchus* of 43% and 39%, respectively. Evidence for predation on *Aedes atropalpus* was found for *Laccophilus* sp. (James 1965). The predation rate for *Hydroglyphus geminus* is in line with a 16% feeding rate in *H. japonicus*. In contrast to current observations, *Rhantus suturalis* had a feeding rate of 99% on *C. tritaeniorhynchus* (Ohba and Takagi 2010).

The prey choice experiments showed a clear feeding preference towards Culicidae, specifically to *Aedes albopictus*. When *A. albopictus* was depleted, a switchover to *Culex pipiens* was observed several times, which is in line with Culler and Lamp (2009) and Ohba and Ushio (2015). *Aedes albopictus* was presumably preferred due to its foraging behaviour, since its larvae tend to feed in the benthic zone in a flexing behaviour. In contrast, *C. pipiens* filter-feeds near the water surface and has a motionless behaviour to avoid predation (Yee et al. 2004; Ohba and Ushio 2015). Three videos demonstrating this behaviour are added as supplementary material (Suppl. materials 2–4). Predacious diving beetles tend to hunt their prey in the benthic zone (Lundkvist et al. 2003); hence, they encounter more *A. albopictus* larvae. Chances are high for introduced *A. albopictus* to encounter a suitable predator, since the distribution of these predacious diving beetles largely overlap with at least two species present in the known PoE of *A. albopictus* in Belgium. Some dytiscids occupy similar habitats as *A. albopictus* (Table 3) and are active all year round, except when freezing (Foster and Friday 2011).

In addition to *Aedes albopictus*, there are also two other invasive mosquitoes in Europe, i.e. *A. japonicus* (Theobald, 1901) and *A. koreicus* (Edwards, 1917) (Deblauwe et al. 2022b). Both species share a similar larval foraging behaviour as *A. albopictus* and, therefore, similar results in predation by dytiscids are expected. These species and especially *A. japonicus*, often occur in more natural habitats in their non-native range (Smitz et al. 2021; Deblauwe et al. 2022b) and, therefore, have an even larger overlap in habitat preference with native Dytiscidae. Most probably the foraging behaviour of these mosquito larvae dictates in large part the suitability of aquatic beetles as native predators.

Coinciding with a higher feeding rate in November, lipid concentration was much lower in November compared to April for *Agabus bipustulatus*, which may indicate a shortage of lipids before winter (Arrese and Soulages 2010). *Agabus bipustulatus* is known to be active during winter (Classen and Dettner 1983) and to hunt underneath ice (pers. observ. K. Scheers). For *A. undulatus*, no differences in weight, lipid, protein and glycogen content were found, which coincides with their disinterest to feed in November. This storage in energy and reduced feeding behaviour could indicate an overwintering strategy in the soil, which is also observed for *A. paludosus* (Classen and Dettner 1983). Average glycogen and protein concentration was doubled in November compared to April for both *Laccophilus minutus* and *Hyphydrus ovatus*. However, the difference was not significant. This observation is in line with their active behaviour in winter, since glycogen is synthesised to sugar alcohols as an adaptation to cold (Arrese and Soulages 2010). Further studies on the feeding behaviour of Dytiscidae should include seasonal effect and age of the used specimens to avoid seasonal bias.

From a European biological control perspective, *Agabus bipustulatus* seems to be the most suitable predator to reduce mosquito larvae, especially *Aedes albopictus* larvae. The species is known to occur in artificial containers (Onyeka 1983; Reyne et al. 2020) and temporary pools (Eyre et al. 1986), has a high dispersal rate (Kehl and Dettner 2007), has a broad habitat spectrum and is very common throughout

Europe (Nilsson and Holmen 1995). In addition, *Aedes cantans* and *Culex pipiens/torrentium* antiserum was found in gut smears of field-caught adult and larval *Agabus bipustulatus* (Service 1977; Onyeka 1983), confirming its suitability as a biological predator against mosquito larvae. Further laboratory and field research on predation of mosquito larvae by *A. nebulosus*, *Hygrotus impressopunctatus* and *Laccophilus minutus* is required to assess whether these species are suitable biological control agents, since these species are largely understudied.

Currently, there is no literature available on the release of diving beetles in Europe. In terms of ecosystem impact, the introduction of additional native diving beetles as proposed in the present study could potentially compensate for loss of biodiversity, especially in biodiversity-poor areas commonly associated with *Aedes albopictus* infestations (Perrin et al. 2022; Giunti et al. 2023). Competition between Dytiscidae will be minimal, since adults are capable of active dispersal (Kehl and Dettner 2007); however, there is potential for competition and cannibalism amongst diving beetle larvae (Inoda and Kamimura 2004). Evaluating these interactions will be essential for predicting the success and sustainability of using Dytiscidae as biocontrol agents.

When considering the introduction of diving beetles as biological control agents against *A. albopictus*, it is crucial to account for several important non-target effects. Although the present study includes various prey species, such as Chaoboridae and *Daphnia* sp., further extensive field studies are necessary to include all naturally occurring prey and predators. This broader assessment will ensure a comprehensive understanding of the ecological impacts. Historical evidence indicates that generalist and specifically non-native predators often proved to become problematic. For instance, the introduction of the cane toad in Australia (Shine et al. 2020) and the mosquitofish in various regions (Jourdan et al. 2021) led to significant ecological disruptions due to their generalist feeding habits and competition with native species. In contrast, the use of native species, such as copepods (Giunti et al. 2023) and *Toxorhynchites* mosquitoes (Malla et al. 2023), could reduce negative ecological impacts.

This form of biological control may synergise with another biological control method that is already widely used in Europe: the use of Bti. This form of integrated vector control may work well with predacious beetles, since they are not affected by Bti (Shaalan and Canyon 2009) and studies showed that predator cues even increased mortality on *Culex pipiens* and *C. quinquefasciatus* when combined with Bti (Op De Beeck et al. 2016; Delnat et al. 2020. In addition, the dytiscids can invade cryptic or domiciliary breeding sites, which often remain undetected or unidentified during Bti application (Achee et al. 2019; Donald et al. 2020).

Rearing of Dytiscidae remains a major challenge due to their high rate of food consumption and their intrinsic cannibalistic behaviour (Inoda and Kamimura 2004; Inoda and Kitano 2013). Dytiscid larvae feed on dissolved body fluids, resulting in food refusal and irreversibly spoiling the water (Inoda and Kamimura 2004). A first solution could be deploying the larval stages in close proximity with *Aedes albopictus* populations to avoid the laboratory rearing to adulthood. Another possibility would be using the rearing technique developed for mass rearing of juvenile lobsters (*Homarus gammarus*), which could resolve the rearing problem by keeping juveniles separately in clean water with a high quality food source (Schmalenbach et al. 2009). Moreover, the terrestrial pupation stage of water beetles in soil is an additional element that is absent in many other reared aquatic animals and which makes rearing on larger scale more difficult. Once resolved, mass-reared Dytiscidae could be released in *A. albopictus* infested areas to aid population control. In addition, species such as *Agabus bipustulatus* have been found to live over two years, meaning a frequent release of these species would be unnecessary (Davy-Bowker 2002).

The results also underline the suitability and possibly important role of native predators in the ongoing battle against invasive species, such as the vectors of mosquito-borne diseases. Good habitat quality and high native predatory insect densities can prevent the establishment of invasive mosquitos (Juliano and Lounibos 2005). Moreover, high species richness of these native predators can assure the presence of suitable species which co-occur in the same habitat and have a compatible foraging behaviour. Restoration of degraded aquatic habitats is an important issue, however, often limited to larger bodies of water and lotic environments. In the light of the management of invasive mosquito species, also small fishless temporary waterbodies should be taken into account in nature management and restoration projects (Liao et al. 2020). Though the habitat overlap between *Aedes albopictus* and Dytiscidae is not 100% secure, we do observe an influence of separation of entomological disciplines: Culicidae specialists aim to find *A. albopictus* for monitoring purposes and do not record other species found in these habitats, while Dytiscidae specialists focus on natural habitats and rarely search specimens in tyres or artificial containers in gardens. From this perspective, it would be beneficial to underline the importance of recording these findings.

During this study, a potential bias was created since the beetles were fed solely under laboratory conditions and, therefore, forced to feed on selected prey, which might differ from their natural preference. They were fed with *Culex pipiens* and *A. albopictus* larvae, both accounting for the diverse feeding strategies of mosquitoes. In addition, *Chaoborus* sp. larvae were included since they resemble mosquito larvae and are very common in lentic waters. *Daphnia* sp., generally found in ephemeral ponds and small waterbodies, were added to include a completely different type of prey. Chironomidae were not included in this study since they prefer waters with sediment, which was beyond the scope of this study. Therefore, it is assumed that prey choices most likely available were added to the study and, hence, reducing the influence of bias.

Conclusion

We provide some evidence on the efficacy of Dytiscidae to predate on *Aedes albopictus* larvae. In total, the feeding rate of thirty aquatic beetle taxa on *A. albopictus* larvae were tested, accounting for almost 25% of the total Dytiscidae diversity in Belgium and one out of two Noteridae species present (Scheers et al., in press). When giving Dytiscidae the choice to feed on taxa other than mosquitoes, such as Chaoboridae and Cladocera, the tested species still preferred Culicidae larvae, with a clear preference towards *A. albopictus* over *Culex pipiens*. The distribution of best-predating species of Dytiscidae in our tests shows a clear overlap with the PoE where *A. albopictus* has been found in Belgium in recent years. Several species which are widespread in Europe, such as *Agabus bipustulatus*, *A. nebulosus*, *Hygrotus impressopunctatus* and *Laccophilus minutus* also show a high predation rate and clear preference for *Aedes albopictus*. *Agabus bipustulatus* seems to be the most suitable predator for *Aedes albopictus*, since this species is known to occur in artificial containers and temporary pools, has a high dispersal rate and is very common throughout Europe. Given these promising results for a nature-based solution, the use of Dytiscidae as a biological control agent against *A. albopictus* should be further investigated.

Acknowledgements

We would like to thank Marre van de Ven and Chaymae Kadi for their contribution to the research. We are grateful to the laboratory and technical staff at the Institute of Tropical Medicine Antwerp, Belgium, especially Luka Wouters, Leen Denis and Karen Jennes for their support of lab experiments. We would like to thank Tom Vermeire for his additional data on Dytiscidae in artificial habitats in Belgium. We also thank Dr. Juliane Hartke for her help during the experiments and Prof. Konrad Dettner for his insights into the seasonal effects. We thank Prof. Norbert Becker for providing us with the *Aedes albopictus* colony.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

This research was funded through the 2018–2019 BiodivERsA joint call for research proposals, under the BiodivERsA3 ERA-Net COFUND programme (project DiMoC – Diversity Components of Mosquito-borne Diseases under Climate Change) and with the funding organisation FWO G0G2319N. The research was also funded through project BIOZ by the Federal Ministry of Health of Germany under the research network programme "Nationales Forschungsnetz Zoonotische Infektionskrankheiten" 2521NIK401 and the MEMO and MEMO+2020 projects (2017–2020) by the Flemish, Walloon and Brussels regional governments and the Federal Public Service (FPS) Public Health, Food Chain Safety and Environment in the context of the National Environment and Health Action Plan (NEHAP) (Belgium). ITM's outbreak research team is supported by the Department of Economy, Science and Innovation of the Flemish government, Belgium. The insectaries at ITM are partially funded through the Department of Economy, Science and Innovation (EWI) of the Flemish Government.

Author contributions

AV: Conceptualisation, Data Curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Validation, Visualisation, Writing – original draft. KS: Conceptualisation, Data Curation, Resources, Writing – review and editing. XV: Resources, Visualisation, Writing – review and editing. RH: Conceptualisation, Resources, Writing – review and editing. AS: Resources, Writing – review and editing. JDW: Resources, Writing – review and editing. ID: Resources, Writing – review and editing. WVB: Resources, Writing – review and editing. FR: Conceptualisation, Methodology, Writing – review and editing. RM: Conceptualisation, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review and editing.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

More information on the aquatic beetle taxa that were collected and used during the experiments

Authors: Adwine Vanslembrouck, Kevin Scheers, Xavier Vermeersch, Rens Hendrickx, Anna Schneider, Jacobus De Witte, Isra Deblauwe, Wim Van Bortel, Friederike Reuss, Ruth Müller

Data type: docx

Link: https://doi.org/10.3897/neobiota.94.121987.suppl1

Supplementary material 2

General overview of larval benthic feeding behaviour of *A. albopictus* (followed) and filter feeding behaviour at water surface of *C. pipiens*

Authors: Adwine Vanslembrouck, Kevin Scheers, Xavier Vermeersch, Rens Hendrickx, Anna Schneider, Jacobus De Witte, Isra Deblauwe, Wim Van Bortel, Friederike Reuss, Ruth Müller

Data type: mp4

Link: https://doi.org/10.3897/neobiota.94.121987.suppl2

Supplementary material 3

The benthic feeding behaviour of *A. albopictus* larvae

Authors: Adwine Vanslembrouck, Kevin Scheers, Xavier Vermeersch, Rens Hendrickx, Anna Schneider, Jacobus De Witte, Isra Deblauwe, Wim Van Bortel, Friederike Reuss, Ruth Müller

Data type: mp4

Link: https://doi.org/10.3897/neobiota.94.121987.suppl3

Supplementary material 4

Filter feeding behaviour of *C. pipiens* at the water surface

Authors: Adwine Vanslembrouck, Kevin Scheers, Xavier Vermeersch, Rens Hendrickx, Anna Schneider, Jacobus De Witte, Isra Deblauwe, Wim Van Bortel, Friederike Reuss, Ruth Müller

Data type: mp4

Link: https://doi.org/10.3897/neobiota.94.121987.suppl4

Supplementary material 5

Predation of *Laccophilus minutus* on *Aedes albopictus* larvae

Authors: Adwine Vanslembrouck, Kevin Scheers, Xavier Vermeersch, Rens Hendrickx, Anna Schneider, Jacobus De Witte, Isra Deblauwe, Wim Van Bortel, Friederike Reuss, Ruth Müller

Data type: mp4

Link: https://doi.org/10.3897/neobiota.94.121987.suppl5

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 205–224 (2024)
DOI: 10.3897/neobiota.94.118480

Research Article

An evaluation of 'pollinator-friendly' wildflower seed mixes in Italy: are they potential vectors of alien plant species?

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Abstract

Wildflower areas are increasingly used in both urban and rural settings to enhance landscape aesthetics and help the conservation of pollinators, whose dramatic decline threatens both ecosystem functioning and agricultural production. Consequently, today many 'pollinator-friendly' wildflower seed mixes can be found on the market. Criteria for the design of these mixes are focused on the ability of the plants to attract pollinators, whereas the identity of the plants themselves in terms of their alien or native status is often not adequately considered. Moreover, seed mixes often lack sufficient floral resources in the early spring and late-season months, which are important to sustain pollinators throughout their whole life cycle. In this study, we analyzed 36 'pollinator-friendly' wildflower seed mixes commercially available in Italy in terms of (1) the abundance of native and alien species, (2) the occurrence of locally alien species with reference to three Italian geographic areas (northern, peninsular, and insular Italy), and (3) the flowering period of native and alien species. Most (83%) of the analyzed seed mixes contain species alien to Italy, with three of them also including invasive alien species. Among native species, many (41%) are locally alien to at least one of the geographic areas. Overall, native species provide earlier blooms than alien species and around 35% of the seed mixes lack blooms in early-spring (March) or prolonged throughout autumn (September-November). These findings highlight the widespread use of alien plant species in 'pollinator-friendly' wildflower mixes, which poses serious risks for biodiversity and habitat conservation, especially when sown in agricultural areas. We suggest a more careful design and use of such wildflower mixes, promoting a wider adoption of native seeds of local origin and a greater attention to the blooming period. If properly designed, wildflower mixes can represent an effective strategy for biodiversity conservation in both urban and rural areas.

Key words: Archaeophytes, biodiversity conservation, exotic plants, flowering period, invasive species, native plants, pollinators, wildflower strips

Academic editor: Sven Jelaska
Received: 9 January 2024
Accepted: 22 June 2024
Published: 5 August 2024

Citation: Nota G, Falla NM, Scariot V, Lonati M (2024) An evaluation of 'pollinator-friendly' wildflower seed mixes in Italy: are they potential vectors of alien plant species? NeoBiota 94: 205–224. https://doi.org/10.3897/neobiota.94.118480

Introduction

Wildflowers are annual or perennial herbaceous species having aesthetically pleasing flowers and being important for biodiversity conservation and pollination services (Bretzel and Romano 2013; Bretzel et al. 2016; Benvenuti et al. 2020). These species are suitable for planting in disturbed areas, such as in urban and agricultural settings, where floristic biodiversity is low due to anthropogenic pressure and agricultural intensification (Benvenuti et al. 2020; Nichols et al. 2022). In addition,

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as these areas are often dominated by autogamous and/or wind-pollinated plants, wildflowers provide important nectar and pollen resources to insect pollinators (Benvenuti et al. 2020). Currently, the decrease in abundance and diversity of pollinators such as bees, bumblebees, wasps and butterflies is a global emergency, which threatens agricultural production and the healthy functioning of natural and semi-natural ecosystems (Carvell et al. 2006; Ouvrard et al. 2018; Hevia et al. 2021). Flower scarcity due to land homogenization and habitat loss and changes in agricultural practices (e.g., pesticides use) are considered the main causes of this decline (Carvell et al. 2006; Goulson et al. 2015).

For these reasons, today many wildflower seed mixes are used to create wildflower areas, with the desire to restore floristically degraded areas and support pollination services. In the cities, such ‘pollinator-friendly’ wildflower mixes are sown in roundabouts, parks, meadows, and green roofs, where they provide an aesthetically pleasing landscape for citizens and contribute to pollinators’ conservation (Blackmore and Goulson 2014; Hicks et al. 2016). In rural areas, farmers often plant wildflower strips along the margins of their fields, aiming to attract wild pollinators to benefit crops (Pontin et al. 2006; Lowe et al. 2021). This activity is also encouraged by policy, such as in the framework of European agri-environmental and eco-schemes, whose implementation resulted in an overall positive effect on pollinator populations (Ouvrard et al. 2018; Ganser et al. 2021; Lowe et al. 2021; Schmidt et al. 2022), although generalist species seem to have benefited the most (Scheper et al. 2013). In addition, wildflower areas could be used to create zones with natural features connecting the city with the farmland, promoting the creation of ecological corridors (Bretzel and Romano 2013; Blackmore and Goulson 2014).

Because of the growing interest for pollinator conservation by policy and people, today many ‘pollinator-friendly’ wildflower seed mixes can be found on the market. Selection criteria for plant species composing the mixes are mainly focused on showy flowers which attract pollinators and at the same time enhance the landscape with their aesthetics. However, other characteristics such as the native or alien status of the plants are often not kept in mind, resulting in many seed mixes containing species alien to the area where they are sold and utilized (Havens and Vitt 2016; Ganser et al. 2021; Barry and Hodge 2023). Alien plants can provide abundant resources to pollinators in both urban (Zaninotto et al. 2023) and agricultural areas (Seitz et al. 2020; Kovács-Hostyánszki et al. 2022), but they can interfere with pollination networks by outcompeting native vegetation and disadvantaging specialist wild bees (Ojija et al. 2019; Seitz et al. 2020; Kovács-Hostyánszki et al. 2022). Moreover, the seeding of alien species can pose risks to the conservation of the native plant communities with which they are in contact, especially in the case of invasive alien species. This is even more problematic in extra-urban areas where farmland can be interspersed with areas of high conservation value such as the Natura 2000 network.

Species are usually defined as alien at the country level. However, there are also species native to the country which can be alien at the sub-country level (hereafter, locally alien) because they were introduced by man in regions where they did not occur naturally. As for the species alien to the country, the introduction of locally alien species may result in problems to biodiversity and natural communities too, particularly on islands, where the distinct and endemic biodiversity may be highly vulnerable to plant invasion (Reaser et al. 2007; Moser et al.

2018). This issue has a great relevance in Italy, which is among the countries with the highest biodiversity in Europe, as it encompasses different biogeographical regions characterized by distinct flora, and hosts a large number of hotspots and endemisms, especially in the Alps and in Sicily and Sardinia islands (Médail and Quézel 1997; Peruzzi et al. 2014; Bartolucci et al. 2024). For instance, several species of the Italian native flora are not present in Sicily and Sardinia, thus their introduction here may adversely affect local biodiversity. At an even finer-scale, the introduction of foreign ecotypes of native species can also be detrimental to native plant communities, as it can modify the genetic structure of populations (Kaulfuß and Reisch 2021).

Another essential characteristic of an effective wildflower mix is to ensure prolonged and diversified bloomings. As a result, not only the aesthetic value of an area increases, but more importantly, pollen and/or nectar would be available throughout the entire biological cycle of pollinators. Particularly, early spring and late-summer and autumn months are critical periods for the survival of pollinators (e.g., early season bumblebees), since the availability of diverse and abundant resources is more limited. Moreover, evidence shows that this could even get worse with climate change due to temporal mismatches between plant flowering period and pollinators' activity season (Memmott et al. 2007, 2010; Hegland et al. 2009). Many authors have emphasized that wildflower mixes often lack plants that flower in such early- and late-season periods and have underlined the need to design seed mixes with more attention to plant phenology (Havens and Vitt 2016; Hicks et al. 2016; Ouvrard et al. 2018; Nichols et al. 2022). Moreover, a prolonged bloom that also covers the period of highest flower scarcity from late fall to winter, would benefit the aesthetics of both urban and rural areas.

Although the attention to the conservation of pollinators is high, with publications reporting lists of flowering plant species suitable for pollinators (e.g., Hicks et al. 2016; Warzecha et al. 2018), only a few studies focused on the possible threats to biodiversity and habitat conservation arising from alien plants available in 'pollinator-friendly' wildflower seed mixes (but see Barry and Hodge 2023). Moreover, the current availability of plants on the wildflower market providing flower resources for pollinators in the early- and late-season periods has been poorly investigated.

To fill these knowledge gaps, we analyzed the species composition of a large number of 'pollinator-friendly' wildflower seed mixes commercially available on the Italian market. Particularly, the specific purposes of this work were to:

1. analyze the abundance of native and alien plant species, with a particular focus on the status of invasiveness in Italy (casual, naturalized, invasive);
2. among those species considered native at the country level, evaluate the occurrence of locally alien species, with reference to three geographic areas in Italy: northern, peninsular and insular Italy;
3. evaluate the flowering period of the native and alien species included in the seed mixes.

With this information, we aimed to highlight possible risks to biodiversity and habitat conservation originating from the presence of alien species in the mixes and to provide indications for improving their botanical composition and for their careful use in urban and extra-urban areas.

Methods

Seed mixes database

Thirty-six seed mixes were selected according to the following criteria:

1) commercial availability, either online or in garden stores, for Italian customers;
2) clear reference as mixes for pollinating insects or as mixes of melliferous plants;
3) plant species listed on labels by their scientific name or for which the attribution to it was certain (mixes with doubtful common names were excluded).

The seed mix compositions were included in a database (Suppl. material 1), following the nomenclature of plant species of the Italian native (Bartolucci et al. 2024) and alien (Galasso et al. 2024) checklists. For the species not recorded in the Italian flora, nomenclature followed World Flora Online (WFO 2024). We considered the 36 mixes to be sufficiently representative of the pollinator-friendly wildflower market as fewer and fewer additional species were found as samples were added to the database, until we obtained a nearly constant species list.

Data analysis

Each plant species was assigned to one of the following five categories according to its status for Italy reported in the Portal to the Flora of Italy (PFI 2023):

1) Natives, i.e., autochthonous species in at least one Italian administrative region (e.g., *Achillea millefolium* L.);
2) Cryptogenics, i.e., doubtfully native plants, whose origin of occurrence in Italy is unknown (e.g., *Papaver rhoeas* L.);
3) Archaeophytes, i.e., alien plants introduced to Italy before 1492; among others, this group includes plants that were introduced by humans as cereal weeds (segetal vegetation) and that today are severely threatened by agricultural intensification (e.g., *Centaurea cyanus* L.);
4) Neophytes, i.e., alien plants introduced to Italy after 1492 (e.g., *Phacelia tanacetifolia* Benth.);
5) Not recorded, i.e., alien plants that are not recorded in the spontaneous Italian flora (= not present in the Portal to the Flora of Italy) (e.g., *Lupinus perennis* L.).

Neophytes were further assigned to one of the following three sub-categories, as reported by the Portal to the Flora of Italy (PFI 2023; definitions are based on Galasso et al. 2018):

1) Casual, i.e., neophytes that may thrive and even produce offsprings occasionally outside cultivation, but that usually disappear because they are unable to form self-maintaining populations; their persistence relies on repeated introductions. (e.g., *Bidens formosa* (Bonato) Sch.Bip.);
2) Naturalized, i.e., neophytes that occur with self-maintaining populations without direct human intervention (e.g., *Phacelia tanacetifolia*);
3) Invasive, i.e., neophyte plants that occur with self-maintaining populations without direct human intervention, produce fertile offspring at considerable

distances from the parent individuals, thus being able to spread over a large area (e.g., *Mirabilis jalapa* L.).

Native species were further classified according to their local native or alien status for three Italian geographic areas, which were defined based on regional administrative borders: (1) northern Italy; (2) peninsular Italy (central and southern Italy); and (3) insular Italy (Sicily and Sardinia islands). Subdivision of Italy into such geographic areas was based on the dissimilarity in the endemic vascular flora among Italian regions according to Peruzzi et al. (2014). Similarly, the endemism criterion was also used in the definition of the floristic realms at the global level by Takhtajan et al. (1986). Other classifications of Italy, such as the ecoregions proposed by Blasi et al. (2014) considering climatic, physiographic, biogeographic and vegetation criteria, might also be appropriate, but their boundaries do not align with the available data on species distribution, which are provided at the regional level by the Portal to the Flora of Italy (PFI 2023). Therefore, for each geographic area, native species were assigned to one of the following four categories, according to the species distribution maps available in the Portal to the Flora of Italy:

1) locally native, i.e., native species that are autochthonous in all the administrative regions of the corresponding geographic area;
2) locally casual alien, i.e., native species that established as casual alien in at least one administrative region of the geographic area, and did not establish as naturalized or invasive in any other of them (e.g., *Sinapis alba* L. in northern Italy; see Suppl. material 2; PFI 2023);
3) locally naturalized alien, i.e., native species that established as naturalized alien in at least one administrative region of the geographic area and did not establish as invasive in any other of them (e.g., *Lobularia maritima* (L.) Desv. in northern Italy; see Suppl. material 2; PFI 2023);
4) locally not recorded alien, i.e., native species that are absent in all the administrative regions of the corresponding geographic area (e.g., *Plantago media* L. is native to northern and peninsular Italy but absent in both the islands; see Suppl. material 2; PFI 2023).

Then, from the seed mixes database, we calculated i) the frequency of each species in the seed mixes, ii) the number of species per each family (APG IV), iii) the proportion of each status category (natives, cryptogenics, archaeophytes, neophytes, and not recorded) in the species list, and iv) the number of species per each status category and seed mix; moreover, we characterized v) the area of origin for neophytes and not recorded species according to the Kew Backbone Distributions (POWO 2024) and calculated vi) the proportion of each locally native and locally alien category in the species list for the three Italian geographic areas. We ran a G-test of independence to test if proportions of locally native and alien categories differed by Italian geographic area ('G.test' function from the 'RVAideMemoire' package in R Statistical Software (R Core Team 2018; Herve 2023).

Finally, we assessed the flowering period of i) natives, ii) cryptogenics and archaeophytes, and iii) neophytes and not recorded species by calculating the number of species that bloom each month of the year. We also performed the same calculation for each of the 36 seed mixes to assess the flowering period covered by each of them. Flowering months were retrieved from different sources, mainly Acta Plantarum (Acta

Plantarum 2024) for the Italian flora and the Missouri Botanical Garden (MBG 2024), Royal Horticultural Society (RHS 2024) and Floraveg.EU (Floraveg.EU 2024) for the species not recorded in Italy. All sources are provided in Suppl. material 1.

Results

Native and alien species abundance

In total, 204 species were identified in the 36 seed mixes analyzed (Suppl. material 1), of which the most frequent in the mixes are reported in Table 1. *Centaurea cyanus* is the most frequent species in the mixes (67%), followed by the alien species *Phacelia tanacetifolia* (50%). Among the 20 most frequent species, natives, neophytes and archaeophytes are similarly represented, with five, five, and four species, respectively. Annual species predominates. Species in the mixes belong to 34 families, of which Asteraceae is the most represented, followed by Lamiaceae and Fabaceae (Fig. 1).

Of the 204 species found, natives are the majority (119 species, 58% of the total), followed by neophytes (39, 19%), not recorded species (25, 12%), and archaeophytes (17, 8%) (Fig. 2). Cryptogenics account for a small fraction (4, 2%). Among neophytes, most are casual, but naturalized and even invasive alien are also present. Particularly, the invasive species to Italy are *Mirabilis jalapa*, *Oenothera glazioviana* Micheli, and *Tropaeolum majus* L. Moreover, some species included in the mixes are considered invasive to other parts of the globe (e.g., *Ageratum houstonianum* Mill., *Gaillardia aristata* Pursh, *Impatiens balsamina* L., *I. walleriana* Hook.f, *Lupinus polyphyllus* Lindl., and *Salvia coccinea* Buc'hoz ex Etl). Alien species (neophytes and not recorded) are mainly native to North America (Fig. 3).

The average number of species per mix is 15, with a minimum of 4 and a maximum of 36 species (Fig. 4). Of the 36 mixes, 6 (17% of the total) have exclusively natives, archaeophytes and cryptogenics, whereas most of them (30 mixes, 83%) have at least one neophyte or not recorded species (5 species on average, minimum 1, maximum 16). More specifically, 15 mixes (42%) include not recorded species and 3 mixes (8%) include invasive neophytes.

Table 1. Species frequency in the mixtures. List of the 15 most frequent plant species available in the seed mixes. Species are sorted by frequency, descendent.

Species	Status	Life cycle	Frequency (%)
Centaurea cyanus L.	Archaeophyte	Annual	67
Phacelia tanacetifolia Benth.	Neophyte	Annual	50
Papaver rhoeas L.	Cryptogenic	Annual	36
Calendula officinalis L.	Archaeophyte	Annual	33
Achillea millefolium L.	Native	Perennial	28
Anethum graveolens L.	Archaeophyte	Annual	28
Nigella damascena L.	Native	Annual	28
Borago officinalis L.	Native	Annual	25
Agrostemma githago L.	Archaeophyte	Annual	22
Glebionis segetum (L.) Fourr.	Native	Annual	22
Leucanthemum vulgare (Vaill.) Lam.	Native	Perennial	22
Bidens formosa (Bonato) Sch.Bip.	Neophyte	Annual	19
Bidens tinctoria (Nutt.) Baill. Ex Sennikov	Neophyte	Annual	19
Eschscholzia californica Cham.	Neophyte	Annual	19
Fagopyrum esculentum Moench	Neophyte	Annual	19

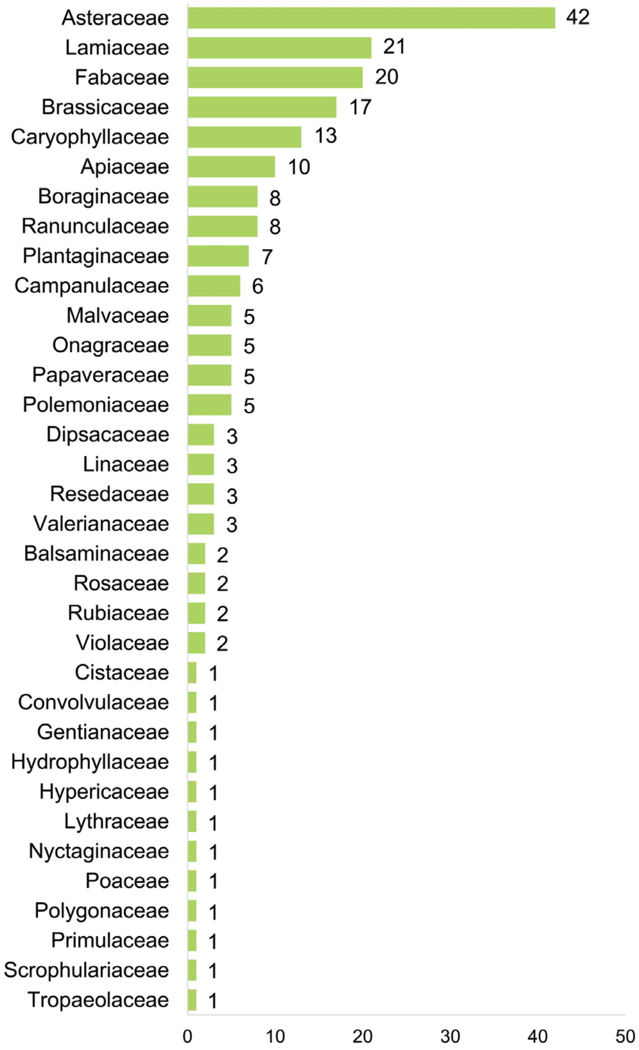


Figure 1. Species families. Number of species included in the mixtures belonging to each family (APG IV).

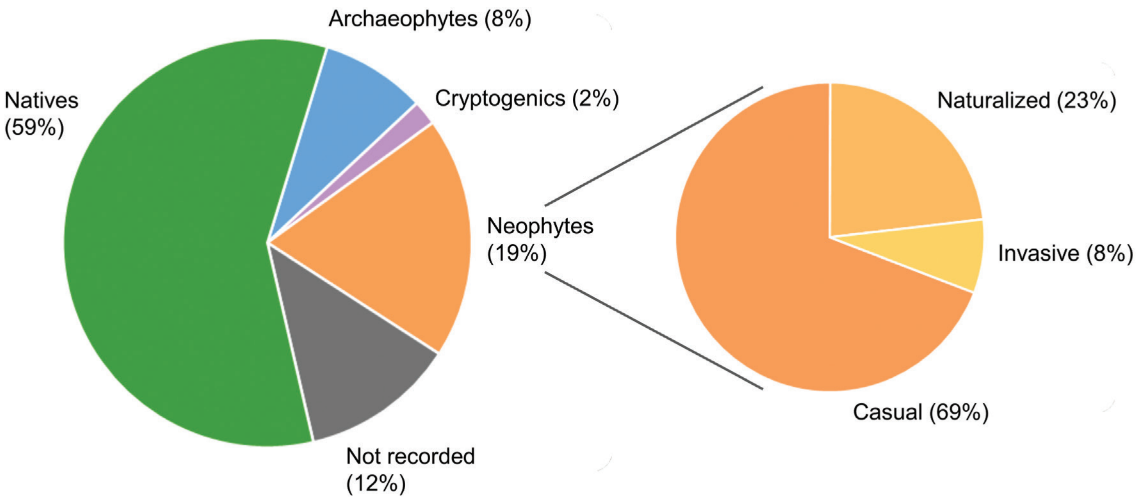


Figure 2. Native and alien species proportions. Proportion of the plant species available in the seed mixes by five categories according to their status for Italy. Neophytes are further divided into proportions of casual, naturalized and invasive species to Italy.

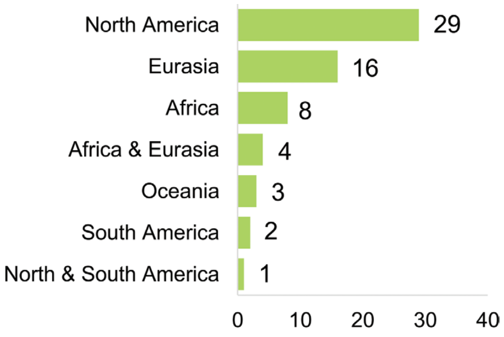
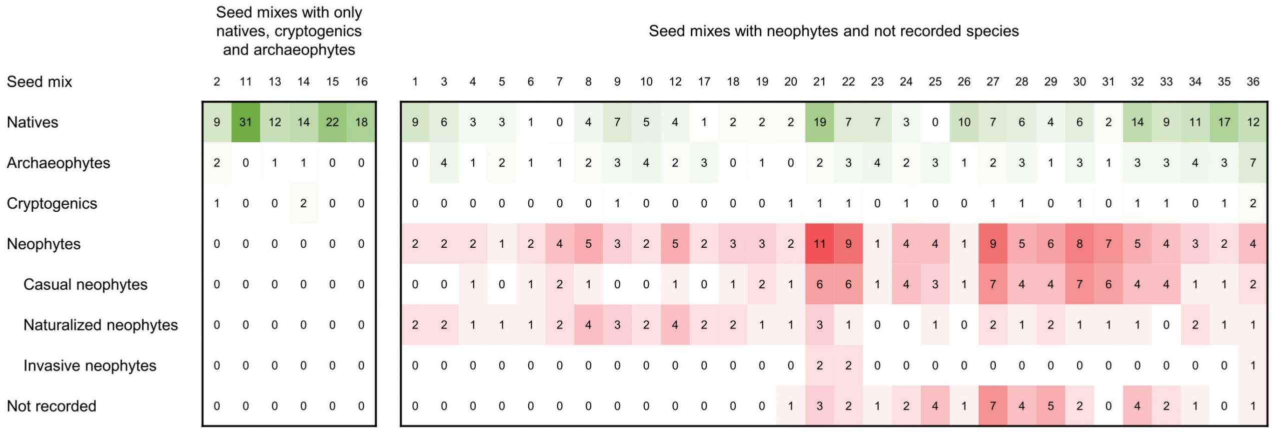


Figure 3. Alien species origin. Number of neophytes and not recorded species according to the region of the world where they are native to. The species *Oenothera glazioviana* Micheli is not included in the analysis of species origin as it is a cultigen originating from *O. elata* × *O. grandiflora* hybrids in Europe (POWO 2024).



	Seed mixes with only natives, cryptogenics and archaeophytes						Seed mixes with neophytes and not recorded species																													
Seed mix	2	11	13	14	15	16	1	3	4	5	6	7	8	9	10	12	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Natives	9	31	12	14	22	18	9	6	3	3	1	0	4	7	5	4	1	2	2	2	19	7	7	3	0	10	7	6	4	6	2	14	9	11	17	12
Archaeophytes	2	0	1	1	0	0	0	4	1	2	1	1	2	3	4	2	3	0	1	0	2	3	4	2	3	1	2	3	1	3	1	3	3	4	3	7
Cryptogenics	1	0	0	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	0	0	1	1	0	1	0	1	1	0	1	2
Neophytes	0	0	0	0	0	0	2	2	2	1	2	4	5	3	2	5	2	3	3	2	11	9	1	4	4	1	9	5	6	8	7	5	4	3	2	4
Casual neophytes	0	0	0	0	0	0	0	0	1	0	1	2	1	0	0	1	0	1	2	1	6	6	1	4	3	1	7	4	4	7	6	4	4	1	1	2
Naturalized neophytes	0	0	0	0	0	0	2	2	1	1	1	2	4	3	2	4	2	2	1	1	3	1	0	0	1	0	2	1	2	1	1	1	0	2	1	1
Invasive neophytes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Not recorded	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	2	1	2	4	1	7	4	5	2	0	4	2	1	0	1

Figure 4. Species composition of the seed mixes. Numbers indicate the number of species per each category. Color scale increases white to green for natives, cryptogenics and archaeophytes and white to red for neophytes and not recorded species.

Evaluation of the native species status in the geographic areas of Italy

Of the 119 species that are native to Italy, 70 species (59%) are native to the entire Italian territory, while 49 species (41%) are absent or introduced by man (as a casual or naturalized alien species) in at least one of the three geographic areas (Suppl. material 1).

Italian geographic areas significantly differ in the proportion of locally native (G-test of independence, G = 32.354, $p < 0.001$), casual (G = 8.303, $p = 0.016$), naturalized (G = 6.531, $p = 0.038$), and not recorded species (G = 58.602, $p < 0.001$). Particularly, in northern Italy, 33% of the species are locally casual (16%) or naturalized (17%) alien taxa (Fig. 5). These species include many Mediterranean plants that are not autochthonous to northern Italy but established in specific ecological conditions after their introduction by man (e.g. *Lobularia maritima*, *Glebionis segetum* (L.) Fourr., *Trifolium resupinatum* L.; Suppl. material 2; PFI 2023). In the other geographic areas, instead, the locally casual and naturalized species are less abundant, with the sum of the two accounting for 11% and 17% in peninsular and insular Italy, respectively. Finally, insular Italy has a larger share of locally not recorded species (27%) compared to the other geographic areas.

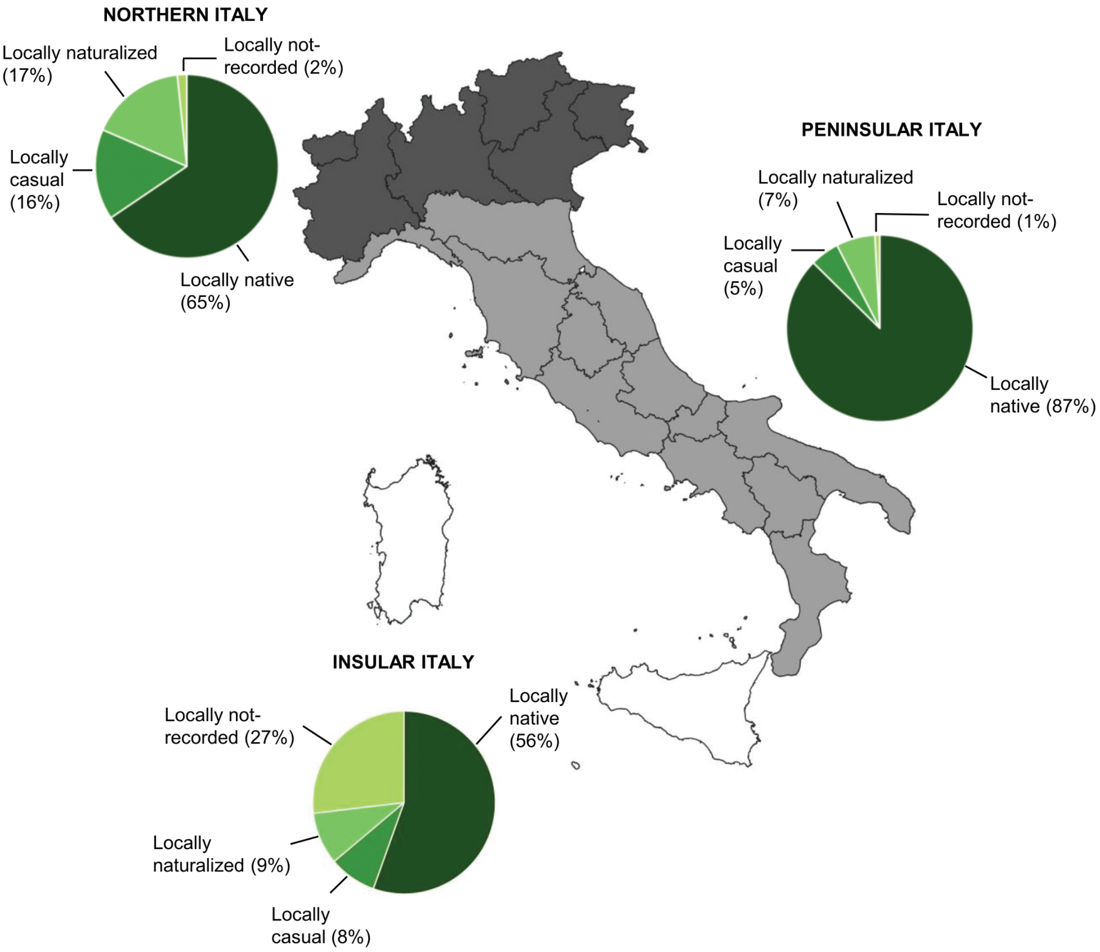


Figure 5. Native species proportion in the geographic areas. Proportion of the native species to Italy available in the seed mixes by four categories (i.e., locally native, casual, naturalized, and not recorded) according to their status for three Italian geographic areas (northern, peninsular, and insular Italy).

Plant species flowering period

The flowering period of plant species available in the seed mixes differs by plant status category (Fig. 6). Natives, cryptogenics and archaeophytes have blooms that potentially cover the whole year, with the peak of blooming in May and June. The distribution of species numbers is slightly skewed to the left, with proportionally more species blooming in early spring than in autumn (Fig. 6a, b). In addition, some native species potentially bloom in the period from November to January too, possibly providing aesthetic benefits during the period of highest flower scarcity (Fig. 7). Among these, three species (*Bellis perennis* L., *Reseda alba* L. and *Trifolium pratense* L.) have a potential continuous flowering along the whole year. Instead, for neophytes and not recorded species, the distribution of species numbers is skewed to the right, no species flower in December and January, and the peak of blooming is in June (Fig. 6c).

According to the flowering period potentially covered by each seed mix (Fig. 8), 39% of the mixes provide blooms throughout the whole year while 100% for the period from April to August. Part of the mixes (36%) lack flowers in early spring (March) or throughout the whole autumn (from September to November). Overall, neophytes and not recorded species provide flower resources later in the season compared to natives, cryptogenics and archaeophytes.

Discussion

Despite the current awareness of the negative impacts that the spread of alien species poses to the environment and humans (IPBES 2023), most (83%) of the commercially available wildflower seed mixes evaluated in this study contained plant species alien to Italy (neophytes and not recorded species). In total, 64 alien plants were found in 36 'pollinator-friendly' wildflower mixes, around 30% of the total number of species. These proportions highlight the widespread use of alien plants on the

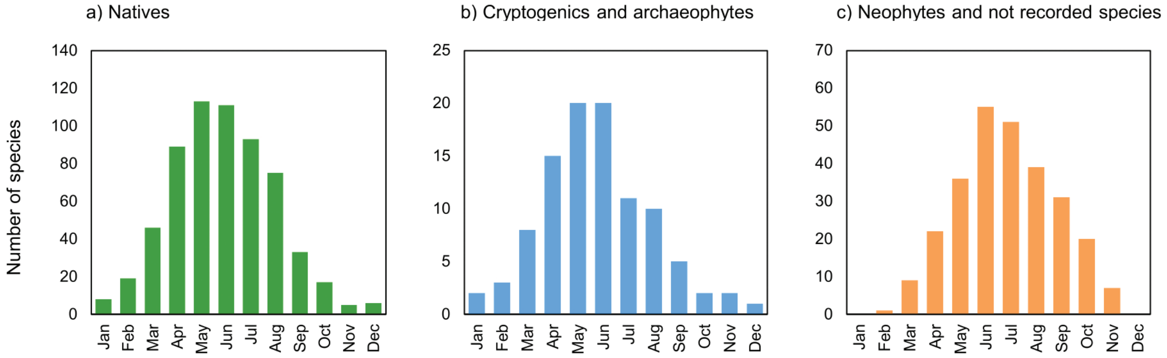


Figure 6. Flowering period of native and alien species. Number of a) natives, b) cryptogenics and archaeophytes, and c) neophytes and not recorded species available in the seed mixes that bloom each month.

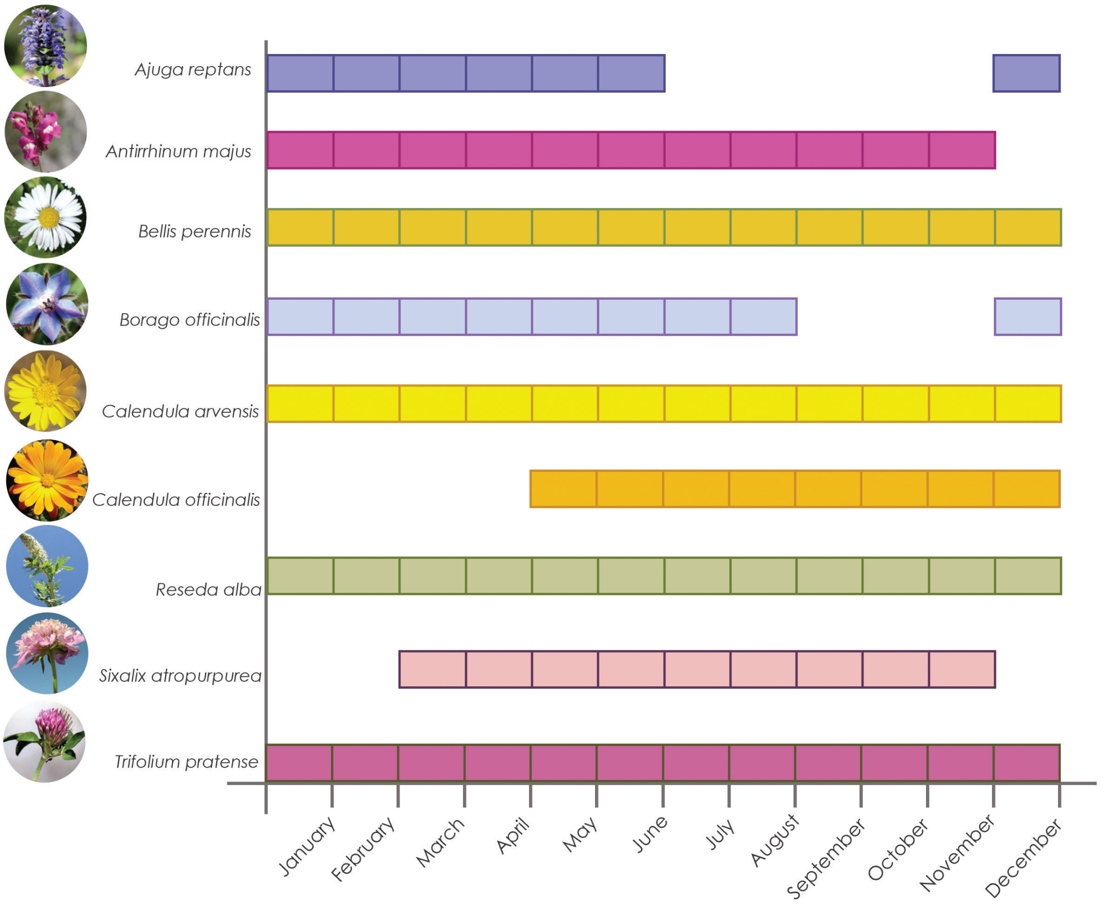


Figure 7. Flowering patterns of native plants with November to January blooms. Flowering patterns of native species available in the seed mixes that potentially flower also in the period from November to January.

Figure 8. Flowering period by seed mix. Number of species that bloom each month for each seed mix. The identification number of the mix is provided in the upper right of each chart.

wildflower market. Alien species are so often included in seed mixes because many of them grow quickly, have pleasant and abundant flowers, are attractive to pollinators, and provide a long flowering period (Seitz et al. 2020; Zaninotto et al. 2023).

Similarly to the findings of Barry and Hodge (2023) for Ireland, the most frequent alien species in our study (50% of the mixes) was *Phacelia tanacetifolia*, native to North America. This species is largely used by farmers and beekeepers in fields and field margins, in mixtures or as monoculture, due to its attractiveness for insect pollinators, especially honeybees (Warzecha et al. 2018; Giovanetti et al. 2022). However, concerns were raised about the potential of the species to become invasive out of its home range (Smither-Kopperl 2018) and possibly compete with native vegetation by affecting native pollination networks (Totland et al. 2006). In Italy, the species escaped from cultivation as a casual species in most of the country and is already naturalized in two regions, i.e., Friuli-Venezia Giulia and Lazio (Suppl. material 2; PFI 2023). Among the alien plants detected in this study, some were invasive to Italy or to other European or world countries. Particularly, three mixes contained invasive species to Italy, i.e., *Mirabilis jalapa*, *Oenothera glazioviana*, and *Tropaeolum majus*, which are mainly used as ornamental plants. The presence of these species in seed mixes is critical as it could favor their spread throughout the country, even in regions where they are not yet established (Suppl. material 2; PFI 2023). Another species of concern is *Lupinus polyphyllus*, which is invasive to northern and central Europe, Australia and New Zealand, where it negatively affects native species richness (Eckstein et al. 2023). This species is naturalized in northern Italy (Suppl. material 2; PFI 2023) and was found in three of the studied mixes. Moreover, *Ageratum houstonianum*, invasive to many countries of the globe except to Europe and two *Impatiens* species (*I. balsamina* and *I. wallerana*), invasive to South America, Oceania and to many tropical and subtropical islands (CABI 2024), are established as casual alien to Italy (PFI 2023). Finally, we also detected species that are not part of the spontaneous flora of Italy (not recorded) but are recorded as invasive in other countries. For instance, *Salvia coccinea* is considered invasive to China (Hao and Ma 2023), Australia (Murray and Phillips 2012), and South Africa (Moshobane et al. 2020) while *Gaillardia aristata* to Hungary, prompting Süle et al. (2023) to ask for its exclusion from seed mixes.

Apart from the obvious negative regard towards invasive species, even the presence of other alien categories (such as not recorded and casual species) in seed mixes could pose a serious threat to biodiversity and habitat conservation, as these plants could eventually establish as naturalized or, more seriously, invasive alien in the next future. Indeed, species invasiveness is extremely difficult to predict and there is a well-known time lag between introduction, establishment and spread of invasive species (Crooks 2005; Gigon and Weber 2005; Pyšek et al. 2020). Moreover, climate change could lead to more favorable environmental conditions for the naturalization of today's harmless alien species, which can gain advantage over native ones (Haeuser et al. 2019; Yang et al. 2022). Thus, caution should be paid when including alien plants in wildflower mixes, especially if they are intended to sow in extra-urban areas such as in fields and field margins and as part of agri-environmental and eco-schemes, where their use is not advisable (Havens and Vitt 2016; Seitz et al. 2020; Barry and Hodge 2023). In urban settings, wildflower areas not only benefit biodiversity, but also provide an aesthetically pleasant space which increases people's well-being (Bretzel et al. 2016). In this context, the use of alien species is of lower concern, but only for those plants whose biology, ecology and mode of interaction with native plants and pollinator populations are well known. In fact, herbaceous ornamentals in urban areas can be an underestimated reservoir of potentially invasive plants (Hu et al. 2023), thus a wider use of natives should be encouraged (Caser et al. 2022).

The legislation does not completely prevent the use of alien species in wildflower mixes, even if they are invasive. At the European level, Regulation EU 1143/2014 lists the invasive alien species of Union concern (Union List) for which keeping, cultivation, selling, and releasing into the environment are forbidden. However, this list misses many invasive species, e.g. plants that are alien to certain European countries but not to others. Other existing national or regional lists may restrict the use of alien species too, but they can miss many invasive plants as well. In Italy, regional black-lists of alien species are available in some but not all administrative regions, while a common national black list is missing (Brundu et al. 2020). For European agri-environmental and eco-schemes, each country establishes its own regulations, and often the use of alien plants is allowed. In Italy, legislation provides a list of plants that can be used for eco-schemes, which includes two neophyte crops (*Helianthus annuus* L. and *Fagopyrum esculentum* Moench) and one invasive alien (*Crepis sancta* (L.) Bornm.) (Suppl. material 2; PFI 2023) (Lettieri et al. 2023). While the use of crop neophytes raises no concerns from a biodiversity conservation perspective, as they have been cultivated by man for a long time and are not competitive in the wild, the inclusion of *C. sancta* in seed mixes could potentially damage native plant communities because of its acknowledged invasive status.

Although some alien species are effective in providing abundant resources to insect pollinators, native plant species can be very good pollen and nectar sources as well (Ouvrard et al. 2018; Warzecha et al. 2018). Among the key species for pollinators identified by Warzecha et al. (2018), the seed mixes of this study included the natives *Achillea millefolium*, *Malva sylvestris* L., *Daucus carota* L., *Echium vulgare* L. and *Linum usitatissimum* L. and the archaeophytes *Calendula officinalis* and *Centaurea cyanus*. Archaeophytes were well represented in the studied seed mixes, with 17 species in total and one species (i.e. *C. cyanus*) as the most frequent. Many of these archaeophytes are annual weeds of cereal crops that were introduced in Europe by man with agriculture (segetal vegetation) and now are considered rare due to agricultural intensification (Albrecht et al. 2016). In this regard, the use of 'pollinator-friendly' wildflower mixes could promote the conservation of such rare arable plants (e.g., *Agrostemma githago* L. and *C. cyanus*).

When evaluating the suitability of native species for sowing, attention should also be paid to their distribution range. This is especially significant in a country as diverse as Italy, where there are different biogeographical regions characterized by a diverse flora. In this study, the native range of many autochthonous species (41% of the species) available in the seed mixes do not cover the entire country, thus their use in certain areas can be problematic. This issue was more pronounced in the insular area, with 45% of the native species that were locally alien. Sicily and Sardinia are the biggest Mediterranean islands, are a macro-hotspot of plant diversity in the Mediterranean basin (Cañadas et al. 2014) and host a rich endemic flora (Médail and Quézel 1997; Peruzzi et al. 2014; Fois et al. 2022). Although there is so far little evidence of extinctions directly caused by introduced plant species on islands (Sax and Gaines 2008), insular biota is considered to be particularly vulnerable to the negative effects caused by the spread of alien taxa (Reaser et al. 2007). To account for the issue of not introducing locally alien species, it would be helpful to develop different mixtures depending on the area where they are intended to be sown. For instance, species native to northern and peninsular Italy only (e.g., *Betonica officinalis* L., *Plantago media* L. etc.) should not be used in seed mixes on the islands. In line with this, Italian legislation for eco-schemes states that only the plants already present in a certain administrative region can be used for seeding wildflower areas in that region (Lettieri et al. 2023).

In addition to the attention for species chorology, the origin of the seeds is another important factor to consider when planning the creation of wildflower areas (Barry and Hodge 2023). However, seed origin indications are usually not available in commercial seed mixes, which typically use foreign ecotypes and cultivated varieties, resulting in the risk of genetic pollution of native vegetation (Mainz and Wieden 2019; Barry and Hodge 2023). According to the EU Directive 2010/60 concerning the use of preservation mixtures, it would be advisable to promote the use of local species ecotypes through the geographical definition of regions of origin, where the seeds are to be collected, propagated and sown (Durka et al. 2017). Today, only seven countries in Europe defined regions of origin within their national borders (Austria, Czech Republic, France, Germany, Great Britain, Norway, and Switzerland). However, the definition of transnational regions of origin in Europe would have a more ecological and biological significance (De Vitis et al. 2017). Moreover, the establishment of a European certification system that guarantees the region of origin, similar to those already established in certain European countries, would be important to ensure the transparency and traceability of the native seed market (Mainz and Wieden 2019). Currently, the native seed market is underrepresented in Italy, with virtually only one company that produces native wildflower seeds and lots of plant species not available on the market. For instance, among the species allowed for use for eco-schemes in Italy, only a small number is actually available (Lettieri et al. 2023). If more of the species native to Italy (consisting of 8080 angiosperm taxa, Bartolucci et al. 2024) were commercially available on the seed market, the presence of alien species in wildflower mixes could be discouraged.

Finally, another key criterion when composing a ‘pollinator-friendly’ wildflower seed mix is ensuring blooms that cover the whole season of pollinators’ activity, especially during critical periods such as early spring and from late-summer to autumn (Hicks et al. 2016; Ouvrard et al. 2018; Nichols et al. 2022). In this study, some of the seed mixes did not cover such critical periods with blooms, resulting in possible pollen and nectar shortages. Moreover, native and alien species differed in the flowering period, with native species characterized by more species blooming in spring and alien species in autumn. This highlights that alien species may play an important role in complementing the flowering period of native species by filling the late-season with feeding resources (Kovács-Hostyánszki et al. 2022; Zaninotto et al. 2023). In this context, the availability on the market of a greater number of native species with a late-season flowering period appears to be a crucial point for reducing the use of alien plants and developing effective native wildflower seed mixes.

Conclusions

This study showed that ‘pollinator-friendly’ wildflower seed mixes, increasingly used in both urban and rural settings, can be potential vectors of alien plant species, resulting in critical risks for biodiversity and habitat conservation. Thus, we underline the importance of taking into account the alien and native status of plant species in the design of such mixes, especially when sown in extra-urban areas. Moreover, another criterion worthy of attention when utilizing a seed mix is to consider the area where it is intended to be planted, avoiding introducing species which can be native to the country but locally alien to certain regions of it. Finally, to guarantee prolonged blooms throughout the pollinators' activity season, it appears crucial to introduce more native species with a late flowering period. Given the growing interest in wildflower areas, as part of green infrastructures in the cities or as wildflower strips targeted by European incentives, this study can help policy makers in suggesting a careful use of the seed mixes while promoting the use of local seeds by the development of the native seed market and the definition of regions of origin. The approach here used to analyze the current situation of 'pollinator-friendly' seed mixes in Italy can be transferred to other countries.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

No funding was reported.

Author contributions

GN, Methodology, Formal Analysis, Writing-Original Draft, Writing - Review and Editing; NMF, Methodology, Writing-Original Draft, Writing - Review and Editing; VS, ML Conceptualization, Methodology, Writing - Review and Editing, Supervision.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Database of seed mixes species composition

Authors: Ginevra Nota, Nicole Melanie Falla, Valentina Scariot, Michele Lonati

Data type: xlsx

Link: https://doi.org/10.3897/neobiota.94.118480.suppl1

Supplementary material 2

Distribution maps of plant species

Authors: Ginevra Nota, Nicole Melanie Falla, Valentina Scariot, Michele Lonati

Data type: pdf

Link: https://doi.org/10.3897/neobiota.94.118480.suppl2

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 225–242 (2024)
DOI: 10.3897/neobiota.94.126714

Research Article

An integrative framework to assess the spatio-temporal impact of plant invasion on ecosystem functioning

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Abstract

Invasive species can alter the structure and functioning of the invaded ecosystem, but predictions of the impact of invasive species on ecosystem functioning are weak. Invasion is determined by the interplay of invasive species traits, the recipient community, and the environmental context. However, efficient approaches to assess the spatial dimension of functional changes in heterogeneous environments and altered plant-plant interactions are lacking. Based on recent technological progress, we posit a way forward to i) quantify the fine-scale heterogeneity of the environmental context, ii) map the structure and function of the invaded system, iii) trace changes induced by the invader with functional tracers, and iv) integrate the different spatio-temporal information from different scales using (artificial intelligence-based) modelling approaches to better predict invasion impacts. An animated 3-D model visualisation demonstrates how maps of functional tracers reveal spatio-temporal dynamics of invader impacts. Merging fine- to coarse-scale spatially explicit information of functional changes with remotely sensed metrics will open new avenues for detecting invader impacts on ecosystem functioning.

Key words: community structure, environmental context, functional tracer, invader-ecosystem interaction, remote sensing, spatio-temporal heterogeneity, spatio-temporal modelling

OPEN ACCESS

Academic editor: Angela Brandt
Received: 2 May 2024
Accepted: 11 July 2024
Published: 7 August 2024

Citation: Werner C, Hellmann C, Große-Stoltenberg A (2024) An integrative framework to assess the spatio-temporal impact of plant invasion on ecosystem functioning. NeoBiota 94: 225–242. https://doi.org/10.3897/neobiota.94.126714

Introduction

Biological invasions of non-native species pose a large threat to biodiversity (IPBES 2019). Many invasive plant species significantly alter the biophysical and biochemical environment, thereby facilitating their own growth (Gaertner et al. 2014), with cascading effects on the structure and functioning of the invaded ecosystems. However, mechanisms of invader impact can be manifold (Ehrenfeld 2010; Sapsford et al. 2020), and challenges to quantify impact occur due to the multiplicity of invading species, context-dependencies, and interactions, as well as intraspecific trait variation (Sapsford et al. 2020). Predicting the effects of biological invasions on ecosystem functioning and services is of uttermost importance for prioritizing management and anticipating undesirable consequences of invasions (Simberloff et al. 2013; Jeschke et al. 2014; Brundu et al. 2020; Essl et al. 2020; Kumschick et al. 2020; Pyšek et al. 2020b; Ricciardi et al. 2021; Gallardo et al. 2024; Vilà et al. 2024). To date, multiple hypotheses and concepts have been developed that capture different aspects of invasion impact, habitat invasibility, and species' invasiveness (Hobbs and Humphries 1995; Alpert et al. 2000; Whitney and Gabler 2008; Enders et al. 2020; Novoa et al. 2020; Catford et al. 2021; Cavieres 2021; Hui et al. 2023), although data to thoroughly test these are often scarce (Gioria et al. 2023).

The ecological impact of invasive species depends on direct interactions between native and invasive plants, which in turn are influenced by native and invasive species' traits (Pyšek et al. 2012, 2020a; Sapsford et al. 2020), or trait differences between both groups (Castro-Díez et al. 2014; Lee et al. 2017; Kuebbing et al. 2018; Dyderski and Jagodziński 2019). Consequently, there is increasing awareness of the relevance of interactions and dynamics for invasion success, such as the interplay between invading species' traits and the recipient ecosystem (Kueffer et al. 2013; Kumschick et al. 2015; Sardans et al. 2017; Novoa et al. 2020). However, new approaches are needed to quantitatively assess these interactions (Gioria et al. 2023).

Species interactions as well as plant-soil feedbacks take place on a spatially confined scale within centimetres to metres in the neighbourhood of the invader (Mitchell et al. 2006), which is further referred to as fine-scale. While the temporal aspect of such feedbacks has been documented (Yelenik and D'Antonio 2013; Gioria and Osborne 2014), studies on impacts at plant-individual scale are clearly underrepresented (Crystal-Ornelas and Lockwood 2020). The spatial arrangement and distribution of native and invasive species is a decisive factor determining whether individuals interact and, potentially, which type of interaction – i.e., competition or facilitation – dominates (Hellmann et al. 2016a, 2016b; Cavieres 2021).

In addition, there is growing recognition that the effect of the invasive species is influenced by the environmental conditions, such as microclimatic or local edaphic conditions (Sapsford et al. 2020; Catford et al. 2021). The environmental context, namely the fine-scale spatial heterogeneity of abiotic and biotic conditions, is an important but often neglected dimension (Jarić et al. 2019; Fenesi et al. 2023), and the spatio-temporal variation of a system is a major source of uncertainty of impact assessments (Probert et al. 2020). Local availability of resources, such as water, nutrients or light, can shift the competitive balance between invasive and native species (Werner et al. 2010; Soliveres et al. 2015; Alba et al. 2019; Haberstroh et al. 2021). Therefore, predicting ecological impacts of invasive species considering context-dependency is still a major challenge (Ricciardi et al. 2021).

We posit that spatio-temporal heterogeneity can represent an environmental property in itself, which can affect invasibility, or else be affected by invasion, and requires better inclusion in invasion ecology. In the past, progress was hampered by a lack of methodological approaches, but now significant progress, e.g. in remote sensing technology, allows capturing high-resolution information on environmental heterogeneity at fine scales where plant-plant interactions take place.

Hence, we advocate combining advances in various disciplines of ecophysiology, invasion ecology, remote sensing, mapping, and modelling. This will open new opportunities to characterize environmental heterogeneity and associated changes in invasive-native species interaction at high-spatiotemporal resolution to better predict invasion dynamics and impact, as outlined in the following.

Concept for integrating fine-scale environmental heterogeneity and functional changes into spatial models of invader-ecosystem interactions

Direct interactions between the invasive and native species, such as above- or belowground facilitation or competition for e.g. resources, spaces, or pollinators will determine the invaders' successful establishment and growth (Fig. 1). These direct interactions are embedded in the environmental context, which is likewise shaping the competitive balance if conditions favour either native or invasive substitute by species. The competitive balance may even shift between competition and facilitation under changing environmental conditions or extreme climatic events (Werner et al. 2010; Grossiord 2020; Cavieres 2021; Haberstroh and Werner 2022). Inversely, some invaders can change environmental properties through positive- or negative feedback loops, thereby often promoting their own invasion success, which can ultimately result in regime shifts (Gaertner et al. 2014) and potentially magnify the impact beyond direct competition through cascading effects (Carboni et al. 2021). Currently, there exists a robust theoretical framework addressing the success of invasiveness as a result of direct and indirect interactions and the interplay between species traits, as well as on the recipient community structure and functioning and the environmental context (Gaertner et al. 2014; Novoa et al. 2020; Pyšek et al. 2020a; Hui et al. 2023; summarized in Fig. 2).

However, detecting and tracing the impacts of invasive species in natural environments have been hindered in the past due to a lack of suitable measurements and integration methods to explicitly quantify the spatio-temporal dimensions involved. This would require not only mapping the invasive species and its spread in natural systems, but also quantifying local changes in different abiotic and biotic processes that are altered by the invader (i.e. quantifying the local impact of the invader). As the latter is a function of both the environmental conditions and the structure and functioning of the native community, both need to be quantified at high spatio-temporal resolution. For each of these aspects, the required tools are at hand, but new integrative analyses are required.

Hence, we posit a way forward (Fig. 3) on how to i) quantify the fine-scale heterogeneity of the environmental context, ii) map the structure and function of the invaded system, iii) trace changes induced by the invader with functional tracers, and iv) apply effective approaches for integration of spatio-temporal information from different scales, e.g. via different (artificial intelligence-based) modelling approaches, for better prediction of invasive species impact.

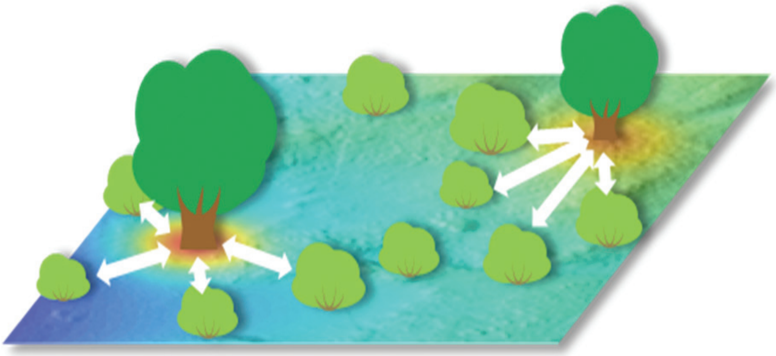

Figure 1. Invasive species directly interact with native species by competition or facilitation e.g. for above and belowground resources, thereby changing the biotic and abiotic environment locally.

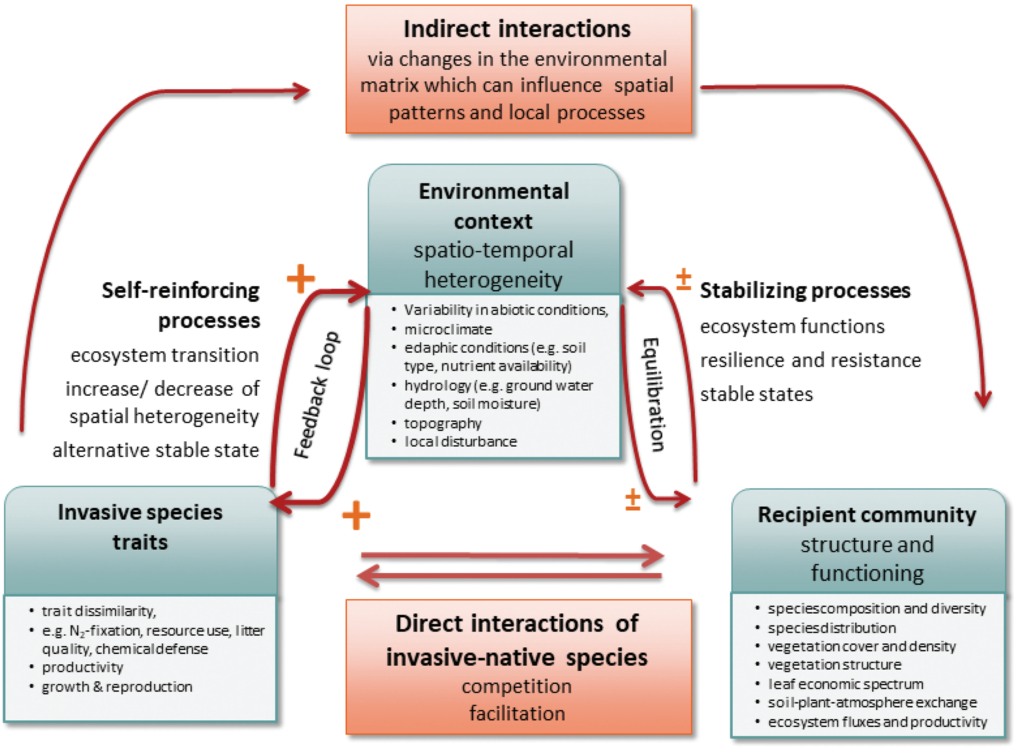

Figure 2. Mechanisms determine plant invasion impact. Plant invasion impact results from direct and indirect interactions between invasive and native species based on the interplay between invasive species traits, structure and function of the recipient community, and spatio-temporal heterogeneity of the environment. Direct interactions between invasive and native species result from competition or facilitation, e.g. for resources, whereas indirect interactions are mediated via subsequent changes in the biotic and abiotic environment, which may favour self-reinforcing processes of the invader or stabilizing processes of the community. Examples of important factors for each category are given in the boxes.

Environmental context

The environmental context is defined as the biogeochemical and physical matrix, which provides the background for both native and invasive species' biotic interactions (Fig. 3). This includes the fine-scale spatio-temporal heterogeneity in, e.g. abiotic conditions like hydrological and edaphic conditions or microclimate, as well as resource patches, e.g. after local disturbance. On the scale of centimetres to kilometres, variation in these conditions can influence plant performance and interactions, thereby creating a multi-layered mosaic of background conditions. An inherent challenge of geospatial analysis is that fine-scale or high resolution data are often collected over small areas, while for large areas only coarse-scale data are available (Millington 2021). However, research rapidly advances regarding the retrieval of two- and three-dimensional geospatial information on the environmental matrix using sensory networks (Allan et al. 2018; Lahoz-Monfort and Magrath 2021; Besson et al. 2022; Sethi et al. 2022) and remote sensing (Lausch et al. 2020; Lahoz-Monfort and Magrath 2021; Skidmore et al. 2021; Timmermans and Kissling 2023). Currently, a broad set of novel digital tools to describe the environmental context is emerging, including UAVs (Unmanned Aerial Vehicles), microsatellites (e.g. PlanetScope), active sensor systems based on LiDAR (Light Detection and Ranging) or SAR (Synthetic Aperture Radar) technology for vegetation structure analysis, and advanced versions of the established Landsat and Sentinel-2 satellites (Crowley and Cardille 2020).

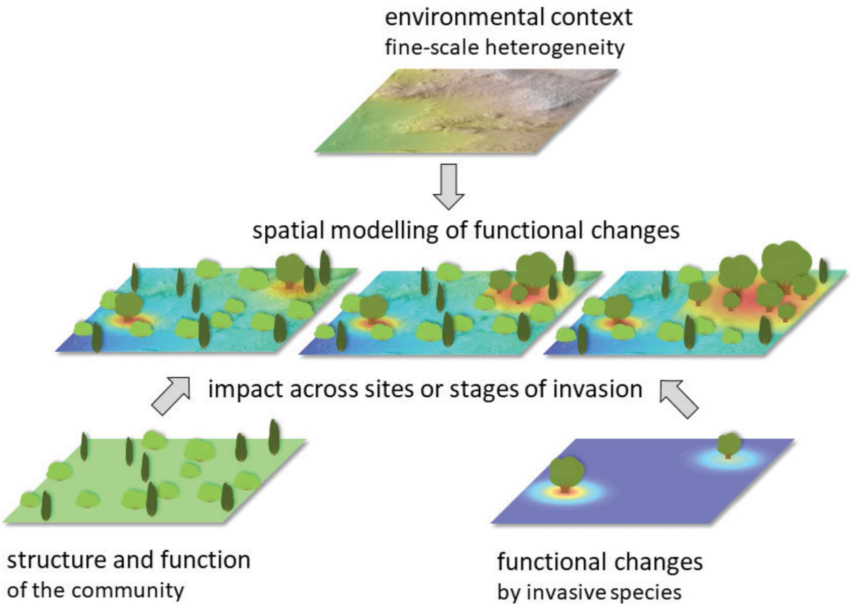

Figure 3. Framework for integrating fine-scale environmental heterogeneity and functional changes into spatial models of invader-ecosystem interactions. Maps of biochemical and biophysical heterogeneous environments can be directly incorporated into predictive models of impact measures across different sites or stages of invasion. Essential complementary spatial data include the location of the invasive species, maps of functional tracers that reflect local changes in key functions induced by the invader, and maps of the recipient community structure and function (e.g. Hellmann et al. (2017)). Technological advances in measurement techniques, sensor networks, and remote sensing will facilitate the collection of high-resolution data on the environmental context, the invasive species, and the recipient community, thereby improving the understanding of invasion dynamics and processes, particularly at the neighbourhood scale.

Structure and function

The environmental context further shapes native species distribution patterns as well as structure and function of the community. Indeed, spatial complexity, in which invasive species interactions take place, can be a proxy for ecosystem structure and dynamics in itself (Parrott 2010). Similarly to assessing the environmental conditions, advances in remote sensing techniques allow for multiscale and multi-temporal mapping of species distribution, and landscape structure and function (Crowley and Cardille 2020), which are exceptionally valuable to describe both the environmental context and structure of the recipient community. LiDAR, SAR, and digital aerial photogrammetry hold great potential to describe ecosystem structural parameters such as vegetation height, cover, density, structural complexity, and population structure (Valbuena et al. 2020). Proofs-of-concept exist for high-resolution automated structural measurements (Calders et al. 2023), while both active and passive sensor systems can be used to map ecosystem functions (Pettorelli et al. 2018).

Moreover, spatially explicit maps of the invader are required, as impact is related to abundance in various forms (Sofaer et al. 2018; Strayer 2020; O'Loughlin et al. 2021). Access to spatial data on invasive plant distribution is improving (Fusco et al. 2023), and distribution maps of the invader can be created and updated across scales using data from UAS (Uncrewed Aerial Systems), aircrafts, and satellites (Vaz et al. 2018; Timmermans and Kissling 2023). Freely available earth observation data are extremely useful to produce such distribution maps, particularly in data poor regions (Truong et al. 2017). Novel approaches to map invasive species and characterize the species composition of the recipient community include methods of Deep Learning (DL) and other methods of machine learning. In invasion ecology, the potential use of DL is manifold (Christin et al. 2019; Hirn et al. 2022; Perry et al. 2022), but its main application is probably species identification and mapping (Christin et al. 2019; Kattenborn et al. 2021; Borowiec et al. 2022; Müllerová et al. 2023).

Functional tracers of invader impact

Ultimately, the impact of the invader, i.e. its effect on the biophysical, biochemical, and biological environment, has to be assessed. One effective way to quantify invasive species impact is the use of functional tracers, which reflect local changes in key functions at fine spatio-temporal scales. The choice of suitable tracers will depend on both the invasive species under study and invaded community properties, and should capture the processes likely altered by the invasive species, for example nitrogen for N-fixing invaders in N-poor environments or water balance for water-spending invaders in water-limited systems (Fig. 2). Stable isotopes, for example, can provide such functional or ecophysiological tracers, as they reflect changes in interactions and altered functional processes at fine spatial resolution (Cheesman and Cernusak 2016), and provide great potential to unravel mechanisms of invasions (McCue et al. 2020). Applied in a spatially explicit manner (i.e. isoscapes (Rascher et al. 2012)), they can trace local changes in plant-plant interaction and in environmental conditions (Hellmann et al. 2017). Maps can be generated for isotopic measurements of the soil environment or leaves of different native species, thereby directly mapping the invader impact on these species (Hellmann et al. 2016a, 2016b; Nielsen et al. 2016; Sena-Souza et al. 2023). For example, atmospheric N_2-input by N-fixing invaders can be traced into the native vegetation by nitrogen isotopes ($\delta^{15}N$, example in Fig. 3). These changes can lead to cascading effects on other ecosystem functions. Besides nutrient cycling, subsequent changes, e.g. in water and/or carbon cycles, may also be involved (Le Maitre et al. 2015; Dzikiti et al. 2017). Competition for water can affect the water-use-efficiency and hydraulic regulation of native species (Haberstroh et al. 2021), with cascading effects on ecosystem water balance (Rascher et al. 2010; Caldeira et al. 2015; Le Maitre et al. 2020). Changes in native species' carbon isotope ratio ($\delta^{13}C$) can resolve changes in water use efficiency in response to competitive or facilitative invader interactions (Hellmann et al. 2016a; Crous et al. 2019; Sena-Souza et al. 2023). Even shifts between facilitation and competition with increasing distance to the invader have been observed (Hellmann et al. 2016a).

Different tracers can be combined (Funk et al. 2017), such as C, N, P concentrations or other biochemical properties that may be affected by the invasion (Drenovsky et al. 2012; Hellmann et al. 2016a; Helsen et al. 2020; Sena-Souza et al. 2023) or which might reveal functional differences (Große-Stoltenberg et al. 2018b; Meira-Neto et al. 2023) along environmental gradients (Crous et al. 2019). Thereby, differences in the spatial dimension of impact can be revealed both between native and invasive species, depending on their susceptibility to these changes, and with respect to different processes involved.

Remote sensing techniques have also proven invaluable in examining functional properties of invasive species (Helsen et al. 2020; Andrew et al. 2014; Dzikiti et al. 2016; Große-Stoltenberg et al. 2018a, 2018b; Ewald et al. 2018; Hacker and Coops 2022; Große-Stoltenberg et al. 2023) as well as plant-plant interactions (Chen et al. 2022). Further, first studies show that mycorrhizal traits (Chaudhary et al. 2022), which may constrain invasion success (Pringle et al. 2009) and/or be altered after invasion (Lekberg et al. 2013), can be inferred from hyperspectral data at leaf (Jantzen et al. 2023) and canopy level (Sousa et al. 2021). Where remote sensing methods reach their limits, novel field-portable instruments and sensor networks are very promising tools to measure ecophysiological information at the individual plant level with very high temporal resolution (Tognetti et al. 2022).

Additionally, advances in wireless, autonomous microsensors, such as leaf wearable sensors of ecophysiological processes (Frey et al. 2023; Reimer et al. 2021), may offer novel sampling strategies. Currently, novel autonomous sensor networks are being developed (e.g. ECOSENSE, Werner et al. 2024; Allan et al. 2018; Besson et al. 2022; Tognetti et al. 2022), enabling high spatial coverage of different functional properties in heterogeneous environments with distributed sensors continuously recording at high temporal resolution. Autonomous sensing is coupled to wireless data transmittance and real-time data assimilation into large databases to streamline the information flow and enable real-time analysis. Though still in its infancy, these novel automated sensing networks may also provide valuable new insights in invasion ecology. Such standardized and automated networks of field sensors are required to validate proxies of ecosystem functioning derived from satellite data, particularly in heterogeneous ecosystems (Naethe et al. 2024). Clearly, challenges apply when sampling at such fine resolution regarding data volume, data heterogeneity, varying data quality, and timely data availability, which requires sophisticated data management and analysis (Farley et al. 2018), as well as appropriate sampling strategies. Despite all technological progress, trade-offs between resolution and extent of analysis will still apply. Nevertheless, these new technologies bear the potential to provide the high spatial coverage required in heterogeneous environments to quantify invader-ecosystem interactions and validate remote sensing data for model transfer and upscaling.

Integration: spatial modelling of functional changes and impact assessment across different stages of invasion

Integration of the information on functional changes by the invader, characteristics of the recipient community, and the environmental context from different sources and at different scales is needed to assess and predict the invader impact on ecosystem functioning along gradients of invasion in heterogeneous ecosystems (Figs 3, 4). Once the functional tracer of impact is identified, the spatio-temporal dimension of the impact is understood, and spatial layers of both ecosystem structure and invader distribution at appropriate scale are available, alterations of ecosystems in the neighbourhood of the invader can be mapped using spatial modelling approaches.

Novel technologies clearly facilitate data sampling at multiple resolutions (see above). This enables explorative analysis of species-environment interactions at multiple scales, which is essential when spatio-temporal dimension of the effect is not known (see Holland and Yang 2016). Integrative approaches include mixed-effect models (Golicz et al. 2023), which have been applied to map invader-ecosystem interactions (Hellmann et al. 2017). To assess model transferability, area of applicability, variable selection, and methods of cross-validation need to be carefully evaluated (Ludwig et al. 2023). Recording data with high spatio-temporal resolution will inevitably lead to large data sets. Again, machine learning approaches, in particular DL, have recently gained popularity to analyze complex spatio-temporal datasets (Wikle and Zammit-Mangion 2023). Within the field of ecology, the versatility of DL is evident (Christin et al. 2019; Hirn et al. 2022; Perry et al. 2022), with its primary utility likely lying in species identification and mapping

(Christin et al. 2019; Kattenborn et al. 2021; Borowiec et al. 2022; Müllerová et al. 2023). Further, methods of DL enable multi-trait retrieval across vegetation types even when data are scarce (Cherif et al. 2023). Recent progress also includes the retrieval of traits using hybrid models, which combine leaf-canopy-atmosphere radiative transfer modelling with Gaussian processes and enable upscaling of trait maps from the local to regional level, including uncertainty estimates (Estévez et al. 2022). Especially in earth system sciences, DL approaches have been used to model system states and analyze systems processes (Reichstein et al. 2019). Future progress regarding model interpretability and explainability is expected if data-driven approaches and physical models are combined (Reichstein et al. 2019), and concepts are developed for automated workflows and pipelines to study ecosystem dynamics (Besson et al. 2022). However, progress in automated data collections and analysis based on artificial intelligence is not a solution per se to answer questions in ecology. It also poses challenges, e.g. in terms of standards, protocols and workflows, data infrastructure and data quality. Thus, defining scope and scale to study ecological phenomena will still be required (de Koning et al. 2023).

In the following, we will use an example to quantify the impact of a N-fixing invader in a N-poor Mediterranean ecosystem as one efficient but not exclusive way to integrate spatio-temporal information and functional tracers for invader impact assessment (Fig. 4).

We used field-based maps of both a N-fixing invasive species and a functional tracer ($\delta^{15}N$), which were joined with airborne LiDAR data on topography (environmental context) and vegetation structure (recipient community) to model functional changes across sites and stages of invasion (Hellmann et al. 2017). Plotting these maps onto fused airborne LiDAR and true colour image data allows visualizing and communicating context-dependent invader-ecosystem interactions at the molecular level, which are otherwise undetectable. The nitrogen isoscapes in Fig. 4 are centred around a N-fixing invasive species and illustrate the effect of dynamic N enrichment by the invader (reddish colours) in a N-poor open dune ecosystem (indicated by the yellowish colours) in three-dimensional space. This N-fixing effect does not occur uniformly around the invader, but is shaped by vegetation structure and topography. This explains, for example, N-transfer from fixation into the non-N-fixing native vegetation (yellowish colours), and the flush of nitrogen into sparsely vegetated areas downhill from the invader, which will slowly be transformed into denser habitats.

Other integrative approaches include the combination of field-based and remotely sensed data on native and invasive species distributions, vegetation structure, Leaf Area Index, or evapotranspiration to, for example, estimate water consumption of an invader in riparian habitats (Nagler et al. 2009; Dzikiti et al. 2017). To transfer and validate approaches across landscapes, information on topography (environmental context) and species composition (recipient community) is deemed essential (Le Maitre et al. 2015).

In summary, the importance of linking ecophysiology with remote sensing data to understand invasion processes has been outlined (Niphadkar and Nagendra 2016), and integrative approaches on mapping invader-ecosystem interactions are at hand (Nagler et al. 2009; Dzikiti et al. 2016; Hellmann et al. 2017). Due to technological progress, automated systems to study ecosystem dynamics at unprecedented scales are being developed and implemented (Allan et al. 2018; Besson et al. 2022; Tognetti et al. 2022), with potential to build digital twins (de Koning et al. 2023) of plant invasion impact, i.e. dynamic virtual representations or models used for simulations

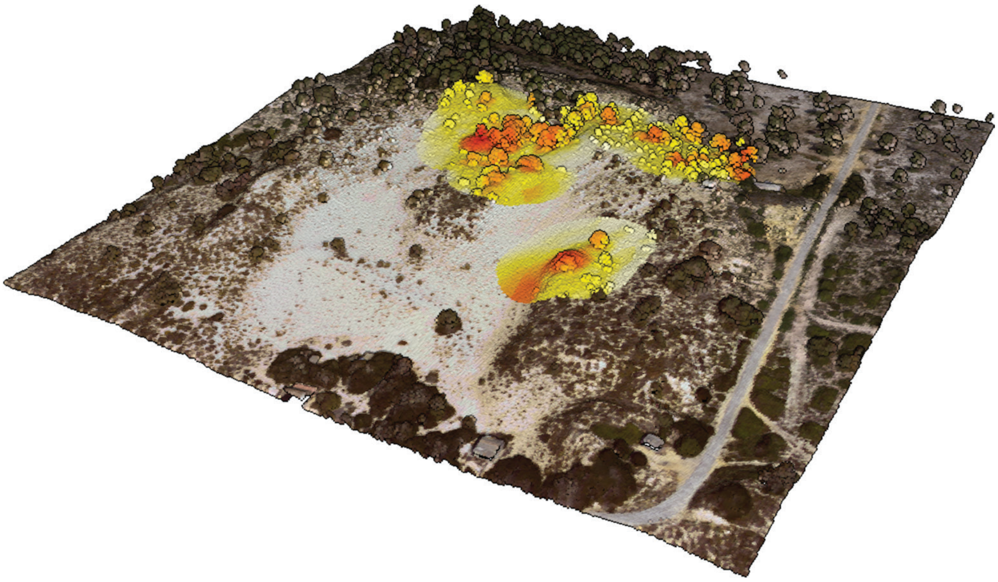

Figure 4. Model visualisation of spatio-temporal dynamics of invader impacts based on the suggested framework. Modelled isoscapes centred around a N_2-fixing invasive plant species using the functional tracer $\delta^{15}N$ and information on the environmental matrix in a nutrient poor ecosystem based on Hellmann et al. (2017). Reddish colours indicate high-levels of atmospheric fixed nitrogen inputs (e.g. dense invader patches and flushes of N into native vegetation); yellow colours indicate lower levels of impact, while whitish colours indicate no impact and are representative for the original status before invasion. The local functional changes do not occur uniformly. Isoscapes are plotted onto high-resolution airborne LiDAR data fused with true colour imagery to illustrate the effect of LiDAR-derived vegetation structure of the recipient community and topography on invader impact in this heterogeneous ecosystem. The 3D map was created using QGIS version 3.30. An animated 3D-video of the model can be found at https://tinyurl.com/4hs23b8p.

and analyses. We envision that these new approaches will also lead to advances in the field of biological invasions, namely to better understand fine-scale invader-ecosystem interactions, test invasion theories, and provide robust validation data for interactions across spatio-temporal dimensions in heterogeneous ecosystems.

Concluding remarks

Spatio-temporal patterns and variation of plant-plant interactions in heterogeneous environments deserve better integration in invasion research. Here we advocate the use of functional tracers for integrating fine-scale interactions between the invasive species, the recipient community, and the environmental context into spatial models to assess context-dependency of invader impact, namely the interplay of direct and indirect invasive-native species interactions. We advocate drawing on the large toolbox of recent methods, which when combined, can open new doors for mapping and predicting changes in ecosystem functioning and for assessing and disentangling the influence of spatio-temporal heterogeneity on invader impacts. By explicitly emphasizing the spatio-temporal variation of plant-plant interactions in invasion ecology, we anticipate major advances for understanding of invasion history, patterns of spread, impact assessment, and prediction of future invasions.

Acknowledgements

Funding was provided by the DFG (WE 2681/8-1, WE 2681/10-1), EUFAR (DeInVader, EUFAR11-06), DAAD (AGS). We thank NERC's Airborne Research Facility and Data Analysis Node for conducting the airborne survey.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

No funding was reported.

Author contributions

All authors developed the framework. CH and CW wrote the first draft, which was revised by CW and AGS. AGS implemented the video animation.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Description of the animated video

Authors: Christiane Werner, Christine Hellmann, André Große-Stoltenberg

Data type: docx

Link: https://doi.org/10.3897/neobiota.94.126714.suppl1

Supplementary material 2

Animated video

Authors: Christiane Werner, Christine Hellmann, André Große-Stoltenberg

Data type: mp4

Link: https://doi.org/10.3897/neobiota.94.126714.suppl2

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 243–259 (2024)
DOI: 10.3897/neobiota.94.119622

Research Article

Dead or alive: the effect of shells and living individuals of *Sinanodonta woodiana* (Lea, 1834) on habitat selection and behaviour of European unionid bivalves

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Abstract

1. Ecosystem engineering freshwater bivalves, burrowing in the substratum and accumulating shell deposits, transform bottom habitats. Especially the invasive Asian bivalve *Sinanodonta woodiana* (SW), due to its rapid growth, large size, and high fecundity, can affect benthic communities. Here, we determined its effect on habitat selection and behaviour of endangered native bivalves, *Anodonta cygnea* and *Unio tumidus*.

2. We conducted laboratory preference assays (Experiment 1: choice between two substrata) exposing the native bivalves to pure sand (control), shells (several densities on the sand surface or burrowed), or living SW. Then, we tested their locomotion and burrowing (Experiment 2) on pure sand and substrata contaminated with shells or living SW.

3. In Experiment 1, native bivalves avoided shells, but not living SW. Burrowed and larger shells were avoided compared with those on the surface and smaller ones, respectively.

4. In Experiment 2, *U. tumidus* exposed to SW delayed activity initiation (in response to living bivalves), increased locomotion (living bivalves, surface shells), and reduced burrowing depth (living bivalves, all shells). *Anodonta cygnea* exposed to SW reduced locomotion speed (living bivalves, shells), and reduced burrowing duration (burrowed shells) and depth (living bivalves, burrowed shells).

5. SW (especially shell beds) constitutes another emerging threat to native bivalves, impairing their burrowing and inducting active avoidance. As SW expands its distribution with climate warming, the range and strength of its impact is likely to increase, reducing the area available to native bivalves, exposing them to environmental dangers (due to burrowing limitation) and deteriorating physical condition (energetic resources used for excessive locomotion).

Key words: Behaviour, biological invasions, Bivalvia, ecosystem engineers, habitat selection, interspecific interactions, species displacement, unionid mussels

Academic editor: Belinda Gallardo
Received: 26 January 2024
Accepted: 12 June 2024
Published: 7 August 2024

Citation: Wiśniewski K, Szarmach D, Kobak J, Kakareko T, Jermacz Ł, Poznańska-Kakareko M (2024) Dead or alive: the effect of shells and living individuals of *Sinanodonta woodiana* (Lea, 1834) on habitat selection and behaviour of European unionid bivalves. NeoBiota 94: 243–259. https://doi.org/10.3897/neobiota.94.119622

Introduction

Bivalves of the Unionidae family are freshwater bottom dwellers of limited mobility (Curley et al. 2021). Through filter-feeding, bivalves can considerably modulate the availability of resources for other organisms by transferring suspended particles to the bottom sediments (Boeker et al. 2016; Pouil et al. 2021). Both living bivalves and their empty shells, accumulating in the environment long after the animal death, constitute unique hard structures affecting community functioning (Gutiérrez et al. 2003). Therefore, bivalves are considered to be ecosystem engineers and their extinction, overpopulation or changes in their taxonomic composition lead to habitat modifications with cascading effects on the aquatic community and ecosystem services provided by these animals (Vaughn 2018).

Freshwater bivalves are threatened globally by human impact, including climate change and pressure from non-native species (Strayer 2008, Blackburn et al. 2014; Bacher et al. 2018). The number of alien bivalve species and sizes of their populations have been growing for several decades (Seebens et al. 2021; Latombe et al. 2022). Accordingly, the pressure from non-native bivalves is considered a major threat to their native counterparts (Mack et al. 2000; Pyšek et al. 2010), especially due to their ecosystem engineering properties and resulting habitat transformations (Bódis et al. 2014a; Douda et al. 2024).

The Chinese pond mussels of the genus *Sinanodonta* are unionid bivalves native to Eastern Asia, but invasive in other parts of the world. Recent genetic studies have shown that invasive lineages belong to three species: (i) *S. woodiana* (Lea, 1834), the "temperate invasive" lineage, native to southern China and invasive in Europe, as well as in western and central Asia, observed probably in Africa (finding needs genetic confirmation (Bensaâd-Bendjedid et al. 2023)); (ii) *S. pacifica* (Heude, 1878) the "tropical invasive" lineage, whose native area is Taiwan and eastern China, whereas it is invasive in North America, southern Asia and Iraq, and (iii) *S. lauta* (Martens, 1877), originating from Japan, the Korean Peninsula and eastern Russia, and invading central and southern Asia (Douda et al. 2024). In Europe, only *S. woodiana* ("temperate invasive" lineage) occurs, likely originating from a single introduction event (Konečný et al. 2018). Therefore, we will focus on this species in the current study. In colder regions (such as central and eastern Europe), its spread was initially limited to artificially heated waters (Urbańska et al. 2012), but, over the past two decades, it has accelerated and extended to habitats of natural thermal regime (Bogan et al. 2011; Bolotov et al. 2016; Lopes-Lima et al. 2017; Bespalaya et al. 2018; Kondakov et al. 2018, 2020; Konečný et al. 2018). Substratum preferences of *S. woodiana* overlap with those of the native European Unionidae (Poznańska-Kakareko et al. 2021) indicating a high risk of competitive tensions between them (Douda and Čadková 2018). This invasive bivalve exhibits a number of competitive advantages over the native Unionidae, including the higher rate of host infection by its parasitic glochidium larvae, faster development and growth rate (Douda et al. 2012; Huber and Geist 2019) and higher fecundity (Labecka and Domagala 2018; Labecka and Czarnoleski 2019).

Another potential mechanism of the impact of *S. woodiana* on native Unionidae can be the transformation of the bottom by living individuals and shell beds formed after the bivalve death (Bódis et al. 2014a; Nakano 2023). *Sinanodonta woodiana* is a large (up to 26 cm) and fast-growing species (Urbańska et al. 2019) reaching high densities. In a Polish lake, densities of 68 ind. m^{-2} and 27.9 kg m^{-2} were observed (Kraszewski and Zdanowski 2007), which is the highest density of this species reported for Europe. In other European countries, the density ranges from a few ind. m^{-2} in Ukraine (Yermoshyna and Pavliuchenko 2021) to c.a. 50 ind. m^{-2} in Hungary and Italy (Benkő-Kiss et al. 2013; Kamburska et al. 2013). Over time, shells accumulate on the bottom surface and in the sediments, outnumbering living individuals and forming a layer significantly changing the substratum quality. More than 280 ind. m^{-2} (counting both valves as one individual) were noted by Bódis et al. (2014a). Shells reduce the near-bottom current velocity, limit the access of light to the bottom, and increase microhabitat heterogeneity (Gutiérrez et al. 2003). Moreover, shells (especially large ones) can create physical barriers limiting bivalve movement and burrowing, and thus degrading the living conditions for these organisms. It is likely that the effect of shell beds formed by *S. woodiana* will be stronger than that of shells originating from the native species present in the environment before the invasion, due to the shorter lifespan of *S. woodiana*. It can live up to a maximum of 12 years (Spyra et al. 2012), compared to the maximum lifespan of 37 and 21 years exhibited by native *Anodonta cygnea* (Linnaeus, 1758) and *Unio tumidus* Philipsson, 1788, respectively (Aldridge 1999). This results in a faster accumulation rate of *S. woodiana* shells on the bottom. Moreover, its shells are larger, thus constituting larger and heavier physical obstacles in the substratum.

Knowledge of the responses of the native bivalves to the presence of *S. woodiana* will help understand the mechanisms and magnitude of its impact, as well as develop methods of dealing with this new threat. The aim of our study was to determine mechanical effects of substratum contamination with living individuals and shells of *S. woodiana* on behaviour (habitat selection, locomotion and burrowing) of two native European unionid bivalves: *A. cygnea* and *U. tumidus*. Their numbers are constantly decreasing worldwide (Lopes-Lima et al. 2017), and they are protected by law in several countries (Van Damme 2011; Lopes-Lima 2014). These species were selected due to their reported coexistence with *S. woodiana* (Lajtner and Crnčan 2011; Beran 2019) and similar habitat preferences (Poznańska-Kakareko et al. 2021). We hypothesized as follows: (1) native bivalves would avoid substrata contaminated with *S. woodiana*. (2) The adverse effect of shell beds on bivalve preferences would result from deteriorated burrowing and/or locomotion. Alternatively, increased locomotion might indicate active avoidance of the substratum contaminated by *S. woodiana*. (3) Empty shell beds would affect native bivalves to a greater extent than living *S. woodiana*. This might be due to (i) variable shell positions in the sediments (horizontally or vertically, on the surface or burrowed) compared to always vertically burrowed living bivalves (see Suppl. material 1: fig. S1), or (ii) the presence of sharp shell edges irritating the foot of moving bivalves. Options (i) or (ii) would be supported by stronger unionid responses to shells present on the sediment surface or burrowed, respectively. (4) The effect of *S. woodiana* shells on native bivalves would differ from that of native shell beds. A presumably stronger effect of the invader (compared to the shells of native bivalves) would result from either interspecific differences in shell structure (resulting in different unionid responses to shells of various species presented at the same sizes and densities) or the larger size of *S. woodiana* shells (resulting in stronger responses of unionids to larger shells).

Materials and methods

Bivalve collection in the field

Anodonta cygnea, *U. tumidus* and *S. woodiana* (shells and living individuals) were collected in early autumn from the sandy/muddy bottom (depth: 1.5–2.5 m) from the Włocławski Reservoir on the River Vistula, Central Poland (52°37'04"N, 19°19'42"E) by scuba divers. This site represents a natural thermal regime for central Europe, and has been recently invaded by *S. woodiana* (Cichy et al. 2016; Douda et al. 2024). Currently, all the study species co-exist at the location and the invader is constantly increasing its abundance and range (personal observation). We obtained *S. woodiana* shells from freshly killed individuals (on the day of collection), while native Unionidae shells were collected as fresh shells (uncorroded, undamaged) lying on the bottom of the reservoir (to avoid killing the native species). The collected bivalves were transported to the laboratory in buckets with substratum and water from the reservoir and tested after two weeks of acclimation.

Stocking conditions

Living bivalves (each species separately) and empty shells were kept in 350-L stock tanks (20–30 individuals per tank) equipped with internal filters and aeration systems, with the bottom covered by a few cm deep layer of sand taken from the collection site. The stock/experimental room was equipped with a photoperiod system (light/dark cycle: 12:12 h) imitating the natural day-night cycle, and air-conditioning which kept the water temperature in the tanks at the level similar to that observed in the reservoir during bivalve collection. We checked the water quality in the stock and experimental tanks using a multimeter Multi340i (WTW GmbH, Weilheim, Germany). The water parameters were within the following ranges: oxygen content: 7.37–7.77 mg ml^{-1} (82.9–87.2%); temperature: 18.4–20.1 °C; pH: 8.01–8.67; conductivity: 643–827 µS cm^{-1}. The bivalves were fed twice a week with a suspension of dried *Chlorella* algae ("*Chlorella* super alga", Meridian company, Poland) in a concentration of 5 mg L^{-1} (Douda and Čadková 2018).

Experiment 1: Habitat selection

Tests were conducted in 30 × 30 × 30 cm tanks divided into halves (Suppl. material 1: figs S1, S2). Each half was filled with a different substratum (see below) to a depth of 10 cm. Then, the tank was filled with conditioned (settled and aerated for at least 48 h) tap water (a 10-cm layer above the substratum surface). One bivalve individual was introduced in the central part of the tank with its ventral side down and anteroposterior axis parallel to the border between the substrata. After 24 h, the location of the tested individual was checked (choosing one of the two substrata). Each configuration of substratum types was repeated 30 times per species. We used the following substratum types:

(1) Sand (grain diameter range: 0.2–1.4 mm; median: 0.63 mm) obtained from the bivalve collection site. This material was earlier found to be preferred by all the species tested (Poznańska-Kakareko et al. 2021). The pure sand was used as a control. The same sand type was contaminated with *S. woodiana* to create other substratum types.

(2) Empty *S. woodiana* shells, small (mean length: 7.6 cm) or large (12.6 cm) (Table 1), composed of two valves connected naturally by the ligamentum. The shells were burrowed under the surface (covered with sand) or laid on the surface of the sandy substratum. We used these two shell position variants because a bivalve usually dies on the surface and, after some time, due to hydrodynamics and sedimentation, its shell becomes burrowed. This is especially visible in the case of mass mortalities, when large quantities of empty shells cover the surface of the bottom (Bódis et al. 2014b). Shell arrangement in/on the substratum (lying on their side or put vertically with their ventral surface down) was random (Suppl. material 1: fig. S1) to reflect their arrangement in the field. Shells were randomly put on the sand within the tank half they were assigned to (Suppl. material 1: fig. S1). Then, those assigned to the burrowed variant were gently and thoroughly covered with sand to fill all the spaces between them.

(3) Living individuals of *S. woodiana* (mean length of 11.6 cm, corresponding to the large shells; Table 1) immobilized by adhesive tape applied to the front of the shell (to prevent their relocation) and burrowed in the sandy bottom at 75% of their length (Suppl. material 1: fig. S4). The immobilized bivalves could not extend their foot and move, which was necessary to keep them in their positions within the assigned half of the tank (Suppl. material 1: fig. S4). However, they could partially open their valves, pull out the siphons, filter water and breathe.

All bivalves and shells were thoroughly rinsed with water before use and biofilm and adhering debris were scrubbed from their surfaces. The sand was rinsed and dried in a laboratory dryer (SLW 115 STD Multiserw-Morek, Poland) at 60 °C for 6 h before use to eliminate any organisms that could potentially affect the results of the experiment. It should be noted that the size defined as large in our study is not of the maximum size of *S. woodiana* (26 cm, Urbańska et al. 2019). These, however, can be generally collected from warmer waters, whereas we used the size range commonly available at the collection site of the thermal regime natural for central Europe.

First, we checked unionid selectivity between the pure sand and various shell densities (small or large, on the surface or burrowed). We started the experiment with a density of 133 ind. m^{-2} (6 shells per tank, two valves counted as one individual), i.e. twice as much as the maximum field density observed in heated waters. Then, we continued with the lower (67 ind. m^{-2}, 3 shells per tank) or higher (200 ind. m^{-2}, 9 shells per tank), depending on the presence or absence of a significant reaction to the initial density, respectively. This allowed us to determine the minimum effective density capable of influencing bivalve behaviour. We also confronted the pure sand with living *S. woodiana* at a density of 133 ind. m^{-2}. We did not use higher densities of living *S. woodiana*, as they would have been unrealistic given the maximum density reported in the wild (Kraszewski and Zdanowski 2007).

Moreover, we confronted the following: (i) burrowed shells vs. shells present on the sediment surface (using small shells at a density of 200 ind. m^{-2}) to check if shell position makes a difference, (ii) living *S. woodiana* vs. large burrowed shells (133 ind. m^{-2}) and (iii) burrowed small vs. burrowed large shells (200 vs. 133 ind. m^{-2}, corresponding to the same total volumes occupied by shells of the two sizes) to check whether bivalves respond differently to shell beds composed of shells of different sizes, (iv) native unionid shells vs. pure sand, (v) native unionid shells vs. small *S. woodiana* shells, to check if unionid responses to shells depend on shell origin.

Table 1. Total length of bivalves and shells [cm].

	Mean	SD	Range
A. cygnea	10.4	0.83	9.0-13.0
U. tumidus	7.2	0.60	6.5-8.5
Native bivalve shells*	7.0	0.88	5.5-9.0
S. woodiana living individuals	11.6	0.84	10.5-13.0
S. woodiana small shells	7.6	1.14	5.5-10.0
S. woodiana large shells	12.6	1.24	10.5-14.5

SD - standard deviation, * - *U. tumidus* with small admixture of *U. pictorum* and *A. anatina*.

Native shell beds were composed mostly of *U. tumidus* shells with a small admixture of *U. pictorum* and *A. anatina* (as they occurred in the field). They were of a size considered in the current study to be small (Table 1) and burrowed in sand (in this form they triggered stronger responses in earlier trials) at a density of 200 ind. m^{-2} (effective density of small shells in earlier trials, see the Results). Treatments (iii)-(v) were conducted using only *U. tumidus*, because both native species responded similarly in earlier trials (see the Results), and we wanted to limit the use of the legally protected and endangered *A. cygnea*.

Furthermore, we tested the habitat preferences of *S. woodiana* for: (i) small burrowed conspecific shells (200 ind. m^{-2}) vs. pure sand and (ii) small burrowed conspecific shells vs. shells of native unionids (200 ind. m^{-2}) to check whether and how this species responds to shell beds. All the pairwise comparisons carried out within Experiment 1 are listed in Suppl. material 1: table S1.

Experiment 2: Bivalve mobility and burrowing

To test the effect of living *S. woodiana* and its empty shells on the locomotion and burrowing of *A. cygnea* and *U. tumidus*, we used tanks (40 × 30 × 35 cm) with a 10-cm layer of sand covered by the conditioned tap water (10 cm above the substratum) (Suppl. material 1: fig. S2). As substrata, we used small *S. woodiana* shells (i) burrowed or (ii) lying on the sand surface, at a density of 200 ind. m^{-2}, as well as (iii) living *S. woodiana* (133 ind. m^{-2}) (Suppl. material 1: table S2). We used *S. woodiana* densities found to be avoided by the native unionids in Experiment 1 (see the Results). In the control treatment, bivalves were tested on (iv) pure sand without shells. A single substratum type was placed in each experimental tank. We introduced a single bivalve to the centre of the tank and recorded its behaviour using a CCTV camera (Samsung SNB-6004, South Korea) for 24 h. The tests were replicated 15 times for each substratum and species. While watching the videos, we determined the following: (i) movement initiation time (time from the bivalve introduction to the first movement), (ii) locomotion duration, (iii) locomotion distance, (iv) locomotion speed (excluding periods of immobility), (v) duration of burrowing activity, (vi) mean burrowing level [%]. Every minute, we estimated the percentage of bivalve burrowing (using a 5-level scale: 0, 25, 50, 75 and 100%) by comparing the length of the part of the shell below the substratum surface with the part of the shell protruding above the substratum (according to Poznańska-Kakareko et al. 2021). Mean burrowing level was calculated according to the following formula:

$$MB = \sum_{i=1}^{4} [25\% * it_i] / \sum_{i=0}^{4} t_i \qquad (1)$$

where: i – burrowing level: 5 steps ranging from 0 (totally exposed on the surface) to 4 (fully burrowed; t_i – time spent by the mussel at burrowing level i.

Statistical analysis

Statistical analysis was carried out using SPSS 26.0 (IBM Inc.). We checked bivalve habitat preferences in Experiment 1 using χ^2 tests of goodness of fit to compare their distribution within a given pair of habitats to a random distribution (assuming equal numbers of individuals selecting each habitat). Because of the high departures of the mobility and burrowing data in Experiment 2 from normality and homoscedasticity assumptions (tested with Shapiro-Wilk and Levene tests, respectively), we compared bivalve behaviour (each species separately) on each substratum contaminated with *S. woodiana* to their behaviour on pure sand using non-parametric Mann-Whitney U tests with a sequential Bonferroni correction for multiple comparisons.

Results

Experiment 1: Habitat selection

Both native species avoided small shells of *S. woodiana* (both burrowed and on the surface) at a density of 200 ind. m^{-2} (Fig. 1a, b, Table 2) and burrowed large shells at a density of 133 ind. m^{-2} (Fig. 1d, Table 2). Large shells on the surface were avoided at a density of 200 (*A. cygnea*) or 133 (*U. tumidus*) ind. m^{-2} (Fig. 1c, Table 2).

Burrowed shells were avoided in favour of shells of the same size and density (200 ind. m^{-2} of small shells) located on the substratum surface (Fig. 1e, Table 2). Large burrowed shells were avoided by *U. tumidus* in favour of small burrowed shells of the same total volume (Fig. 1f, Table 2).

The bivalves did not discriminate between living *S. woodiana* and pure sand (Fig. 1g, Table 2). *Unio tumidus* moved to the habitat formed by living *S. woodiana* avoiding large shells burrowed in the substratum, whereas *A. cygnea* did not discriminate significantly between these habitats (Fig. 1g, Table 2).

Unio tumidus showed a tendency to avoid shells of the native species, though it was non-significant (Fig. 2a, Table 2). Moreover, *U. tumidus* did not discriminate between shells of the native species and those of *S. woodiana*.

Sinanodonta woodiana avoided conspecific shells and did not discriminate between them and shells of the native unionids (Fig. 2b, Table 2).

Experiment 2: Bivalve mobility

Time from the introduction to the first movement of *A. cygnea* was not affected by the presence of shells and living individuals of *S. woodiana* (Fig. 3a, Table 3). *Unio tumidus* delayed the start of their activity in the presence of living *S. woodiana* (Fig. 3a, Table 3). Shells and living individuals of *S. woodiana* did not affect significantly the duration and distance of locomotion of *A. cygnea* (Fig. 3b, c, Table 3).

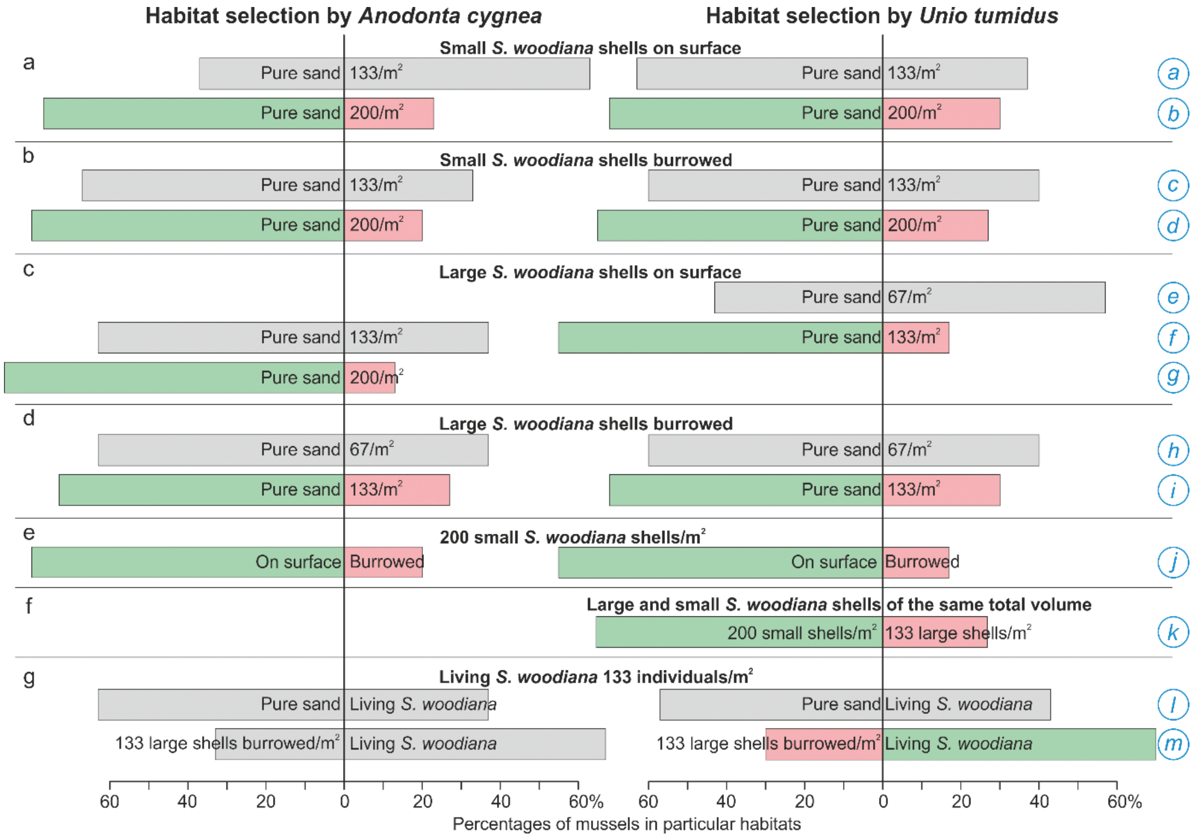

Figure 1. Habitat selection by *A. cygnea* and *U. tumidus* in the presence of substrata contaminated by *S. woodiana* in Experiment 1. Selected and avoided substrata are marked in green and red, respectively. The grey colour indicates non-significant differences. Blue letters in circles on the right refer to specific statistical tests presented in Table 2.

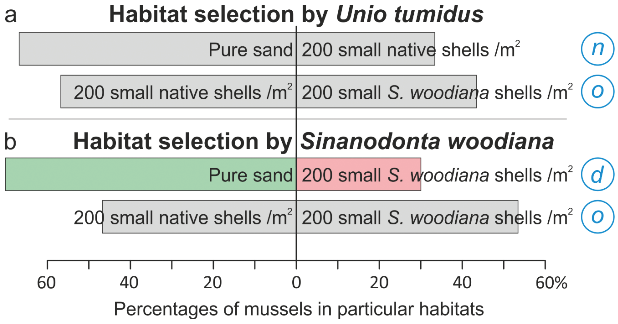

Figure 2. Habitat selection of *U. tumidus* and *S. woodiana* in the presence of burrowed shells of native and invasive bivalves in Experiment 1. Selected and avoided substrata are marked in green and red, respectively. The grey colour indicates non-significant differences. Blue letters in circles on the right refer to specific statistical tests presented in Table 2.

However, in the presence of all forms of *S. woodiana* contamination, distances travelled by *A. cygnea* were slightly shorter and movement duration longer, resulting in a significantly slower crawling speed compared to that observed in the pure sand (Fig. 3d, Table 3).

Unio tumidus increased duration and distance of their locomotion in the presence of living *S. woodiana* or its shells on the surface (Fig. 3b, c, Table 3). In the pure sand and with burrowed shells, *U. tumidus* usually did not move horizontally at all, but burrowed immediately. Due to the total lack of locomotion of *U. tumidus* in the pure sand, it was not possible to calculate their speed on this substratum.

Table 2. Statistical analysis of habitat selection by *A. cygnea*, *U. tumidus* and *S. woodiana* in Experiment 1 (χ^2 tests of goodness of fit comparing bivalve distribution within a given pair of habitats to the random distribution assuming no selection). Statistically significant differences are indicated by bold font and asterisks. χ^2 – test statistic, P – statistical significance.

	Substrata			***Anodonta cygnea***		***Unio tumidus***		***Sinanodonta woodiana***	
				χ^2	*P*	χ^2	*P*	χ^2	*P*
a	control (pure sand)	vs.	133 small SW shells m^{-2} on surface	2.13	0.144	2.13	0.144	–	–
b	control (pure sand)	vs.	200 small SW shells m^{-2} on surface	8.53	**0.003***	4.80	**0.028***	–	–
c	control (pure sand)	vs.	133 small burrowed SW shells m^{-2}	6.53	0.068	4.80	0.273	–	–
d	control (pure sand)	vs.	200 small burrowed SW shells m^{-2}	10.80	**0.001***	6.53	**0.011***	4.80	**0.028***
e	control (pure sand)	vs.	67 large SW shells m^{-2} on surface	–	–	0.53	0.465	–	–
f	control (pure sand)	vs.	133 large SW shells m^{-2} on surface	2.13	0.144	13.33	**<0.001***	–	–
g	control (pure sand)	vs.	200 large SW shells m^{-2} on surface	16.13	**<0.001***	–	–	–	–
h	control (pure sand)	vs.	67 large burrowed SW shells m^{-2}	2.13	0.144	1.20	0.273	–	–
i	control (pure sand)	vs.	133 large burrowed SW shells m^{-2}	6.53	**0.011***	4.80	**0.028***	–	–
j	200 small SW shells m^{-2} on surface	vs.	200 small burrowed SW shells m^{-2}	10.80	**0.001***	13.33	**0.000***	–	–
k	200 small burrowed SW shells m^{-2}	vs.	133 large burrowed SW shells m^{-2}	–	–	6.53	**0.011***	–	–
l	control (pure sand)	vs.	133 living SW m^{-2}	2.13	0.144	0.53	0.465	–	–
m	133 large burrowed SW shells m^{-2}	vs.	133 living SW m^{-2}	3.33	0.068	4.80	**0.028***	–	–
n	control (pure sand)	vs.	200 small burrowed native shells m^{-2}	–	–	3.33	0.068	–	–
o	200 small burrowed native shells m^{-2}	vs.	200 small burrowed SW shells m^{-2}	–	–	0.53	0.465	0.13	0.715

Experiment 2: Bivalve burrowing

Anodonta cygnea spent a shorter time on burrowing in the substratum containing burrowed shells compared to the control sand (Fig. 4a, Table 3). There was no effect of *S. woodiana* habitats on the duration of burrowing activity of *U. tumidus*.

The mean burrowing level of *A. cygnea* was reduced in the presence of burrowed shells and living *S. woodiana* (Fig. 4b, Suppl. material 1: fig. S2, Table 3). *Unio tumidus* responded to all types of *S. woodiana* habitats by reducing its burrowing level.

Discussion

In accordance with our first hypothesis, we reported avoidance of *S. woodiana* shells by native unionids. On the other hand, living individuals of the invasive species were not avoided even at a density twice as high (133 ind. m^{-2}) as the maximum densities observed so far in the field (Kraszewski and Zdanowski 2007). However, living *S. woodiana* did influence unionid behaviour: their presence delayed initiation of activity and increased horizontal locomotion of *U. tumidus*, reduced locomotion speed in *A. cygnea*, and reduced burrowing of both species. An increased locomotion was also exhibited by *U. delphinus* Spengler, 1783 in the presence of the invasive clam *Corbicula* sp. (Ferreira-Rodríguez et al. 2018). It appears that the presence of shells induced displacement of native unionids, whereas living *S. woodiana* impaired the habitat quality for the natives, which tried to counteract by changing their activity. The increase in activity may be induced by searching for a habitat free of competitors, but its side effect may be the displacement of native bivalves to suboptimal environments, where they will be exposed to increased water flow or predatory pressure (Block et al. 2013). Another consequence of increased locomotion can be the depletion of energetic resources. As

Table 3. Statistical analysis of locomotion and burrowing of *A. cygnea* and *U. tumidus* in Experiment 2. Bivalve behaviour in the presence of *S. woodiana* shells (200 ind. m^{-2}, on the surface or burrowed) and living *S. woodiana* (133 ind. m^{-2}) was compared to the behaviour of individuals exposed on the control pure sand with pairwise Mann-Whitney U tests. Statistically significant differences are marked with asterisks and those that are still significant with the sequential Bonferroni correction are marked in bold font. Z – test statistic, P – statistical significance.

	Variable	Substrata			*Anodonta cygnea*		*Unio tumidus*	
					z	*P*	z	*P*
a	Movement initiation time	control (pure sand)	vs.	shells on surface	2.30	0.022*	0.62	0.534
				shells burrowed	1.52	0.129	0.77	0.443
				living individuals	0.23	0.818	3.59	**<0.001***
b	Locomotion duration	control (pure sand)	vs.	shells on surface	1.33	0.184	2.67	**0.008***
				shells burrowed	1.16	0.245	1.79	0.073
				living individuals	1.05	0.293	2.40	**0.017***
c	Locomotion distance	control (pure sand)	vs.	shells on surface	1.00	0.319	2.67	**0.008***
				shells burrowed	1.22	0.223	1.79	0.073
				living individuals	0.78	0.438	2.40	**0.017***
d	Locomotion speed	control (pure sand)	vs.	shells on surface	2.49	**0.013***	–	–
				shells burrowed	2.44	**0.015***	–	–
				living individuals	2.10	**0.035***	–	–
e	Duration of burrowing activity	control (pure sand)	vs.	shells on surface	0.73	0.467	1.81	0.071
				shells burrowed	3.32	**0.001***	0.56	0.575
				living individuals	1.83	0.067	1.14	0.254
f	Mean burrowing level	control (pure sand)	vs.	shells on surface	1.06	0.290	2.64	**0.008***
				shells burrowed	3.11	**0.002***	2.61	**0.009***
				living individuals	2.63	**0.009***	4.54	**<0.001***

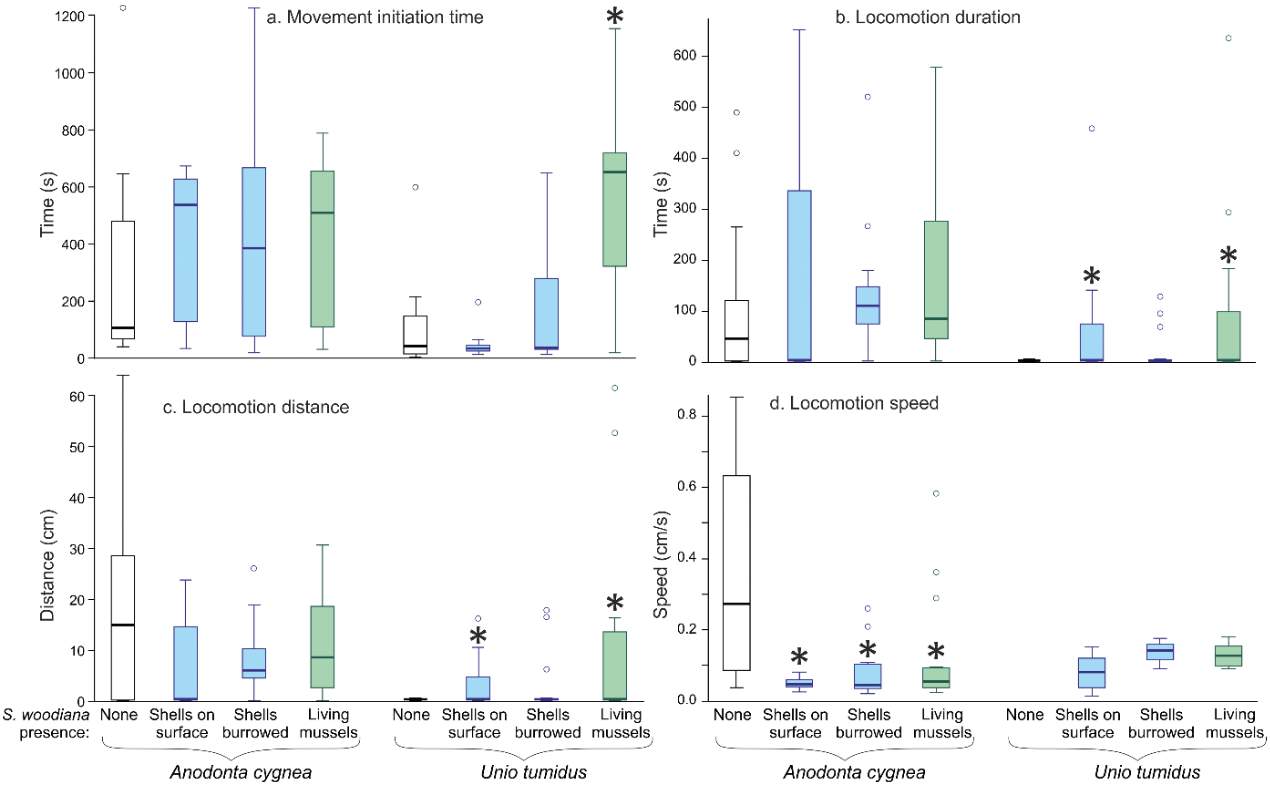

Figure 3. Mobility of *A. cygnea* and *U. tumidus* in Experiment 2: in pure sand (white bars), in the presence of *S. woodiana* shells (small shells, 200 ind. m^{-2}, blue bars) and in the presence of living *S. woodiana* (133 ind. m^{-2}, green bars) **a** movement initiation time **b** locomotion duration **c** locomotion distance and **d** locomotion speed. Asterisks indicate statistically significant differences in behaviour compared to that observed in the pure sand (see Table 3a-d for details of statistical test results). Boxplots present medians (horizontal lines), 1st and 3rd quartiles (boxes), 1.5*interquartile range (whiskers) and outliers (circles).

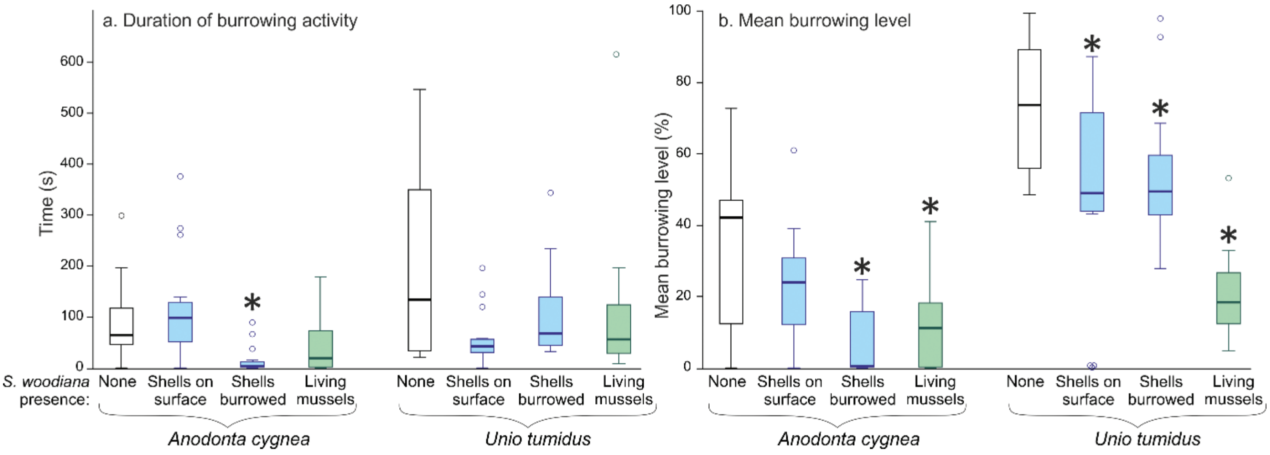

Figure 4. Burrowing of *A. cygnea* and *U. tumidus* in Experiment 2: in pure sand (white bars), in the presence of *S. woodiana* shells (small shells, 200 ind. m^{-2}, blue bars) and in the presence of living *S. woodiana* (133 ind. m^{-2}, green bars) **a** duration of burrowing activity **b** mean burrowing level (expressed as the percentage of bivalve length, see formula (1)). Asterisks indicate statistically significant differences in behaviour compared to that observed in the pure sand (see Table 3e-f for details of statistical test results). Boxplots present medians (horizontal lines), 1st and 3rd quartiles (boxes), 1.5*interquartile range (whiskers) and outliers (circles).

similar changes in behaviour took place in the presence of empty shells, they most likely resulted from mechanical properties of shells (acting as physical obstacles), rather than from infochemicals released to the water column by living *S. woodiana*.

As expected (third hypothesis), empty shells had a more aversive effect on native bivalves than living *S. woodiana*. The key result here is that the native bivalves avoided burrowed shells of *S. woodiana* to a greater extent than shells lying on the surface or living bivalves. The strongest effect of burrowed shells, immobilized in sediments, suggests that they are more difficult to push away by a moving mussel (compared to loose shells on the surface). Moreover, sharp shell edges, absent in living individuals, may irritate the foot of bivalves and discourage them from entering such a substratum. This was confirmed by the fact that *U. tumidus* did not increase their locomotion activity in the presence of burrowed shells, as they did among shells on the surface or with living *S. woodiana*. Thus, burrowed shells not only prevented mussels from entering the area, but also made it more difficult to leave a shell habitat when already present around the moving unionid. On the other hand, increased locomotion of *U. tumidus* among shells on the surface associated with their avoidance of such habitats indicates the active selection of shell-free habitats. The locomotion of *A. cygnea* in the presence of shells in or on the substratum resulted in similar distances as without shell beds, but at the cost of slower speed. This suggests a greater effort needed to obtain the same final effect, though, despite this, mussels continued to move in the presence of shells, also suggesting the active avoidance of shell beds by this species.

Avoidance of burrowed shells is related to their effect on the bivalve behaviour. We did note the negative effect of burrowed objects (empty shells and living *S. woodiana*) on the burrowing of both native species, especially *A. cygnea*. Moreover, *A. cygnea* spent less time on burrowing in shell beds, probably to avoid excessive energy expenditure. Restricted burrowing may be dangerous for unionid mussels, as being immersed in the substratum is their natural position, enabling their filtration, as well as reducing predation risk and the probability of dislodgement by water movements (Tallqvist 2001; Saloom and Duncan 2005). This indicates that the post-mortem effect of *S. woodiana* is two-faceted: it modifies the horizontal and vertical mobility of bivalves, as predicted by our second hypothesis. This can substantially worsen the environmental conditions for native bivalves. We found that large shells are more aversive than small ones at the same volumetric quantities. This supports the idea that shells act as physical objects interfering with the movement of living mussels (a single large shell is more difficult to push away or bypass than a group of small ones). Thus, the impact of the invader is likely to be greater than that experienced by the native species before the invasion due to the much larger size of *S. woodiana* compared to native unionids, as predicted by our fourth hypothesis.

Native unionids can also create shell beds (Bódis et al. 2014a), which, as we have shown in our study, can exert a similar impact on living bivalves. However, their formation is slower than in the case of *S. woodiana*, which is capable of reaching high abundance and large body size in a relatively short time (Urbańska et al. 2019). Taking into account the thermophilicity of *S. woodiana* (Yermoshyna and Pavliuchenko 2021), it should be expected that, with progressing climate warming, mussels will reach larger sizes, higher density and biomass (Kraszewski and Zdanowski 2007; Spyra et al. 2012; Mehler et al. 2024) in temperate waters. This will increase their impact on local communities (Gutiérrez et al. 2003; Bellard et al. 2012) to an unprecedented level, not experienced so far by the native bivalves. It may be manifested by intensified competition for space, given that *S. woodiana* and the native unionids have similar substratum preferences (Poznańska-Kakareko et al. 2021), and by reduction in available area of optimum substratum due to the presence of extensive shell beds (Gutiérrez et al. 2003; Bódis et al. 2014a).

We have shown that *S. woodiana* also avoids habitats transformed by shells of its own species. This behaviour suggests that small scale spread of *S. woodiana* may be additionally stimulated by changes generated by this species in the environment, resulting in the occupation of a greater bottom area at an invaded site. Such a transformation of the environment by an ecosystem engineer associated with a negative feedback on its own living conditions is similar to the activity of cormorants (*Phalacrocorax* sp.), which pollute their surroundings (trees on which they nest in large numbers) with corrosive excrements and then move to new, not yet destroyed habitats (Ishida 1996).

Our results highlight that apart from competition for host-fish, food resources or living space, *S. woodiana* poses a further threat to native unionid bivalves by altering their horizontal and vertical movement behaviour. Whilst our tests were carried out in strictly controlled, specific conditions (i.e. stagnant water on sandy bottom), long-term negative impacts of living *S. woodiana* on native unionids can be expected to be even stronger than demonstrated in our study. We can expect that additional factors, such as water flow, different substratum or temperature would modify the relationships and behaviours observed in our study (Sullivan and Woolnough 2021). In our study, we only considered the physical effect of shells and living individuals of *S. woodiana*, showing the stronger impact of the former. In the environment, living *S. woodiana* may have additional adverse effects on native bivalves, such as competition for food or host fish, or reduction in water oxygenation. To fully understand the interactions between invasive and native Unionidae bivalves, would require to conduct longer tests, both in laboratory and in the field, examining additional potential mechanisms of impact.

Acknowledgments

We would like to thank Maja Grabowska for her help in conducting the experiments. We are deeply grateful to Mrs Hazel Pearson for improving the English language of our text. To conduct these experiments, we obtained permissions from the Regional Directorate for Environmental Protection in Bydgoszcz, Poland: WOP.6401.5.7.2020.LK for collecting, keeping and conducting research on *A. cygnea* (a protected species in Poland), and WOP.672.4.2020.MO for keeping and conducting research on the invasive *S. woodiana*.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

No funding was reported.

Author contributions

KW: Conceptualisation, Methodology, Resources, Investigation, Formal analysis, Visualisation, Writing - Original draft, Writing - Review and Editing; DS: Methodology, Resources, Investigation, Writing - Review and Editing; JK: Conceptualization, Methodology, Validation, Formal analysis, Data Curation, Visualization, Writing - Review and Editing; TK: Resources, Investigation, Writing - Review and Editing; ŁJ: Resources, Investigation, Writing - Review and Editing; MPK: Conceptualization, Methodology, Validation, Visualization, Formal analysis, Supervision, Project administration, Writing - Review and Editing

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supplementary material

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Data type: docx

Explanation note: **table S1.** Full list of pairwise comparisons conducted to check unionid habitat selection in Experiment 1. **table S2.** Full list of treatments, response variables and statistical comparisons to check changes in unionid locomotion and burrowing in response to *S. woodiana* presence in Experiment 2. **fig. S1.** Photographs of the experimental setup. Experiment 1: Habitat selection by bivalves in various configurations: (A) pure sand (control) vs. 133 ind. m-2 of small *S. woodiana* shells on surface; (B) pure sand vs. 200 ind. m-2 of small shells on surface; (C) pure sand vs. 133 ind. m-2 of small burrowed shells; (D) pure sand vs. 200 ind. m-2 of small burrowed shells; (E) pure sand vs. 67 ind. m-2 of large shells on surface; (F) pure sand vs. 133 ind. m-2 of large shells on surface; (G) pure sand vs. 200 ind. m-2 of large shells on surface; (H) pure sand vs. 67 ind. m-2 of large burrowed shells; (I) pure sand vs. 133 ind. m-2 of large burrowed shells; (J) 200 ind. m-2 of small shells on surface vs. burrowed; (K) pure sand vs. 133 ind. m-2 of living *S. woodian*a; (L) 200 ind. m-2 of burrowed shells of native bivalves vs. shells of *S. woodiana*. **fig. S2**. Schemes of experimental sets concerning habitat selection (Experiment 1) and locomotion and burrowing (Experiment 2). **fig. S3.** Percentage of time spent in different levels of burrowing (Experiment 2) by *A. cygnea* and *U. tumidus* on different substrata. Error bars – standard deviation. **fig. S4.** Photo of immobilized *Sinanodonta woodiana*.

Link: https://doi.org/10.3897/neobiota.94.119622.suppl1

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 261–288 (2024)
DOI: 10.3897/neobiota.94.122337

Research Article

Perseverance of management is needed – Efficient long-term strategy of *Reynoutria* management

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Abstract

One of the most problematic invasive species in Europe are knotweeds from genus *Reynoutria* (*Fallopia*) which have significant negative impact on the native communities as well on human activities. Therefore, they are a target of many control programmes. Due to their high regeneration potential, their management is problematic, and only chemical treatment is reported to be sufficiently effective.

The aim of this paper was to describe and analyse the patterns of *Reynoutria* invasion under long-term chemical treatment with glyphosate-based herbicide in The Morávka river floodplain, Czech Republic. The data covers 17 years of management which started with the European project "Preservation of alluvial forest habitats in the Morávka river basin". We focus on (i) assessment of *Reynoutria* distribution during long-term management, (ii) analysis of the change of distribution according to the habitat, and (iii) discussion of the optimal management strategy based on the long-term data.

Distribution data was obtained using GNSS field mapping. Before the start of the study in 2007, *Reynoutria* stands covered 29% of the study area (96.9 ha). As a result of systematic whole area chemical management, the extent decreased to 19.6% (65.3 ha) in 2009, and even reached 14.5% (48.2 ha) in 2013, three years after its end. Due to implementation of local chemical management in the following years, the area of *Reynoutria* was maintained at similar level, with minimum value 41.8 ha in 2018 and a slight increase in recent mapping in 2023.

Beside the extent, the structure and coverage of invaded sites was analysed. There was a clear trend of fragmentation of larger polycormons with high coverage into many smaller and less dense ones as a result of chemical spraying. The average size of *Reynoutria* stand decreased from 0.61 ha in 2007 to half in 2013 (0.32 ha) to 0.15 ha in 2023. Testing of the effects of time, habitat, and biotope did not reveal significant differences of changes of extent and abundance over different environments (forest, open, bare ground), which indicates that there are no differences in reaction to management in the studied habitat and vegetation types.

Our study provides a robust and unique overview of the invasion, reinvasion, and suppression dynamics for an important invasive species. If herbicide management is used, chemical treatment must be quite long-term as even three years of intensive glyphosate foliar spray application was not sufficient for the complete eradication of *Reynoutria*.

Therefore, we propose the following procedure for effective chemical management of *Reynoutria*:

1) In largely infested sites, the first step is to reduce the distribution of *Reynoutria* stands to isolated polycormons. This phase can last 3–5 years.

Academic editor: Gerhard Karrer
Received: 19 April 2024
Accepted: 25 July 2024
Published: 20 August 2024

Citation: Švec P, Perglová I, Fröhlich V, Laštovička J, Seidl J, Růžičková K, Horáková I, Lukavský J, Ferko M, Štych P, Pergl J (2024) Perseverance of management is needed – Efficient long-term strategy of *Reynoutria* management. NeoBiota 94: 261–288. https://doi.org/10.3897/neobiota.94.122337

2) After reaching the state of sparse distribution of *Reynoutria*, we recommend herbicide application only in periods of every 3–5 years depending on the local context and rate of regrowth.

3) At sites exposed to soil disturbances, where the soil is contaminated by fragments of *Reynoutria* rhizomes, there is a need to apply herbicide immediately to target newly resprouting individuals.

Key words: *Fallopia*, GNSS, knotweed, long-term management, mapping, neophyte, protected area, Roundup

Introduction

The spread of non-native plant species has a negative impact on the conservation of native communities and is one of the most serious threats to ecosystem biodiversity (Pyšek et al. 2020; IPBES 2023). Invasibility and the level of invasion differs among different habitat types (Chytrý et al. 2005) and is generally the highest in riparian stands and riverbanks (Chytrý et al. 2009; Pyšek et al. 2010; Fristoe et al. 2021). In terms of invasion ecology, floodplains exhibit most of the characteristics that support invasion of non-native species: strong diaspore introduction, frequent disturbances, and major human impact (Richardson et al. 2007).

One of the most problematic invasive neophytes in the Czech Republic are species from the genus *Reynoutria* (syn. *Fallopia* sp.) (Pyšek et al. 2022). *Reynoutria* sp. have a significantly negative impact on the native communities, including soil environment (Hejda et al. 2009, 2021; Pergl et al. 2023). Their first spread into wild nature was reported in the territory of today's Czech Republic in the late 19th century (Mandák et al. 2004). Due to the negative impacts of *Reynoutria* on native species, it is desirable to carry out their control programmes especially in environmentally valuable locations, as their total mass eradication is practically impossible (Delbart et al. 2012; Cottet et al. 2015; Halas et al. 2018). *Reynoutria* is managed mechanically, chemically or by a combination of both methods. Mechanical treatment is not very effective in the long term (Scott and Marrs 1984; Jones et al. 2020a; Bzdega et al. 2022; Kadlecová et al. 2022); chemical treatment using a Glyphosate active ingredient is reported to be the most effective (Kadlecová et al. 2022; Hocking et al. 2023). Biological control on *Reynoutria* is tested, but not yet very effective (Shaw et al. 2011). The effectiveness of less common methods such as plastic sheet covering, stem injection, digging or ploughing varies largely and there are many regional guidelines targeted on practical use, even in regional languages (see e.g., Child and Wade 2000; Mantzou 2008; Csiszár and Korda 2017; Bzdega et al. 2022) which are the useful source of information for local managers. Long-term efficiency of these methods such as hot water/steam and/or covering by a plastic sheet is not available, and their efficiency in the short run is suspicious (Jones et al. 2020a; Dusz et al. 2021). The study by Mantzou (2008) provides a comprehensive list of methods with their cost available mostly for human-made stands, but again without any longer efficiency evaluation. It has to be kept in mind, that there can be regional/country restrictions on use of herbicides.

Even though the use of herbicides is mostly rejected by the public compared to mechanical methods, application of herbicides leads to more effective control of *Reynoutria* and can therefore lead to a less negative overall effect on the environment in terms of its pollution (Pergl et al. 2020a; Hocking et al. 2023).

The wide and frequent use of herbicides can be linked to a negative effect on surrounding ecosystems through a direct effect on pollinators, the effects on non-target organisms, and the effects of herbicide residues in the soil and water. Therefore, there is a continuous effort to minimize the use of herbicide applied by spraying. Unfortunately, for large *Reynoutria* stands the foliar spraying is the only effective management, and therefore we need to identify ways to limit the amount of used herbicide.

The effectiveness of management of invasive species depends largely on the level of invasion. Several studies have shown that the success of eradication depends on time, extent, abundance and regulation of the propagule pressure (Pluess et al. 2012). Even though there are continuously invested financial and human-labour resources to manage the invasive species, the effectiveness of individual projects at a large-scale perspective is under-evaluated. However, the need for prioritization of management is needed at different regional scales and also at the level of species, as some species can be tolerated due to high management costs, low effect of management and low environmental risk (Pergl et al. 2016). Such assessments rarely exist. Long-term data is scarce as most of the projects are limited to a duration of approximately 3–5 years (Kettenring and Adams 2011; Pergl et al. 2020b).

As *Reynoutria* is one of the most problematic species in Europe, there have been many projects devoted to developing efficient and sustainable control methods (Kadlecová et al. 2022). One was the European project “Preservation of alluvial forest habitats in the Morávka river basin”, which took place in 2007–2010 (LIFE-Morávka 2007; Barták et al. 2010). The main objective of this was to suppress the population of *Reynoutria* in the study area. It was the largest continuous coordinated control programme of this species in the Czech Republic at the time.

Distribution of *Reynoutria* can be mapped relatively easily by the methods of aerial photographs (Martin et al. 2018) or by the methods of direct field mapping (Blahuta et al. 2016). Currently, a common mapping method is the use of Global Navigation Satellite System (GNSS) based on the use of Global Positioning System (GPS) (Teunissen and Montenbruck 2017). Negrea et al. (2022) used their own spectral UAV (Unmanned Aerial Vehicle) data combined with in-situ measurements to estimate the expansion potential of *Reynoutria japonica* with the Inverse Distance Weighted (IDW) method being used for interpolation. Jovanović et al. (2018) observed Southeastern Europe for *Reynoutria* niche modelling. For the purpose of data collection, Moore et al. (2006), Molitoris (2013), Jovanović et al. (2018) used GPS measurements. Dyrmann et al. (2021) used neural network classification for Camera Assisted Roadside Monitoring to observe invasive alien plant species (including *Reynoutria*).

Based on the outlined context of invasion of *Reynoutria* and the possible management options, our study (i) assesses the distribution of *Reynoutria* in the Morávka riverside during long-term management, (ii) analyses the change of distribution according to the habitat, and (iii) discusses the optimal management strategy based on the long-term data.

Material and methods

Study area

The study area of 334.1 ha is located in northern Moravia between the municipalities of Frýdek-Místek and Vyšní Lhoty (the outermost municipality), covering the floodplain landscape in the vicinity of the Morávka River (river kilometre 1.1–11.5) in the elevation range 298–380 m (Figs 1, 2). Orographically, the territory belongs to the Sub-Beskydy Highlands. The Morávka is a watercourse of the third

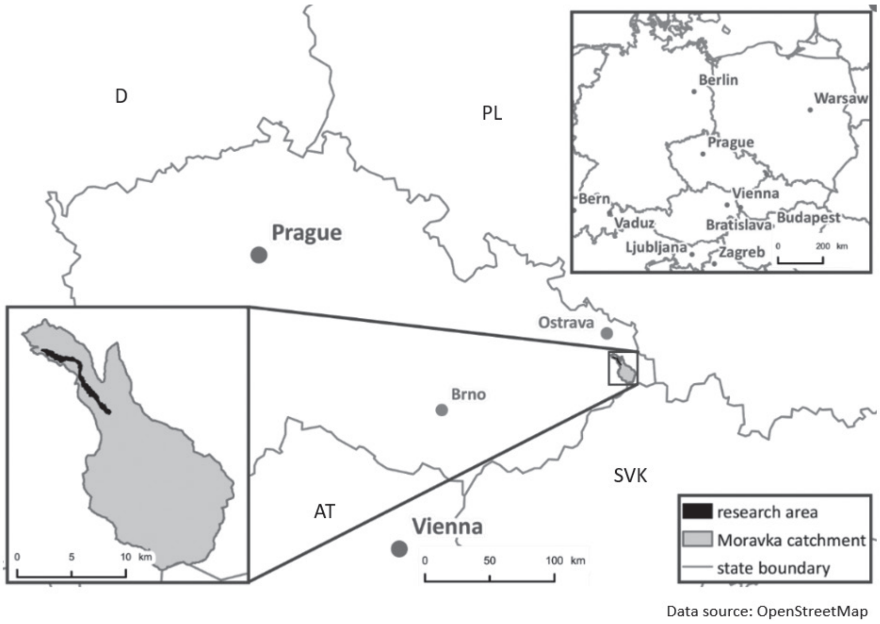

Figure 1. Study area.

order, belonging to the Odra River catchment and the Baltic Sea drainage basin. The Morávka is a rightward tributary of the Ostravice River; its total length is 29.4 km and it has an average annual flow at its mouth of 3.7 $m^3.s^{-1}$.

The Morávka River (Fig. 2, Suppl. material 4) drains the north-western part of the Moravian-Silesian Beskydy, part of the flysch zone of the Outer Western Carpathians (Chlupáč 2002). The bedrock is built of rhythmically alternating positions of claystones and sandstones. The Morávka River forms extensive accumulations of gravel alluvium in its floodplain, in which, especially during major floods, it changes its watercourse (Škarpich et al. 2013). The Morávka floodplain represents the most extensive area in the Czech Republic in which the river is still wild. In order to protect the remaining fragments of the originally up to 300 m wide riverbed, a complex of several protected areas: the Profil Morávky Natural Reserve, the Niva Morávky Natural Reserve, and the Skalická Morávka National Natural Reserve, have been gradually declared part of the Niva Morávky European Site of European Importance. Since the beginning of the 20th century, systematic modifications have been carried out in order to stabilise the riverbed. One of the most major interventions in the fluvial regime of the Morávka was the construction of the weir and the Žermanice Reservoir inlet in Vyšní Lhoty (1953–1964) in river kilometre 11 and the Morávka Waterworks (1961–1967) in river kilometre 19 (Škarpich et al. 2013).

The study area (Fig. 2) has been determined to respect the floodplain forest boundary. It includes the protected sites the “Profil Morávky” (49.6 ha) and the “Niva Morávky“ (74.6 ha) Natural Reserves and most of the “Skalická Morávka” (102.0 ha) National Natural Reserve. It therefore covers a larger part of the Special Area of Conservation (SAC) the Niva Morávky (total area 367.4 ha). The protected areas are maintained by the Nature Conservation Agency (NCA) of the Czech Republic and the Regional Council of the Moravian-Silesian Region (RCMSR).

The vegetation cover of the floodplain involves mostly floodplain forests and naturally similar habitats, including gravel bars and floodplain. In the more remote parts of the floodplain there are human settlements and arable land.

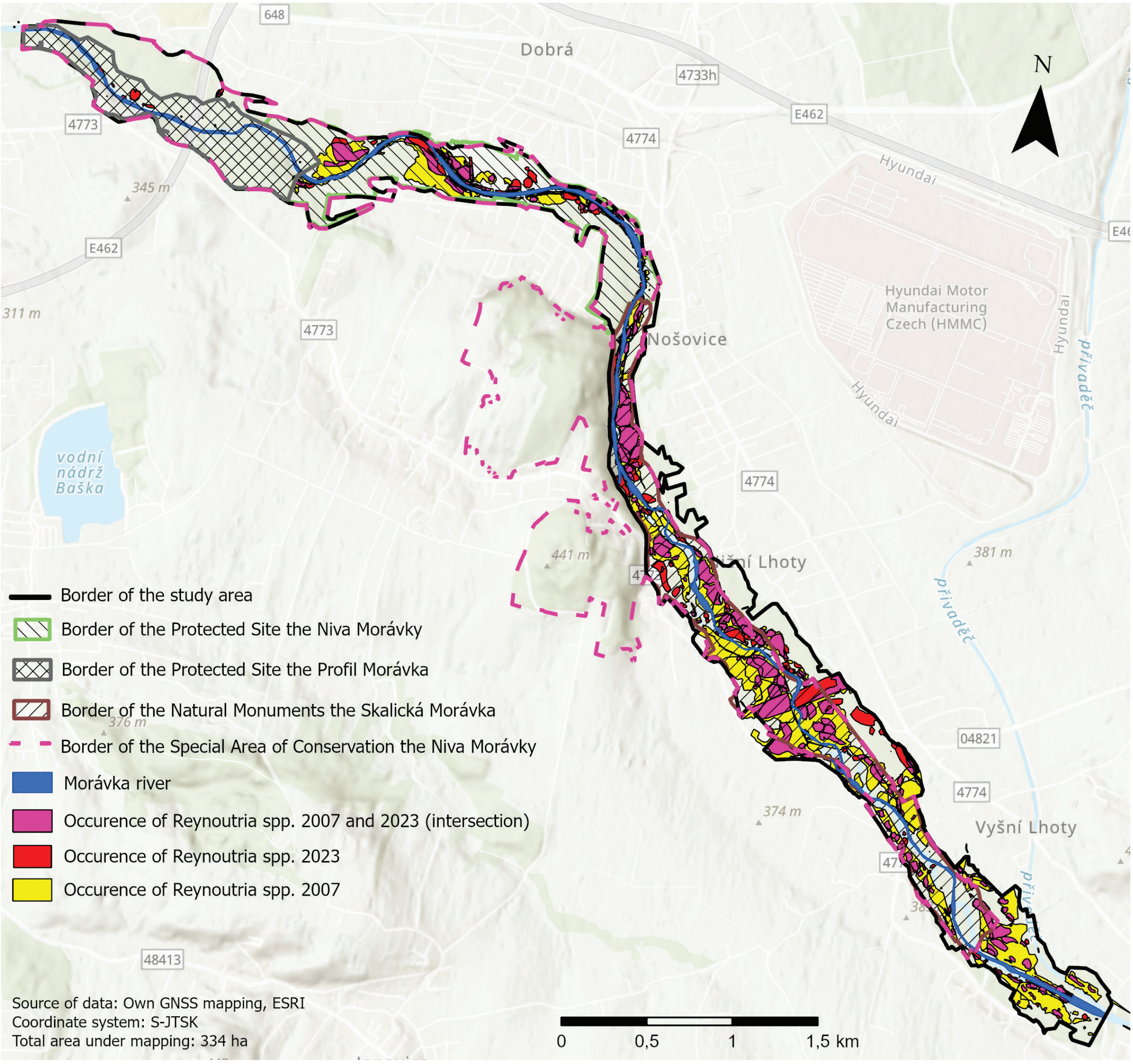

Figure 2. Study area in detail.

The species composition of the floodplain forests roughly corresponds to the natural potential vegetation of bird cherry-ash woodland (*Pruno-Fraxinetum*), which in places on the valley slopes change to Carpathian sedge oak-hornbeam woodland (*Carici pilosae-Carpinetum*) or wet oak-beech woodland (*Carici brizoidis-Quercetum*) (Neuhäuslová et al. 1997). According to the 2018 Corine Land Cover (CLC) database (Feranec et al. 2016), the structure of the study area was as follows: 83.7% deciduous forest, 16.1% grassland, mainly meadows, and 0.3% other land. The structure of land cover has undergone substantial changes during the 20th century. While in the first half of the 19th century the area was mainly made up of gravel deposits, there was a significant increase in forest land during the 20th century (Trnčák 2012; Škarpich et al. 2013).

The first record of *Reynoutria* in the study area of the Morávka riverside comes from the 1940's (Talpa 1948), and today all the three species of *Reynoutria* genus occur here. The large-scale invasion of *Reynoutria* was probably conditioned by several circumstances. These circumstances included the recurrent disturbances by floods, which in the Morávka floodplain, unlike many other Czech rivers, can be manifested over a relatively wide area of the river floodplain, and in particular the change in the hydrological regime and regulation of the Morávka flow channel. Old aerial photographs (1950s) and older maps (imperial copies of stable cadastre from the first half of the 19th century) show that many of today's floodplain forests and non-forested parts were used as pastures with solitary trees (Trnčák 2012; Škarpich et al. 2013).

Study species

Invasive knotweed from genus *Reynoutria* (*Fallopia*) growing in the Morávka river basin includes *Reynoutria japonica* (native to Japan), *Reynoutria sachalinensis* (native to East Asia) and their hybrid *Reynoutria ×bohemica*. They are perennial, up to 3 m tall, shrub-like herbs that often form connected, impenetrable stands. Within their primary range, *Reynoutria* grows naturally and secondarily in nutrient-rich environments, e.g., near rivers, on young lava flows in alpine environments, and in ruderal vegetation (Beerling et al. 1994; Forman and Kesseli 2003; Mandák et al. 2004). *Reynoutria* negatively affects the species diversity of plant communities (Bímová et al. 2004; Gerber et al. 2008; Maurel et al. 2010), but also the diversity of fauna (Gerber et al. 2008). Their dense and interconnected stands prevent light penetration to the leaves of native herbs (Beerling et al. 1994; Siemens and Blossey 2007), which are mostly of a smaller size.

Reynoutria species reproduce mainly vegetatively within the introduced range. Nevertheless, there are reports of repeated crossing between the species, which is indicated by higher genetic diversity in the stands (Mandák et al. 2005; Suda et al. 2010; Bzdega et al. 2016). The study area around Morávka River was identified as one of the hotspots of such hybridization (Suda et al. 2010). Therefore, although we are aware of possible different reactions of individual *Reynoutria* species and even genotypes to management (Bailey et al. 2007), we were not able to discriminate individual taxa/genotypes for the purpose of our work and consider all *Reynoutria* only at genus level.

The control of *Reynoutria* in the study area

In the study area, the European project "Preservation of alluvial forest habitats in the Morávka river basin" (LIFE-Moravka 2007) was carried out in 2007–2010. The main objective of the project was to suppress the *Reynoutria* population in the study area. It combined mechanical and chemical treatment throughout the study area.

The control of *Reynoutria* was done with a 7–10% solution of the herbicide Roundup Biaktiv in years 2007 to 2010 (glyphosate-based herbicide). The herbicide was applied with a backpack sprayer predominantly in August and September. In locations with high *Reynoutria* coverage, *Reynoutria* was cut mechanically before herbicide application whereas regenerating plants were treated with a backpack sprayer. With regard to the elimination of environmental risks in the proximity of the Morávka reservoir, foliar spraying was replaced by injecting of herbicide directly into the stems of *Reynoutria* in this area; a 20–30% concentration was used. The application of herbicide was done once or twice each season depending on the success of the first treatment (Barták et al. 2010; Halas et al. 2018). Throughout the project duration (2007–2010), this systematic whole area chemical management was carried out every year in the entire study area.

After the end of the LIFE project, which significantly reduced the surface area of the stands, different parts of the study area were treated locally, in different years, depending on financial resources and conservation needs. Most of the study area consists of nature reserves whose authorities (RCMSR and NCA) have continued with chemical treatment using a 4–7% solution of the Roundup Biaktiv herbicide applied by a backpack sprayer. The first application took place after GNSS mapping in 2013 over the entire area of all nature reserves (226.2 ha). In the Niva Morávky Natural Reserve and the Profil Morávky Natural Reserve (124.2 ha), further chemical spraying was carried out in 2014, 2017 and 2019–2023. In the Skalická Morávka National Natural Reserve, the application was carried out once a year for three years, in 2016–2018 on river kilometre 9.4–10.6 (treated area 46.0 ha), and in 2020–2022 on river kilometre 5.5–9.4 (treated area 60.0 ha). The area from the southern boundary of the Skalická Morávka National Natural Reserve to the Žermanice Reservoir inlet was repeatedly chemically treated by the Povodí Odry (Odra River Basin Authority).

Reynoutria is also found in the upper parts of the watercourse below the Morávka Reservoir and above it up to river kilometre 21.1. This poses a risk for the introduction and distribution of *Reynoutria* rhizomes and repeated further spread. At the same time, repeated irregular chemical control took place in these parts as well.

Data

In-situ measurement by GNSS

Data on the occurrence of *Reynoutria* was obtained using GNSS field mapping. For the purpose of our mapping, we used the GPS system. GPS mapping was conducted in the study area in 2007 prior to the start of management, in 2009 during management, and in 2013 prior to the start of local management. In 2015, 2018, and 2023, it continued during local management. The GPS mapping was done in early summer (June and July) before the application of herbicide. In 2007 and 2009, a TOPCON FC-100 PDA was used in combination with a Navilock BT-338 external GPS module. In 2013, 2015, 2018, a JUNO 3D device by Trimble with an integrated GPS antenna was used. In 2023, we used a Xiaomi Redmi 7 smartphone with an integrated GPS antenna. All measurement methods used were autonomous. The error in this type of measurement is in the order of units of metres (Blažek and Švec 2010; Tomaštík et al. 2017; Teunissen and Montenbruck 2017). For the field mapping, ArcPad software (ESRI 2022) by ESRI was used. And in 2023 we used the mobile application Field Maps (ESRI 2023). These applications allow mapping of both the geometric and attribute components of the data. A project was created in ArcPad and Field Maps software in which attributes and a mapping form were defined. The attributes listed in Table 1 were recorded as part of the mapping.

The measurement was carried out in such a way that a mapper walked around the perimeter of each *Reynoutria* polycormon. During the mapping, a polygon edge was automatically recorded every second from the GPS in the ArcPad app. This created areas of different sizes and shapes. *Reynoutria* was mapped so that the mapped areas were homogeneous in terms of the defined parameters: Coverage, Vitality, Moisture type and Vegetation cover type (see Suppl. materials 1, 3). For point occurrences, *Reynoutria* was defined as a point and the radius was entered into the attribute, which were then used for creating the circle polygon.

Table 1. Mapped variable attributes.

Monitored attribute type	Monitored attribute category
Coverage (%)	0.01–0.1; 0.11–1.0; 1.1–10.0; 10.1–50.0; 50.1–100
Moisture type	dry; normal; wet
Vitality	low; average; high
Vegetation cover type	forest; open stands; bare ground habitats

During the mapping, we recorded the coverage percentage of *Reynoutria* according to selected coverage intervals, assessed *Reynoutria* vitality and habitat moisture according to selected criteria (Table 1). Vitality was defined by three categories, that is, plant size, vegetation compactness, and signs of plant damage due to chemical treatment were assessed (see Suppl. material 2). Plants with high vitality were massive, reaching more than 3.0 m in height, and often formed continuous stands. *Reynoutria* that were considered as average plants were up to ca 2.5 m tall. Plants relatively short, spindly, often with deformed leaves and usually isolated were mapped as stands with low vitality.

The moisture-type attribute was categorized according to relief, land cover, and vascular plant species representation. Areas with lowlands, oxbow lakes, pools, clay soils were mapped as wet habitats. Normal habitats were located on flat relief without frequent influence of flood waters, away from river channels and pools. Normal habitats were characterised by loose, organic soils, lacking wetland and xerophytic plant species, whereas mesophilic herbs were common. Dry habitats were mapped on elevated sites, mainly on gravel bars, accompanied by a dry coarse-grained substrate (see Suppl. material 3). The vegetation cover type was grouped into: forest, open stands (meadows, grasslands, sparse shrubby vegetation) and bare ground habitats (mostly gravel river stands).

Due to the high dynamics of changes in the relief and course of the channel in the Morávka floodplain, we vectorised the Morávka river in the years that most closely corresponded to each year of mapping based on archival aerial photographs and orthophotomaps.

Auxiliary data

Aerial photographs and land cover datasets were used as auxiliary data. The Corine Land Cover (CLC) database was used for the land cover data, the orthophoto Web Map Service (WMS) layer and Base topographic map of the Czech Republic at a 1:10 000 scale WMS by State Administration of Surveying and Cadastre in the Czech Republic was used for the aerial photographs.

Orthophoto

The Orthophoto is a map service by State Administration of Surveying and Cadastre in OGC (Open Geospatial Consortium) WMS 1.3.0 (https://geoportal.cuzk.cz/(S(zwhj1uzsovk24saxpcdkjjfy))/Default.aspx?lng=EN&mode=TextMeta&side=wms.verejne&text=WMS.verejne.uvod&head_tab=sekce-03-gp&menu=311). The Orthophoto is derived by orthorectification from the aerial photographs product. The product has spatial resolution within 0.2 m. Data is available in JPEG with JGW (world file) in several coordinate systems: S-JTSK, ETRS89-TM33N a ETRS89-TM34N. The temporal resolution of the dataset is two years.

Base Map of the Czech Republic at 1:10 000

The base topographic map of the Czech Republic at a 1:10 000 scale (ZTM 10) is a map service provided by State Administration of Surveying and Cadastre in OGC WMS 1.3.0 (https://ags.cuzk.cz/arcgis1/services/ZTM/ZTM10/MapServer/WMSServer?). The ZTM 10 includes planimetry (settlements and individual objects, hydrology, communication networks, administrative and cadastral boundaries, boundaries of protected areas, height and planimetric control points, soil surface, vegetation), altimetry (terrain steps, contour lines) and lettering.

Corine Land Cover (CLC)

The CLC is an open dataset for land cover of European countries provided by the Copernicus Programme. Data is available for download from 1990, 2000, 2006, 2012, and 2018. Datasets are in the vector and raster format with 100 m Minimum Mapping Width (MMW) and the minimum mapping unit (MMU) is 25 ha. Data is distributed over 44 thematic classes. Datasets are downloadable at Copernicus website https://land.copernicus.eu/en/products/corine-land-cover.

Data processing

Geodata was processed in ArcGIS and QGIS. It was then analysed in SPSS Statistics and Statgraphics software. From the geometric component of the *Reynoutria* polygons, we calculated their surface area. After we finished data processing, a script using the ModelBuilder tool in the ArcMap programme was created. The script successively created 19 buffer zones increasing in 20 metres up to a distance of 380 metres from the Morávka River. For each buffer zone, the cumulative surface area of the areas invaded by *Reynoutria* was calculated (see Table 5).

Before the core tests the normality of the *Reynoutria* stand size data was tested in the Statgraphics programme using the Kolmogorov-Smirnov test. Normality was tested for each year under study. The normality of *Reynoutria* stand sizes for each year was not confirmed. In all years examined, the p-value was less than 0.001.

Furthermore, we carried out statistical testing of the significance of the difference between surface area sizes without *Reynoutria* in the studied years at regularly increasing distances from the Morávka River as part of spatial data analysis. Areas without *Reynoutria* were calculated from the difference between the total size of the buffer zone and the area with *Reynoutria*, always for a specific distance from Morávka. The change of size patterns of stands with *Reynoutria* over time was tested by Friedman test. This test was selected as the used variables were colinear and normality tests were not significant (Gibbons and Chakraborti 2010).

Since these were categorical variables, we calculated a Chi-square test of independence between the selected pairs of attributes. To perform the calculation well, we aggregated the attributes Area and Coverage. Aggregation was performed to meet the Chi-square test criterion that the smallest expected frequency had to be equal to or greater than 1. Also, the maximum 20% of the areas could have an expected frequency less than 5 (Greenwood and Nikulin 1996). Thus, the aggregated values of the attributes Area and Coverage were obtained. For the Area attribute, two interval values of 0.01–0.07 ha and 0.08–8.10 ha were created. Aggregation of the Coverage attribute produced area categories of 0.01–1.00% and 1.01–100.00%.

To ascertain the effect of biotopes and type of vegetation on the pattern of change of extent of *Reynoutria* polygons over time, linear models (R. 4.3.1) were used. The extent was taken as dependent variable, levels of abundance were 0.05, 0.50, 5.00, 25.00, and 75.00. To analyse the effect of time we specifically analysed the significance of interactions with years.

Results

Detected changes in the distribution of *Reynoutria*

The following numbers of *Reynoutria*-invaded areas were mapped in each year of GPS mapping: 2007, N = 160; 2009, N = 171; 2013, N = 149; 2015, N = 352; 2018, N = 530; 2023, N = 345. Analysis of data collected between 2007 and 2023 (Fig. 3, Table 2) shows that in 2007 *Reynoutria* covered 29% of the total surface area of the area under study (96.9 ha). Due to chemical treatment, the total area size decreased to 19.6% (65.3 ha) in 2009. In 2013 (3 years after the end of systematic whole area chemical management), the *Reynoutria* area size was 14.5% (48.2 ha) and from this year on, a switch to targeted local management of *Reynoutria* stands was made. In 2015, the *Reynoutria* area remains at similar levels as a result of partial chemical treatment. The area size increased by 0.9 ha to 49.1 ha. 2018 saw a decrease in the *Reynoutria* area size to 41.8 ha. The area reduction was due to repeated local management. Surprisingly, in 2023, the size increased to 52.6 ha and there was a return of *Reynoutria*, although partial chemical treatment was continued. We attribute the increase in the area size to the passage of 5 years since the last chemical treatment in part of the Skalická Morávka National Natural Reserve and other parts of the study area.

As a result of *Reynoutria* management, the structure and coverage of *Reynoutria* stands also changed, as shown in Fig. 4 and Table 2. While in 2007, stands with coverage above 50.1% (50.5 ha) covered the largest area, no *Reynoutria* stands with such high coverage were recorded in 2013. In 2015, these stands reappeared and covered an area of 0.6 ha. In 2013, *Reynoutria* stands with a coverage of up to 0.1% occupied the largest area, but in 2015 there were already stands with coverages of 1.1–10.0%. In 2018, although there was an overall decrease in total area size, surprisingly the largest area of *Reynoutria* stands were already in the 10.1–50.0 coverage category. In 2023, total area size of *Reynoutria* increased again and the most prevalent stand category was the 0.11–1.00% coverage category.

Table 2. Area size of invaded areas of *Reynoutria* and their proportion in the study area by coverage intervals. The data in bold show the highest values of the area of *Reynoutria* in a given year.

	Total area size and proportion of areas with *Reynoutria* in coverage intervals (ha) and (%)											
	2007		**2009**		**2013**		**2015**		**2018**		**2023**	
Coverage (%)	**ha**	**%**	**ha**	**%**	**ha**	**%**	**ha**	**%**	**ha**	**%**	**ha**	**%**
0.01–0.1	–	–	8.1	2.4	**24.4**	**7.3**	4.7	1.4	5.3	1.6	10.1	3.0
0.11–1.0	–	–	17.9	5.4	15.0	4.5	18.1	5.4	8.9	2.7	**18.4**	**5.5**
1.1–10.0	22.7	6.8	**30.7**	**9.2**	8.9	2.7	**19.6**	**5.9**	8.9	2.7	12.7	3.8
10.1–50.0	23.7	7.1	6.4	1.9	–	–	6.1	1.8	**13.2**	**4.0**	7.1	2.1
50.1–100.0	**50.5**	**15.1**	2.2	0.7	–	–	0.6	0.2	5.4	1.6	4.4	1.3
Invaded area (ha)	**96.9**	–	**65.3**	–	**48.2**	–	**49.1**	–	**41.8**	–	**52.6**	–
Invaded area (%)		**29.0**		**19.6**		**14.5**		**14.7**		**12.5**		**15.8**

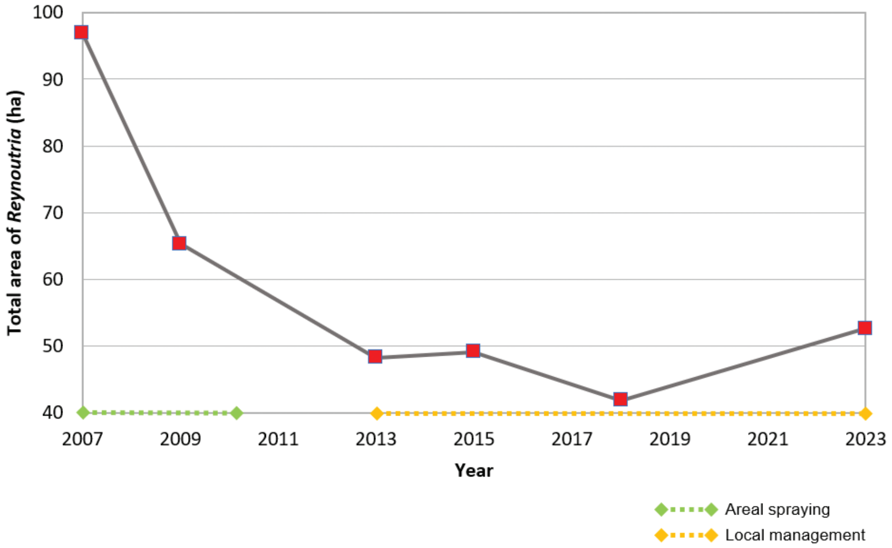

Figure 3. Development of the total area size invaded by *Reynoutria* in the study area between 2007–2023.

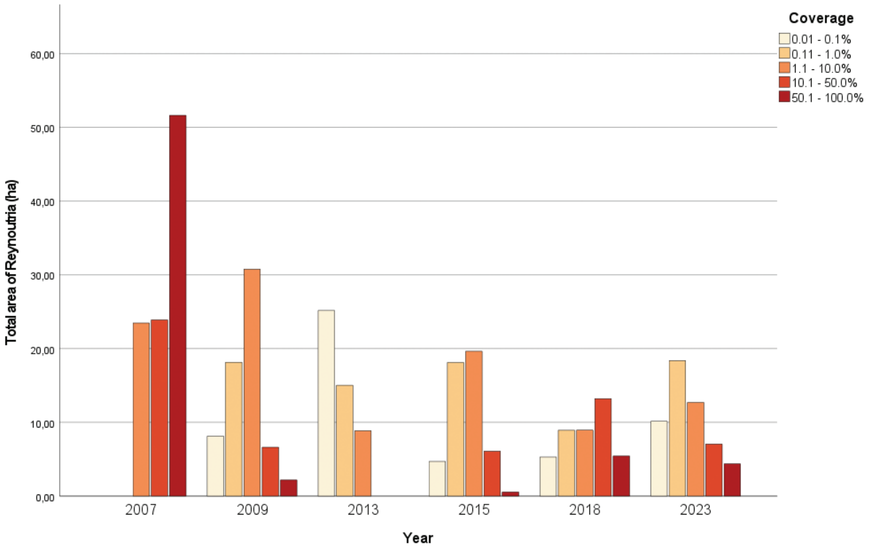

Figure 4. Changes of the area size invaded by *Reynoutria* by coverage intervals.

In addition to the changes in *Reynoutria* coverage, the average area of each stand has also decreased with time elapsed since herbicide application (Figs 5, 6). The graph shows that most areas are less than 1 ha in all years under study, and except for 2007, the largest invaded areas are up to 3.2 ha in size. The average area size decreased from 0.61 ha in 2007 to 0.38 in 2009, to 0.32 in 2013, to 0.14 ha in 2015 and to 0.08 ha in 2018. However, the average area size increased again to 0.15 ha in 2023, which is comparable to 2015. After the end of systematic whole area chemical management in 2010, the fragmentation of *Reynoutria* areas increased. This was evidenced by both the 2015 and 2018 mapping (Table 3). There was a slight increase in the total area of *Reynoutria* in 2015, and an increase in the area of stands with higher *Reynoutria* coverage (Figs 5, 6), although this decreased in 2018 due to local management. However, the trend of fragmentation was still evident. By 2023, the fragmentation trend was no longer confirmed and there was an increase in average area size, although chemical control was ongoing in part of the area.

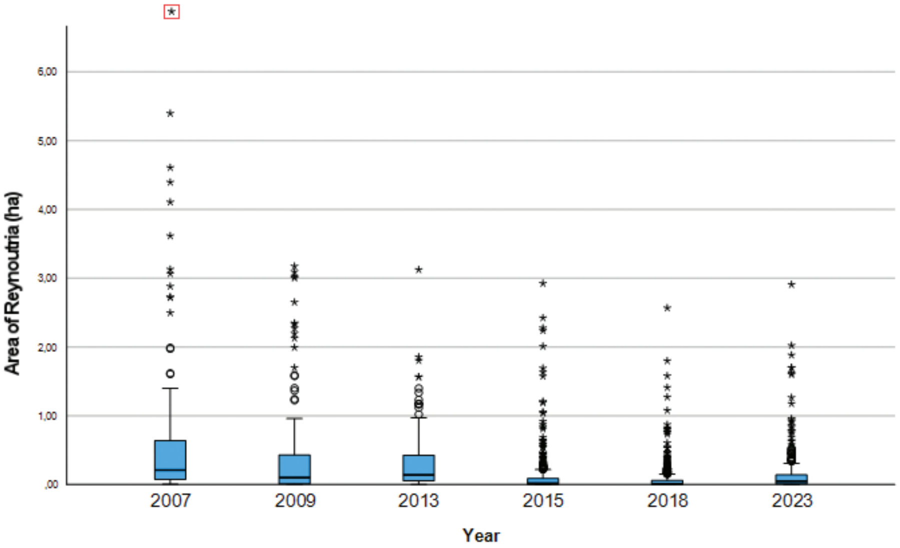

Figure 5. Development of the size of mapped stands invaded by *Reynoutria*. Outliers are represented by a circle and extreme outliers by an asterisk. The asterisk in the red square shows one area from 2007 of 8.1 ha which is outside the scale range.

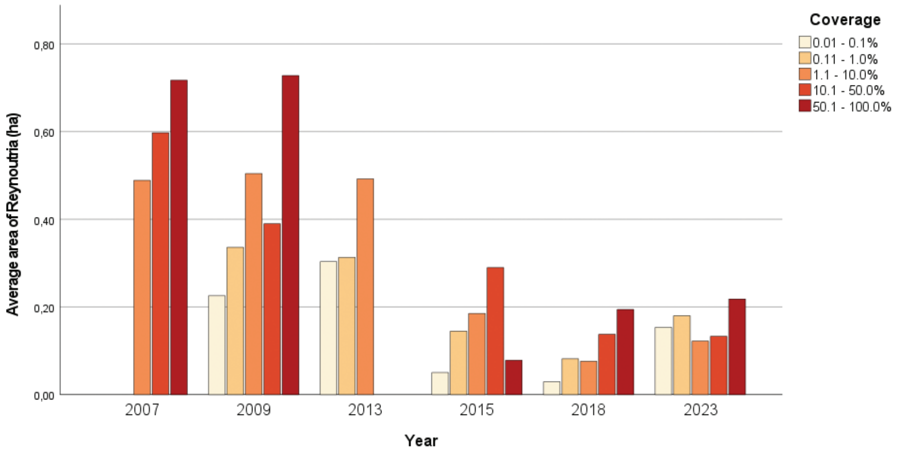

Figure 6. Average sizes of mapped stands invaded by *Reynoutria* by coverage intervals.

During the mapping, the structure of vegetation type where *Reynoutria* occurred was also monitored in selected years (Table 1, Fig. 7). Fig. 7 shows that the habitats where *Reynoutria* occurred have changed as a result of ongoing management. In 2007, 73.1% of the area invaded by *Reynoutria* was forest, 25.4% was grassland and 1.4% was gravel bars (no vegetation). In 2015, there is an overall decrease in the occurrence of *Reynoutria* in the study area, but there is a change in the structure; forest stands represent 77.4%, grassland 22.5% and gravel bars 0.4%. In 2023, *Reynoutria* was already present in 82.7% in forest stands, 13.5% in grassland and 0.8% on gravel bars. According to the 2018 CLC, deciduous forest stands accounted for 83.7% of the area invaded by *Reynoutria*, grassland (mainly meadows) for 16.1% and other areas for 0.3%. The structure of these areas thus corresponds to the structure of *Reynoutria* vegetation in 2023 (Fig. 7). This indicates that the chemical control was more successful when used on grassland than in the forest.

However, testing of the effects of time, habitat and biotope did not reveal significant differences of changes of extent and abundance over different environments.

Table 3. Number of areas invaded by *Reynoutria* in each coverage interval.

Coverage (%)	2007	2009	2013	2015	2018	2023
0.01–0.10	–	36 (21.1%)	83 (55.7%)	93 (26.4%)	180 (34.0%)	66 (19.1%)
0.11–1.0	–	54 (31.5%)	48 (32.2%)	125 (35.5%)	109 (20.6%)	102 (29.6%)
1.1–10.0	48 (30.0%)	61 (35.7%)	18 (12.1%)	106 (30.1%)	117 (22.1%)	104 (30.1%)
10.1–50.0	40 (25.0%)	17 (9.9%)	–	21 (6.0%)	96 (18.1%)	53 (15.4%)
50.1–100.0	72 (45.0%)	3 (1.8%)	–	7 (2.0%)	28 (5.3%)	20 (5.8%)
Total	**160** (100%)	**171** (100%)	**149** (100%)	**352** (100%)	**530** (100%)	**345** (100%)

Note: The value in parentheses represents the percentage of occurrences of *Reynoutria* in a specific category.

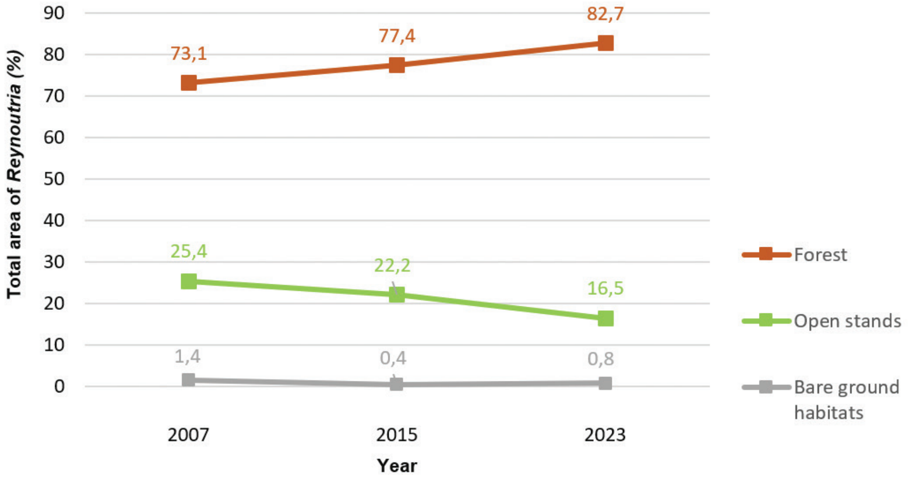

Figure 7. Extent of *Reynoutria* based on Vegetation cover type.

There were no significant interactions between year, abundance, moisture type (F = 1.86, DF = 2,1536, n.s.), and vegetation cover type (F = 0.71, DF = 2,846, n.s.), which indicates that there are no differences in reaction to management in the studied habitat and vegetation types. Significant effect of year (F= 24.2, DF = 1,1537, $p < 0.001$) shows that the extent of *Reynoutria* sites changes. Additional comparisons are shown in Table 4.

The results of GNSS mapping of *Reynoutria* stands from 2007–2015 were published using the web-based map application http://gisak.vsb.cz/knotweed/. This application allows comparison of the changes in the distribution/spread of *Reynoutria* stands with the recorded attributes (stand coverage, moisture, vitality, ID number) in each year using OpenStreetMap (Fig. 8).

Analysis of the attributes of invaded habitats

In the mapped years 2009, 2013, 2015, 2018 and 2023, the dependence between pairs of attributes was tested using Pearson's Chi-square test of independence in a contingency table. The tests intended to statistically demonstrate the dependence between the categorical attributes were mapped (Table 1), and we were specifically interested in the dependence on Moisture type. The test P-values are shown in Table 4. We aggregated the attribute Area into the categories of small *Reynoutria* stand area (0.01–0.07 ha) and large *Reynoutria* stand area (0.08–8.1 ha).

For the Coverage – Vitality attribute pair, we demonstrated statistically significant dependence or relationship in all years under study. This dependence is logical.

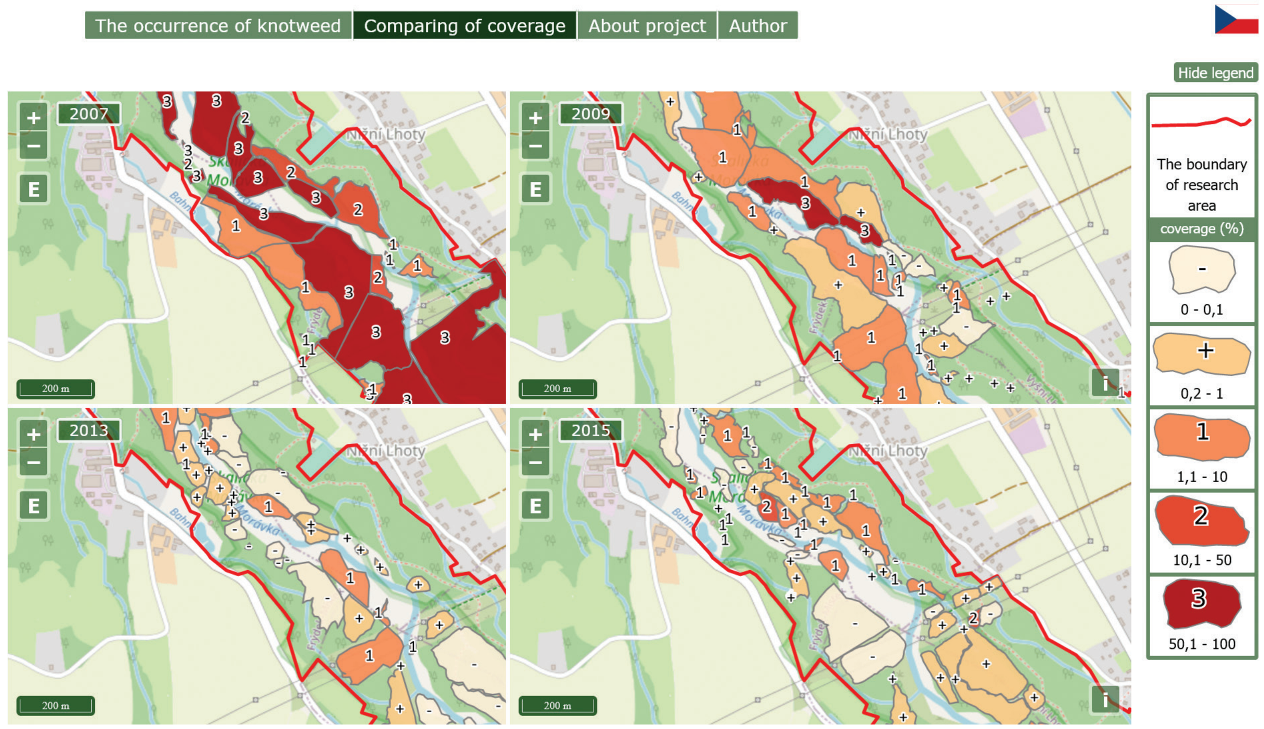

Figure 8. Example of the web-based map application "*Reynoutria* occurrence". The section shows a comparison of *Reynoutria*-invaded habitats, including their coverage in the vicinity of the 8th river kilometre near the village of Nižní Lhoty.

Table 4. P-values of Chi-square test of independence of pairs of attributes.

P-value	2009	2013	2015	2018	2023
Area – Coverage	0.289	0.229	0.960	**0.003**	**0.002**
Area – Moisture type	**< 0.001**	**0.011**	0.437	0.066	0.156
Area – Vitality	0.698	0.708	0.696	**0.046**	0.556
Coverage – Moisture type	**< 0.001**	0.279	**0.015**	**< 0.001**	**< 0.001**
Coverage – Vitality	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Vitality – Moisture type	**0.002**	0.098	0.070	**< 0.001**	**0.026**

Note: For p-values in bold, dependence was demonstrated with 95% confidence (2009, N = 171; 2013, N = 149; 2015, N = 352; 2018, N = 530; 2023, N = 345).

Areas with *Reynoutria* stands with high coverage also had high vitality and vice versa. For the Area – Moisture type pair we showed a dependence in 2009 and 2013. Dry habitats have only a local occurrence in the river floodplain and were therefore not frequently represented or occurred less frequently among large areas (0.08–8.10 ha). Normal habitats were more common among small areas (0.01–0.07 ha) in 2009 and 2013. Aggregate area size therefore depended on Moisture type in these years.

For the Coverage – Moisture type attribute pair, dependence was demonstrated in all years examined except for 2013. In these years, the aggregated Coverage depended on Moisture type. Areas with higher coverage of 1.1–100.0% were more likely to occur in the moist habitat type in these years. Conversely, areas with lower coverage of 0.01–1.00% occurred less frequently in the wet habitat type.

In the Vitality – Moisture type attributes we demonstrated a relationship in 2009, 2018 and 2023. The Vitality attribute was therefore dependent on the Moisture type habitat in which the *Reynoutria* was situated in those years. The wet habitat type had stands with high vitality. On the other hand, in the dry habitat type there were stands with lower vitality. The Area – Coverage attributes were only able to show a relationship between the two most recent years of measurement, i.e., in 2018 and 2023. This suggests that the more extensive the stands were, the higher the *Reynoutria* coverage was and vice versa. For the Area – Vitality attribute pair, a relationship was only demonstrated in 2018.

Using spatial analysis, we evaluated the distribution and size of *Reynoutria* stands as a function of distance from the river. For this purpose, we successively created buffer zones around the river with increments of 20 metres, i.e., 19 zones in total (Table 5). The results of the analysis showed that the *Reynoutria* stands extended the furthest from the watercourse (up to 360 m) in 2007, before the start of the plant chemical treatment. During the management, the extent of *Reynoutria* stands was reduced, so that in 2013 they extended to no more than 280 m from the watercourse. Since 2015, *Reynoutria* has been pushed back further from the watercourse to a distance of up to 320 m. However, the growth of *Reynoutria* areas within 220 m from the watercourse is minimal in all years.

Table 5 shows that at least 25% of the *Reynoutria* stands are located within 40 m from the Morávka River. More than half of all stands were located within 80 m from the stream, except for in 2013, when this distance was even lower (60 m). In total, 75% of *Reynoutria* stands were located within 120 m, except for in 2013 when this distance was 100 m. Thus, the cumulative area sizes show that *Reynoutria* is concentrated in relatively close proximity to the river and the river is the main factor in the occurrence of *Reynoutria*.

Table 5. Cumulative area size of *Reynoutria* at regular distances from the Morávka River. The values below are in hectares (ha).

Year		2007	2009	2013	2015	2018	2023
Extent of zones from the river	0–20	16.47	11.71	7.78	6.25	6.61	10.36
	0–40	30.87	22.34	16.20	13.07	13.78	18.86
	0–60	45.15	32.21	25.06	20.83	20.57	26.23
	0–80	58.24	40.91	32.49	28.34	26.23	32.21
	0–100	69.01	47.99	38.00	34.47	30.67	37.15
	0–120	77.27	53.83	42.00	39.22	33.83	40.59
	0–140	83.64	58.33	44.74	42.84	36.26	43.87
	0–160	87.94	61.44	46.18	45.30	37.42	46.20
	0–180	90.80	63.41	47.05	47.03	38.24	47.92
	0–200	92.66	64.48	47.59	48.01	38.90	49.17
	0–220	93.82	65.00	47.86	48.65	39.57	50.11
	0–240	94.42	65.22	48.08	48.84	40.24	50.90
	0–260	94.83	65.26	48.22	48.93	40.85	51.52
	0–280	95.51	65.26	48.24	49.04	41.38	52.08
	0–300	96.11	65.26	48.24	49.08	41.58	52.40
	0–320	96.49	65.28	48.24	49.08	41.73	52.60
	0–340	96.76	65.28	48.24	49.08	41.82	52.62
	0–360	96.85	65.28	48.24	49.08	41.82	52.62
	0–380	96.85	65.28	48.24	49.08	41.82	52.62
Quartile (%)	25	24.21	16.32	12.06	12.27	10.46	13.16
	50	48.43	32.64	24.12	24.54	20.91	26.31
	75	72.64	48.96	36.18	36.80	31.37	39.47

Note: The size of the *Reynoutria* areas smaller than half of the total size of the *Reynoutria* areas are shown in red. Areas larger than half of the total size of the *Reynoutria* areas are shown in white. Green cells show the areas where their size is no longer increasing and *Reynoutria* was no longer present there.

Conclusion and discussion

The goal of this paper was to describe and analyse the patterns of invasion of *Reynoutria* and, more importantly, the effectiveness of its management. The data covers 17 years of continuous management which provides a robust and unique overview of the invasion, reinvasion, and suppression dynamics. Given the large area and long timespan, our results can be appreciated by many stakeholders working on the control or suppression of *Reynoutria*. We hope that this paper will stimulate publishing more studies based on long-term efficiency of management of this important invasive species. *Reynoutria* is a problematic and persisting invasive species in most of Europe, North America, and Asia and therefore the species has been widely studied. Because of its negative impact on biodiversity as well as other effects, there are numerous studies focused on its regeneration, spread, and management (Kabat et al. 2006; Chmura et al. 2013; Kadlecová et al. 2022) as well as studies focused on its ecology, genetics and taxonomy (Bímová et al. 2003; Mandák et al. 2005; Parepa et al. 2014; Lavoie 2017). Even in Europe, where there is a reluctant attitude toward biocontrol, the species has been subject to various attempts to establish successful biocontrol for many years (Shaw et al. 2011). Individual countries, municipalities, and stakeholders spent enormous resources for its control, and *Reynoutria* is classified as one of the costliest species in Europe (Haubrock et al. 2021). While the initial aim of many nature conservation organisations, NGOs and municipalities was to eradicate knotweeds completely, it is now becoming more and more evident that full large-scale control is almost impossible and the aim is therefore to mitigate its negative impact and prevent further spread.

Many studies focus on the methods of management of *Reynoutria* (see overview in Kadlecová et al. 2022). The studies offer a range of methods from purely mechanical methods (mowing, grazing, covering soil with plastic, heat treatments, disposing the material to compost), to chemical treatment (various chemical substances and timing), to biocontrol (Shaw et al. 2011). Unfortunately, economic evaluation of the methods is only rarely available (Hocking et al. 2023). The results from long-term sustainable control are missing, as the studies cover only a limited time span or are based on small study sites, or even use only experimental data from laboratory or experimental gardens (Kettenring and Adams 2011; Jones and Eastwood 2019). Nevertheless, the practical nature protection and land managers need the long-term studies that can be applied on a large scale (Pergl et al. 2020b). It has been repeatedly shown that the patterns can differ significantly between short and long-term studies even if they are based in the same area, and sometimes provide the opposite results (Lepš 2014; Čuda et al. 2017).

As knotweeds are widely recognized as problematic species and thus are widely managed, they become a suitable model system for various schemes of planning and prioritizing management. Apart from this, the three closely related *Reynoutria* species are also often studied due to their differences in their invasion potential and regeneration ability (Pyšek et al. 2003; Schmiedel et al. 2016; Kadlecová et al. 2022). However, we are not aware of any data on management valid for more than five years published for any of the species. Our study focuses on the use of herbicides, which presents only one type of management from the large list of available methods (Bzdega et al. 2022). In addition to the data presented in our study, it would be highly beneficial to evaluate a large-scale and long-term study, including the effectiveness of various methods of *Reynoutria* management.

Our data shows that within a few years after the management of the area started, the extent of the *Reynoutria* stands sharply decreased and the large polycormons were split to small and less dense ones. Within the first five years of the management, the extent of *Reynoutria* decreased to 49.7% of initial values (96.9 ha) in 2013, and the size of the average stands (polycormons) decreased to 0.32 ha. Since *Reynoutria* is a species with a high negative impact on vegetation when growing in large stands (Hejda et al. 2009, 2021), reducing its size at invaded sites has been important for nature protection. Nevertheless, *Reynoutria* exhibits high regeneration potential, which required a repeated chemical treatment, applied locally since 2013. Despite a slight increase in 2015, there has been a significant reduction in the *Reynoutria* area to 41.8 ha in 2018, i.e., 43.1% of initially invaded area. However, despite ongoing local management in subsequent years where some sites were locally treated the total area of *Reynoutria* spp. increased to 52.6 ha in 2023. We attribute this increase to the time lag of 5 years since the last coordinated herbicide application.

Another factor in the increase in the total species area may also be the increased flood flows (cca 70 m^3/s) reached during the 2020 floods. These flows cause overlaying of the river channel and its course, and the disappearance and creation of new gravel bars and islands. As a consequence, *Reynoutria* stands are disturbed and their rhizomes fragmented and further spread. These fragments serve *Reynoutria* to regenerate, as described above (e.g., Bímová et al. 2003).

Not only the size of the stands (polycormons) significantly changed, but also the coverage of *Reynoutria* rapidly decreased. At the time of the start of the chemical spraying, the study area was mainly *Reynoutria* stands with high coverage and the two categories of areas with the lowest coverage were not at all represented. This changed dramatically as a result of three years of systematic whole area chemical management. There was a significant reduction in areas in the case of higher coverage sites as shown in Fig. 4, and in 2013 even no more high coverage sites were recorded (coverage categories 10.1–50.0% and 50.1–100.0%). However, since 2015 the trend has been reversed and in 2018, despite ongoing chemical interventions in parts of the area, the largest area was already in the 10.1–50.0% coverage category. In the last year of monitoring (2023), the largest area was occupied by *Reynoutria* with coverage between 0.11–1.00%, which is probably the result of mixed effect of local chemical treatment and floods in 2020, as mentioned above.

The area size of most *Reynoutria* stands (except for in 2007) prior to the start of eradication was less than 1 ha throughout the management period. Nevertheless, we identified areas of several hectares in each of the years studied (Fig. 5). The smallest average area size of *Reynoutria* (0.08 ha) was recorded in 2018, which was also the year with the largest number of mapped areas (530). At the same time, *Reynoutria* occurred in more than half of them with a coverage of less than 1% (Table 3). This is indicative of the increased fragmentation of areas with *Reynoutria* stands that has been occurring since 2015 (Fig. 5, Table 3). In 2018, fragmentation increased further due to chemical treatment with herbicide. As more time passed since chemical treatment, the number of areas decreased again to 345 in 2023, which made it comparable to 2015 (352).

The mapping results indicated that there was a change in the total area of habitat covered with *Reynoutria*, which was a result of chemical treatment (Fig. 7). However, the non-significant interactions show that the change for each occurrence is similar across all types, i.e., *Reynoutria* responds to chemical control in the same way in all habitats.

Such changes of invaded population structure are important for neighbouring species. In a study by Hejda et al. (2021) it was found that stands with low to moderate cover of knotweed have approximately more than twice as many species as dense *Reynoutria* stands. In our study area, reduction of the *Reynoutria* cover induced regeneration of the species diversity, as documented in the study by Halas et al. (2018). On phytocoenological plots where the herbicide was applied more frequently, the ruderal plant species, e.g., *Crepis biennis*, *Impatiens parviflora*, *Lactuca serriola* and *Solidago canadensis*, prevailed. On the contrary, in places where the herbicide was not applied so often, a frequent occurrence of autochthonous plant species of a submontane floodplain forest, e.g., *Carex remota*, *Dentaria glandulosa* and *Veronica montana* could be observed apart from some ruderal plant species. However, with the time passing since the last herbicide application, the abundance of both native and non-native plant species has increased, and so the coverage of the native plant species. Nevertheless, as a side negative effect, growth deformations of many native herbaceous species were detected even one year after the last application of herbicide (Halas et al. 2018).

In spatial analysis we found (Table 5) that at least 25% of *Reynoutria* stands occur within 40 m from the Morávka river. More than 50% of the stands occurred within 80 metres, except for in 2013, when this distance was only 60 metres. Similar to the study by Shen et al. (2015), we found that the closest vicinity of the riparian zone has the greatest impact on the observed phenomenon, in our case the spread of invasive neophytes. From 2007 to 2013, the distance from the watercourse where *Reynoutria* stands were still recorded decreased from an initial 380 m in 2007 to 280 m in 2013. In the following years, *Reynoutria* stands expanded again to 320 m. However, from a distance of 220 m from the Morávka watercourse, the cumulative growth of *Reynoutria* areas was low.

All field measurements of the spatial extent of *Reynoutria* were made using autonomous GNSS measurements based on ZTM 10 and Orthophoto digital mapping, which were chosen due to the complexity of the field and forest cover where phase measurements would be difficult to implement. Similar GNSS mapping was also carried out by Moore et al. (2006), whose mapping concept was similar. However, their results do not include a statistical evaluation of the *Reynoutria* areas and so our results cannot be compared with their study in this respect. The accuracy of autonomous GNSS measurements is in the order of units of metres (up to tens of metres in adverse conditions) and is influenced by several factors such as observational conditions and the state of the atmosphere, the presence of vegetation, buildings, the relief shape, etc. (Blažek and Švec 2010; Tomaštík et al. 2017; Teunissen and Montenbruck 2017). Currently, some low-cost apparatuses for GNSS measurements on Android platforms are being tested (Dabove et al. 2019; Halaj and Kačmařík 2022). This includes the possibility of using differential GNSS (DGNSS) or real-time kinematic (RTK) correction measurements (Netthonglang et al. 2019; Tomaštík et al. 2021). Mapped areas of *Reynoutria* stands are published for selected years in the *Reynoutria* occurrence web-based application (http://gisak.vsb.cz/knotweed/). An alternative to ground-based mapping of invasive neophytes is the currently increasingly used Remote Sensing. Both satellite and aerial data is used to study invasive neophytes. Currently, UAV data, as noted by Martin et al. (2018), Michez et al. (2016), Dorigo et al. (2012), Negrea et al. (2022), are increasingly used. The advantage of this approach is the speed of data acquisition, without the need for direct mapping in the field, and the possibility to map even difficult or inaccessible locations. Another advantage is the possibility of automated processing of invasive neophyte occurrence data using different classification methods or machine learning (Müllerová et al. 2013; Jones et al. 2020b; Dyrmann et al. 2021). Therefore, we would like to continue and expand the study of the issue in the study area by comparing the results of GNSS mapping (using satellite data) and UAV imagery.

Implications for practical management

The most important point in the whole *Reynoutria* management is to work in long-term scale. It is important to note that any management action must be followed by reasonable land use. It is useless and is only a waste of resources to make any random and single control actions (cutting, herbicide application) without a concept of future land use that will restrict *Reynoutria* reinvasion. Based on the results from other studies (e.g., Jones et al. 2020a; Kadlecová et al. 2022) we have come to conclusion that the only effective control management is application of herbicide. Therefore, in areas where regional restrictions allow its use, this is the preferred type of management. However, chemical treatment must be quite long-term. Even three years of intensive herbicide application was not sufficient for the complete control of *Reynoutria*. Following field mapping in 2013 (i.e., 3 years after the end of the chemical treatments) it was clear that *Reynoutria* would start to expand again without further management of the area. In several locations, particularly in hydrically normal habitats in the floodplain forest, *Reynoutria* was forming vigorous stands without necroses. Without further intervention, we anticipated its successful regeneration and expansion in the study area. These assumptions were confirmed, because despite further local treatment carried out gradually in all parts of the site, the areas with *Reynoutria* increased by 4.4 ha over the next ten years (2013–2023). However, these interventions have reduced *Reynoutria* coverage in invaded areas and *Reynoutria* remains under control due to management.

Therefore, the key message resulting from our study concerns the structure of long-term management by herbicides. Based on the presented data and gained field experience, we propose the following procedure:

1. In largely infested sites, the first step is to reduce the distribution of *Reynoutria* stands to isolated polycormons (ca 1×1 m^2) . This phase can last 3–5 years.
2. After reaching the state of sparse distribution of *Reynoutria* (although several large clones may remain at a site), apply the herbicide only in period of every 3–5 years depending on the local context and rate of regrowth. More frequent applications increase economic costs and increase the risk of negatively affecting the surrounding flora and fauna. Longer breaks between individual applications allow significant regeneration of stands. It is recommended to maintain the reduced *Reynoutria* sites by long-term land-use such as mowing or grazing. However, the risk of potential spread of *Reynoutria* biomass must be considered in such cases.
3. At sites exposed to soil disturbances (e.g., after floods, road constructions), where the soil is contaminated by fragments of *Reynoutria* rhizomes, there is a need to apply herbicide immediately to target newly resprouting individuals. New and young resprouts are highly susceptible to herbicide (due to low nutrient stock) and such application has higher efficiency leading to full eradication.

The proper application of herbicide is also crucial. Glyphosate dosage/application rates are discussed by Delbart et al. (2012), Jones and Eastwood (2019), or Kadlecová et al. (2022). Also, several literature sources recommend to reduce above-ground biomass prior to herbicide application (Jones and Eastwood 2019; Hocking et al. 2023).

We are aware that herbicide application can be a conflicting issue for the public and also for some parts of nature protection. However, it is clear that the use of herbicides is needed for some highly resprouting species (Csiszár and Korda 2017; Pergl et al. 2020a). In some cases (e.g., *Reynoutria*), it was shown that herbicide application is the most effective approach in the context of costs and side effects on the environment (Hocking et al. 2023). Properly used chemicals also limit the risks of accidental spread of the rhizomes and contaminated soil by mechanical methods (e.g., digging or excavating) and importantly for the managers, they are also cheaper. Similarly, improperly applied herbicides may negatively affect the neighbouring biota as well as decrease the efficiency of the management. In addition, correct herbicide application becomes more important in the era of changing climate conditions with increased CO_2 levels and rising drought and heat, when herbicides may become less effective (Varanasi et al. 2016; Bzdega et al. 2022). The local conditions and possible restrictions on the use of chemicals are always to be considered, as well as interaction with the public.

To compare the patterns of management effectivity, more long-term studies on management of the species and from other regions/types of habitats are needed. To fulfil such needs, detailed monitoring of the management actions and their results is recommended with specification of the management methods used, costs of treatment, etc. In this study, we were unable to properly cover the issue of costs and effort of management, as management was done by several bodies. Despite this, we believe that the presented study offers a valuable contribution to the proper management of *Reynoutria* species.

Acknowledgements

Our appreciation is extended to the editor and the reviewers whose comments contributed to the improvement of the manuscript.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

Jan Pergl and Irena Perglová were supported by the project DivLand – Centre for Landscape and Biodiversity from the Technology Agency of the Czech Republic (SS02030018), and a long-term research development project (RVO 67985939) from the Czech Academy of Sciences.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Example of mapped *Reynoutria* coverage by each mapped category

Authors: Pavel Švec, Irena Perglová, Václav Fröhlich, Josef Laštovička, Jakub Seidl, Kateřina Růžičková, Ivana Horáková, Jan Lukavský, Martin Ferko, Přemysl Štych, Jan Pergl

Data type: PNG

Explanation note: 1 – Coverage of less than 0.1%. 2, 3 – Coverage of 0.11–1.0%. 4 – Coverage of 1.1–10.0%. 5 – Coverage of 10.1–50.0%. 6 – Coverage of 50.1–100.0% – In this category of coverage, the area is almost impassable.

Link: https://doi.org/10.3897/neobiota.94.122337.suppl1

Supplementary material 2

Example of the impact of chemical control on *Reynoutria*

Authors: Pavel Švec, Irena Perglová, Václav Fröhlich, Josef Laštovička, Jakub Seidl, Kateřina Růžičková, Ivana Horáková, Jan Lukavský, Martin Ferko, Přemysl Štych, Jan Pergl

Data type: PNG

Explanation note: 1, 2 – Chemically treated area one year after herbicide application (July 2008). The success rate of chemical control is high, *Reynoutria* regenerates sporadically after spraying. Overall coverage increases and new species with ruderal tendency appear. The photo foreground shows the invasive species *Impatiens parviflora* and *Impatiens glandulifera*. 3, 4, 5 – Example of various forms of malformation and necroses after chemical eradication of *Reynoutria*. The height of *Reynoutria*, including the leaf forms, is greatly altered, with the formation of various "deformed" forms.

Link: https://doi.org/10.3897/neobiota.94.122337.suppl2

Supplementary material 3

Example of the mapped Moisture types

Authors: Pavel Švec, Irena Perglová, Václav Fröhlich, Josef Laštovička, Jakub Seidl, Kateřina Růžičková, Ivana Horáková, Jan Lukavský, Martin Ferko, Přemysl Štych, Jan Pergl

Data type: PNG

Explanation note: Habitat moisture was categorised by relief, soil cover, and vascular plant species representation. 1 – Areas with lowlands, oxbow lakes, pools, clay soils were mapped and classified as wet habitats or stands. 2 – Normal habitats were located on flat relief without frequent floodwater influence, away from river channels and pools. Normal habitats were characterized by loose, humic soils, lacking wetland and arid-loving plant species, with mesophilous herbs being common. 3 – Dry habitats were mapped on elevated sites, primarily on gravel bars, accompanied by dry coarse-grained substrate.

Link: https://doi.org/10.3897/neobiota.94.122337.suppl3

Supplementary material 4

The Morávka River and its surroundings in the study area

Authors: Pavel Švec, Irena Perglová, Václav Fröhlich, Josef Laštovička, Jakub Seidl, Kateřina Růžičková, Ivana Horáková, Jan Lukavský, Martin Ferko, Přemysl Štych, Jan Pergl

Data type: PNG

Explanation note: 1 – The Morávka River represents a uniquely preserved Carpathian-type stream in the Czech Republic. Especially in the preserved locality Profil Morávky, there are unique pools with clear water. 2 – There are also rock thresholds and rapids. 3 – After the flood, the riverbed is "cleaned" of the vegetation and the position of the riverbed changes. The photo taken after the flood in 2010. 4 – Due to deep erosion, the riverbed is deepened about 10 m below the river floodplain in the lower part of the stream. This process is still ongoing. 5 – The middle part of the stream in the Vyšní Lhoty area differs from the lower part of the stream.

Link: https://doi.org/10.3897/neobiota.94.122337.suppl4

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 289–310 (2024)
DOI: 10.3897/neobiota.94.124500

Research Article

Exotic species swapping: Reciprocal movement of animal species among regions of the Americas

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Abstract

The movement of exotic species, both intentional and unintentional, is among the top threats to global biodiversity and native taxa. Research has frequently explored species movement between the eastern and western hemispheres, focusing on the number of species moving from east to west. Here we use qualitative and quantitative information from a compiled exotic species compendium (CABI Digital Library) to produce a conservative picture of the exchange of nonnative animal species, trends in movement of various taxa among regions, and the trade relationships that could contribute to species' movements strictly within four major regions of the western hemisphere (North America, South America, Central America, and the Caribbean). Species exchange between regions in the western hemisphere (285) were higher than documented invasions from all regions of the eastern hemisphere with the exception of Asia, the largest region in the study (348). Among the broad taxonomic categories, arthropods and fish dominated the counts of exchanged species in every region, largely due to trade related to food production, aesthetics, or sport. Perhaps due to the importance of trade-related movement vectors for the dominant taxa, country GDP was positively related to export of exotic species. Therefore, the magnitude and importance of species exchanges among countries in the western hemisphere has been underestimated, with factors like proximity and economic trade connections likely leading to more species translocations.

Key words: Economic activity, exotic species, international trade, species translocation, vectors

Academic editor: Michael McKinney
Received: 1 April 2024
Accepted: 22 June 2024
Published: 21 August 2024

Citation: Ray HA, Tristano EP, Work KA (2024) Exotic species swapping: Reciprocal movement of animal species among regions of the Americas. NeoBiota 94: 289–310. https://doi.org/10.3897/neobiota.94.124500

Introduction

The long history of global colonization by European powers has resulted in transport of species around the world and produced historical records of species' movements. These records include domesticated animals such as pigs, game species, or species introduced by Acclimatization Societies, which released animals with the express purpose of having them naturalize in colonized regions. From the 900s to 1900s, explorers and colonists released these species explicitly to become naturalized for food and aesthetic enjoyment, a phenomenon coined "ecological imperialism" (Crosby 2004). The nuisance effects of these species' movements are well documented (Crosby 2004). However, the lessening of these types of introductions in more recent times may misrepresent the high number of species introductions that continue to occur throughout many parts of the world (Blackburn et al. 2015; Early et al. 2016; Pyšek et al. 2020). For example, Blackburn et al. (2015) argue that, while European Acclimatization Societies are traditionally at the heart of our understanding of invasive birds, global bird introductions cannot be entirely attributed to European influence and, like many other taxa, are mostly accidental. Moreover, as global trade increased in the 20th and now the 21st century and nations outside of Europe have risen in economic status, patterns of species invasions have become less Euro-centric and more cosmopolitan (Early et al. 2016; Turner et al. 2021) and the numbers of species invasions have only increased (Pyšek et al. 2020).

The origins and directions of these modern invasions may correlate with national GDP (Hulme 2009) and the trade of invasive species may be higher between countries that have developed strong economic ties, such as between the United States and the countries of Central and South America and the Caribbean (e.g., the US is currently Brazil's second most important import and export partner; World Bank 2023). It is also likely that these connections have been present for some time. Although the US did not formally colonize the rest of the Americas, it has long exerted strong economic influence in the region (Gill 2019), which may have led to high levels of invasive species trade along with traditional imports and exports between the US and other countries in the western hemisphere. There is less information about movement of nonnative species within the Neotropics than there is about European import and export of nonnative species to the region, but archaeological evidence suggests that transport of vertebrates to the Caribbean from the mainland Americas may have begun prior to European colonization and has continued to the present (Kemp et al. 2020).

In these exchanges, the presence of movement vectors, the specific characteristics of individual species, and the characteristics of the receiving sites all can contribute to successful species invasions. In general, species that are linked in some way to human activity are more likely to move between continents and countries (Jeshke and Strayer 2006; Gippet and Bertelsmeier 2021; Olden et al. 2021; Turner et al. 2021). This linkage may be direct and intentional, as when people import plants and animals for their use as pets, ornaments, food, sport, or biocontrol (Simberloff 2013; Chan et al. 2019; Olden et al. 2021). In particular, species used for food and nonfood resource production (e.g., silviculture) have been, and continue to be, moved around the world extensively (Garnas et al. 2016; Chan et al. 2019). Most of these intentional releases are of attractive or useful plants or vertebrates, such as fish, birds, and mammals (Chan et al. 2019; Jarić et al. 2020; Gippet and Bertelsmeier 2021). However, introductions may also be linked indirectly to human activity as species may hitchhike along with human movement or human trade and shipping (Hulme 2009; Tatem 2009; Olden et al. 2021; Turner et al. 2021). Species moved intentionally often are large enough to be observed easily, but the species that hitchhike on these larger species often are much smaller and less conspicuous (Dale et al. 2020; Jarić et al. 2020). Both intentional and unintentional introductions may happen repeatedly, producing high propagule pressure (Jeshke and Strayer 2006; Turner et al. 2021), a phenomenon only made worse by online trading which may produce diffuse shipping of species with less regulatory oversight (Gippet and Bertelsmeier 2021; Olden et al. 2021).

Of course, intentional movement of attractive species or hitchhiking on such species does not ensure a successful invasion; plasticity of behavior, lifestyle, and physiology as well as high productivity greatly increase, although do not guarantee, the likelihood of invasion success. The ability to change investment in reproduction, such as crabs that may produce more or fewer broods with changing resource availability, can allow populations in new habitats to persist in lean, and grow under, flush conditions. Omnivory can reinforce the ability to capitalize on variable resources to support population growth and expansion (Havel et al. 2015; Geburzi and McCarthy 2018). Parthenogenic reproduction and early maturity can allow populations to grow quickly from introductions of only a few individuals. Wind or water dispersal of organisms with limited movement ability, such as some insects or plankton, may aid in the spread within the new habitat as does resiliency to survive in the hold of an airplane or the ballast water of a ship (Garnas et al. 2016; Pyšek et al. 2020). Characteristics like the ability to attach to a vessel, such as fouling invertebrates on ships, or the production of planktonic larvae that can travel in ballast also increase the likelihood of introductions (Simberloff 2013; Geburzi and McCarthy 2018). Tolerance to a wide array of environmental conditions, such as variable temperature and presence of pollutants, may increase survival in new habitats (Kelly 2014; Havel et al. 2015; Geburzi and McCarthy 2018). In the receiving habitat, a novel disturbance may facilitate, but not guarantee, invasibility. A typical disturbance in a habitat that is regularly disturbed, such as storm-induced turbulence in an estuary, may not increase the likelihood of a successful invasion, but a novel disturbance, such as the introduction of aquaculture into a coastal region, might (Simberloff 2013; Geburzi and McCarthy 2018). Islands, in particular, are prone to invasion, perhaps due to missing top predators, large grazers, or regular massive disturbances from storms. Due to lower species richness, the proportion of their biota that are invasive increases with isolation from the mainland (Simberloff 2013; Moser et al. 2018). Again, these characteristics do not ensure invasion, but may increase the likelihood of success.

Movement and establishment of invasive species ranks high, along with habitat loss/degradation and climate change, in the threats to the world's biodiversity (McKinney and Lockwood 1999; Dueñas et al. 2021). As a result of the huge number of species transported around the world with European colonists for food, building materials, or medicine (Mack and Lonsdale 2001) and current global trade, much of invasive species literature has focused on the transport species favored by these colonists or on the inter-hemisphere transfer of species with trade. As a result, the literature is dominated by studies of species of Palearctic origin, with relatively few studies of exotic species of Nearctic origin and even fewer originating in the Neotropics (Florencio et al. 2019). Despite this paucity of research, human movement and trade have, in fact, occurred in the western hemisphere and likely contributed to species' movements due to proximity. In this work, we leveraged datasets made available online to explore the invasion patterns of different species at regional as well as countrywide scales. This effort was made possible by the recent advent of online data storage, management, and accessibility. For this project, we used data from the Exotic Species Compendium in the CABI Digital Library, which includes contributions from the US Department of Agriculture and several other governmental, non-governmental, and private organizations (https://www.cabi.org/isc/about). We used the quantitative and qualitative information available in those databases to evaluate: 1) the extent to which reciprocal trades occurred between countries in the western hemisphere, 2) whether there were spatial patterns in reciprocal trades and whether some regions traded more, and 3) whether there were taxonomic patterns in reciprocal trades and whether some taxa moved more. We predicted that movements of species between countries in the western hemisphere have been common and widespread and that taxa associated with movements of people (animals associated with agricultural and ornamental plants, animals used as food or sport, and animals used as pets) would be among the species most likely to move.

Methods

Data collection

To evaluate the movement of nonnative species within regions of the Americas, we collected lists of exotic species for each country in North, Central, and South America and in the Caribbean from the CABI Invasive Species Compendium (CABI 2021). This website provided lists of nonnative species compiled for countries, as well as information on taxonomy, distribution, biology, ecology, movement vectors, and threats to native species and ecosystems. The information in this database was collected from a variety of published sources, cited, and corroborated by contributing scientists around the world. We compiled these individual lists into one large dataset of nonnative species that occur in at least one country in the Americas. Then we searched the CABI ISC species pages to record where each species originated, in which countries it occurred, when it may have moved, and by what vectors it may have moved. We eliminated species that originated from outside of the Americas or that had an unclear origin (in particular, widespread marine species). Because many species occurred in multiple countries within a region, we also recorded origins and destinations by region: South America (Colombia to Chile), Central America (Guatemala to Panama), Caribbean (Bahamas to Trinidad and Tobago), and North America (Canada to Mexico). To facilitate analysis, we also grouped species by phylum for invertebrates and by class for vertebrates.

Mapping

To put the western hemisphere data into context, we plotted the total number of species that have invaded the western hemisphere from other countries in the western hemisphere (the Americas), but also from Australia and New Zealand, Asia, Europe, Middle East, and Africa. When the origin information was broad or not clear, we assigned them to a Not Specified category.

We used the R package circlize (Gu et al. 2022) in RStudio (R Core Team 2023) to create a circular diagram to visualize the relative contribution of invasive species from different regions of the world to different regions of the Americas.

To visualize patterns in the origin and end movement of invasive species, we constructed webs of species movement using the R package bipartite version 2.19 (Dormann et al. 2008) in RStudio (R Core Team 2023). We split the data into the levels ‘region of origin’ and ‘receiving region’ using the following regions: (1) North America, (2) South America, (3) Central America, and (4) Caribbean. We also selected four countries as case studies, the US, Cuba, Costa Rica, and Brazil, to highlight the number of taxa that they sent to other countries. The purpose of these webs was to visually characterize the strength of those exchanges. Thicker bars that connect the two levels represent more documented taxa that were sent to the corresponding region. We also mapped the origin and invasion patterns of genera represented by more than one species in the database (*Pomacea*, *Anolis*, *Eleutherodactylus*, *Cichlasoma*, *Lepomis*, *Poecilia*, and *Pterygoplichthys*) using the R package ggplot2 version 3.4.4 (Wickham 2016). These spatial analyses were performed in RStudio (R Core Team 2023).

Statistical analyses

To evaluate whether the regions differed in the number of nonindigenous species that arrived within their borders, we compared the numbers of these species that entered the four different regions to a null hypothesis of equal movement among regions with chi-square tests. To evaluate whether some taxa were more likely to move, we compared the number of nonindigenous species among the different taxa in the database to a null hypothesis of equal movement among taxa with a chi-square test. A country or region with a lot of international trade or traffic might be expected to both import and export more species, so we compared the total number of species exported from one region to the next (e.g., from North America to Central America) with its reciprocal (e.g., from Central America to North America) with linear regression. However, species at different taxonomic levels might move using different vectors, so we repeated this regression analysis using the different phyla or classes for which there were sufficient numbers of species in a taxonomic category for analysis. These analyses were performed in RStudio (R Core Team 2023).

To understand how the regions differed in the types of species that they were receiving, we used nonmetric multidimensional scaling (nMDS) ordination of fourth root-transformed variables that represented the counts of species in each taxonomic group in each country of the Americas. This ordination was based on a resemblance matrix of Euclidean distances between countries (Clarke and Gorley 2006). Then we coded the countries by region and we determined whether regions differed in the taxonomic groups introduced with analysis of similarities (ANOSIM), a nonparametric analysis that compared the regions using a similarity matrix (Clarke 1993). To evaluate whether regions differed, ANOSIM ranked the similarities between regions and produced a global R value, which can range from <0 (similarity within regions is greater than between regions) to 0 (similarities within and between regions are equal) to 1.0 (regions are dissimilar). We conducted the nMDS and ANOSIM analyses with PRIMER version 6 (Clarke and Gorley 2006).

Finally, we examined whether trade might have affected species movement. We compared the number of species that moved by different vectors to a null hypothesis of equal movement by all vector types using chi-square tests. To evaluate the potential effect of trade activity on species movement, we collected the national Gross Domestic Product (GDP) from The World Bank (2023) for each country in the Americas with reported values, as not every country and territory in the Americas had a reported GDP. To test for a relationship between trade activity and invasive species transport, each country's GDP (if reported) was compared to the number of species exported by that country via linear regression analysis. This analysis was performed in R 4.0.5 (R Core Team 2023).

Results

Where did species move?

For species coming into North America, South America, Central America, and the Caribbean, Asia contributed the greatest number of imported species (348 invasions). However, nearly as many of the species imported into these western regions originated within the Americas (285 invasions, Fig. 1). These imports were greater than the numbers of exotic species originating from Africa (128), Europe

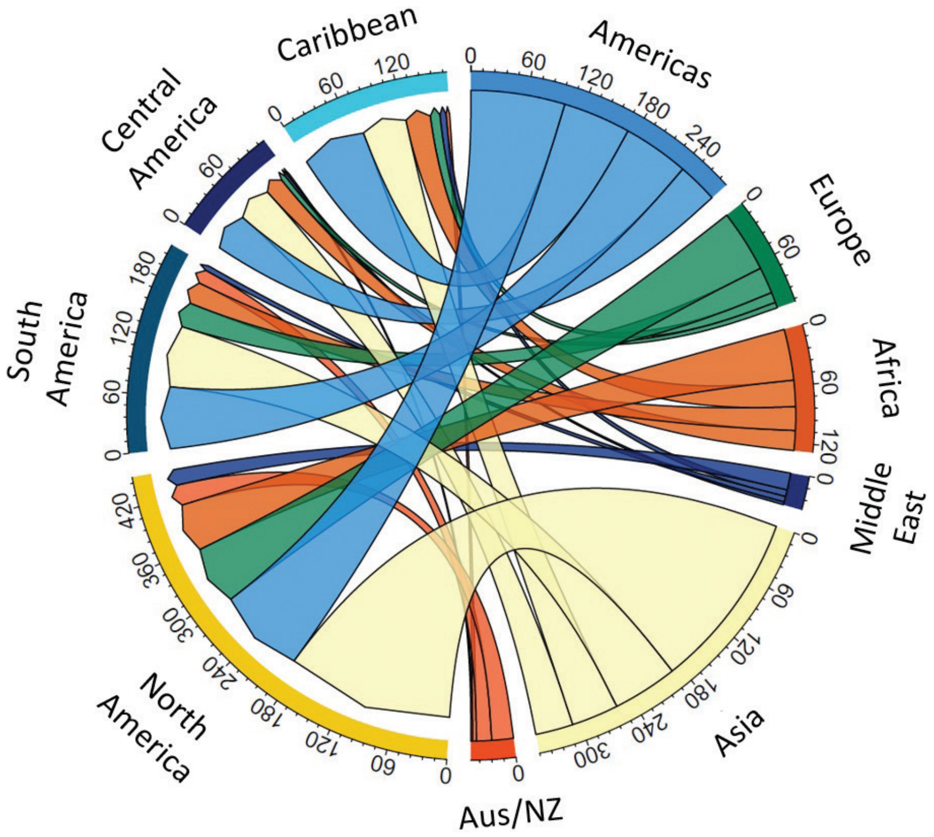

Figure 1. Origin of exotic animal species found in four regions of the Americas. North America has been the largest recipient of exotic species (453), followed by South America (214), Caribbean (172), and Central America (115).

(111), Australia/New Zealand (48) or the Middle East (34). However, the trend in species' origin differed for invertebrates and vertebrates. Proportionately more vertebrate invasions originated within the Americas, whereas relatively more invertebrate invasions originated outside of the Americas (Fig. 2). The records of these introductions ranged from the years 1800 to 2020 and many species were introduced multiple times. The minimum difference between the first and last introduction record was one year and the maximum was 204 (mean = 52.6 ± 39.0 years).

Across all taxa, the number of species that were exported from a region was comparable to the number of species imported to that region (Regression: $r^2 = 0.57$, $F_{1,4} = 7.76$, $p = 0.05$, Fig. 3). However, this symmetrical relationship broke down for each of the individual taxonomic groups analyzed (Regression: Arthropods: $r^2 = 0.40$, $F_{1,4} = 4.38$, p = 0.1; Molluscs: $r^2 = 0.03$, $F_{1,4} = 0.13$, $p = 0.74$, Fish: $r^2 = 0.45$, $F_{1,4} = 5.02$, $p = 0.14$, Herps: $r^2 = 0.18$, $F_{1,4} = 0.90$, $p = 0.40$; Fig. 4).

Did all regions of the Americas contribute equally to this trade?

All regions traded species, but regions differed in the number of species that they contributed to the database (Chi-square: $X^2 = 228.33$, df = 3, $p = 2.7\times10^{-12}$). North and South America contributed the largest number of exported species, and the number of species in the database that originated in these two regions were roughly equal (116 vs. 112). Compared to the large continents to the north and south, the Caribbean exported approximately half the number of species (52) and Central America approximately one quarter (27) of the number of species exported by

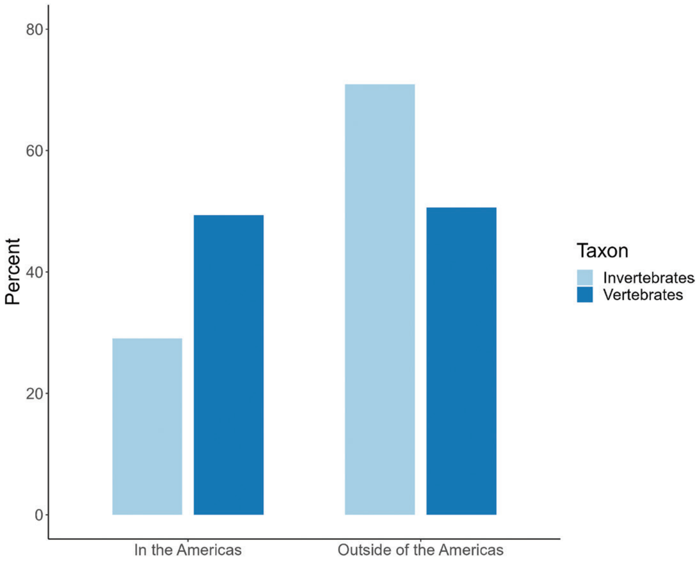

Figure 2. The proportion of invertebrate and vertebrate species indigenous to one of the countries in the Americas or indigenous to a country outside of the Americas (Eurasia, Africa, or Oceania) that have moved into a country within the Americas outside of their original range.

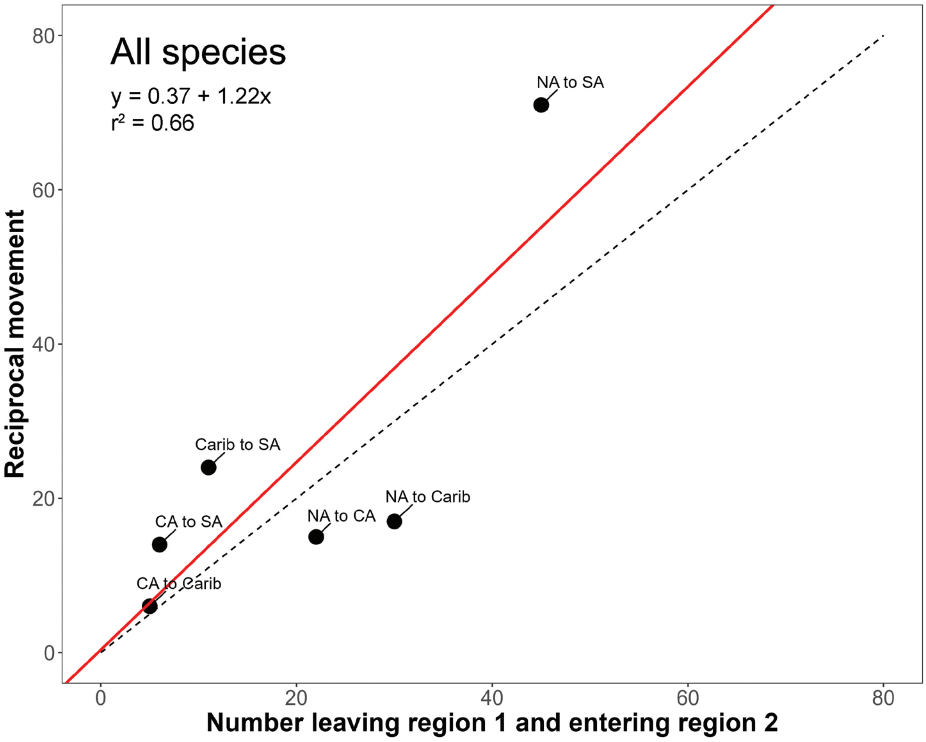

Figure 3. Reciprocal swaps of animal species in aggregate. Solid line is the regression line, whereas the dotted line is the 1:1 line, indicating equal numbers of species swapped between regions (North America - NA, South America - SA, Central America - CA, Caribbean - Carib).

their neighboring regions (Fig. 5). Many of the Caribbean exports occurred between Caribbean Islands.

Of the four countries highlighted in our analysis, all exported species widely, sending species to 24–44 countries. This export was lopsided; for example, the US sent the largest number of species to the rest of North America (Canada and Mexico), but it was the largest receiver of species from Cuba, Costa Rica, and Brazil by far (Fig. 6).

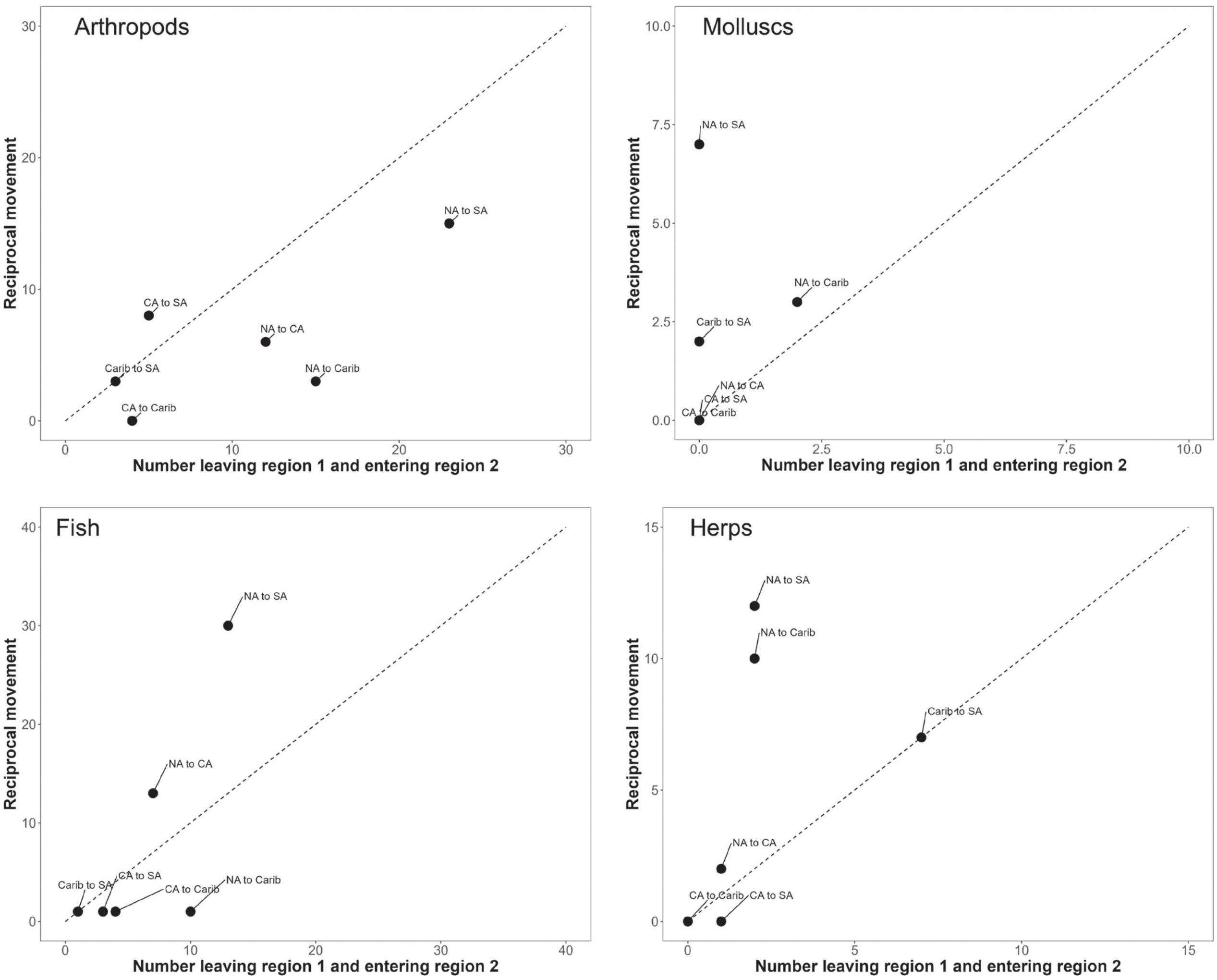

Figure 4. Swaps of individual taxa were not reciprocal. Dotted line is the 1:1 line, indicating equal numbers of species swapped between regions (North America - NA, South America - SA, Central America - CA, Caribbean - Carib).

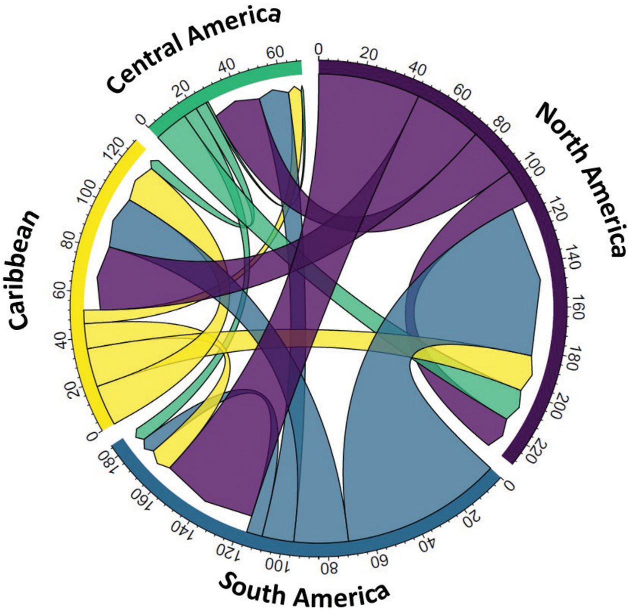

Figure 5. Exchange of animal species between North America, South America, Central America, and the Caribbean. The largest exchanges were between North and South America, with South America being the highest exporter of exotic species.

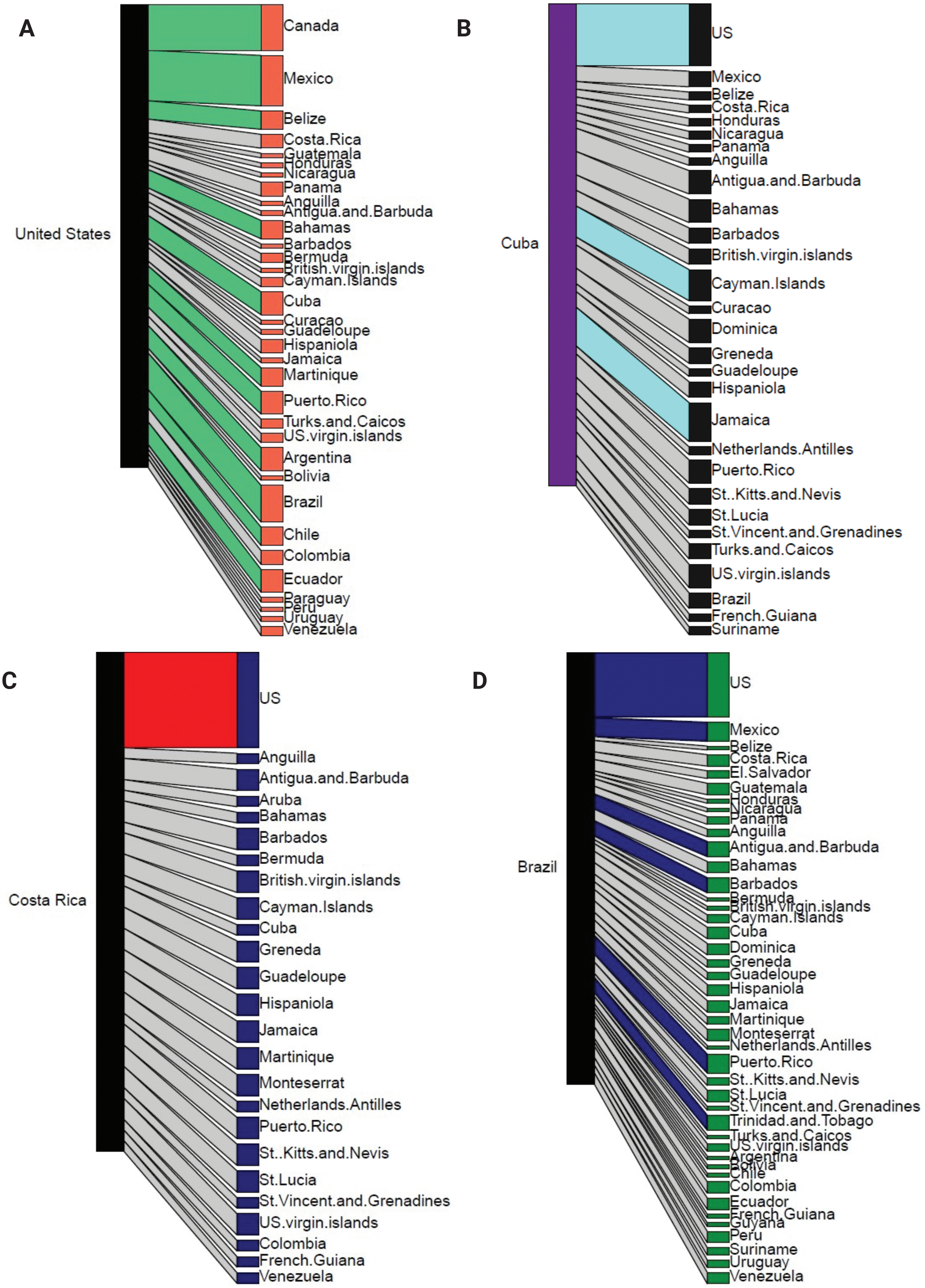

Figure 6. Largest animal species-exporting countries in each of our four major regions **A** United States **B** Cuba **C** Costa Rica, and **D** Brazil Bars with color represent interactions with at least five species sent to the receiving country. All three of the non-North American countries sent the most species to the United States.

Were all taxa equally represented in the movements between regions?

The taxa differed in their representation in the database (Chi-square: $X^2 = 2410.4$, df = 8, $p = 5.9\times10^{-48}$) with a greater number of arthropods and fish than other taxa in the countries' nonindigenous species lists (Fig. 7). Invasions into North America were dominated by arthropods, fish, and reptiles, but arthropods comprised a majority of the invasions into the other three regions (Fig. 7), producing a different taxonomic composition of the nonindigenous species that moved between regions within the Americas (nMDS: stress = 0.11, ANOSIM: global $r = 0.348$, $p = 0.001$, all pairwise comparisons between regions $p < 0.045$, Fig. 8).

The largest number of arthropod exchanges occurred between North America and South America, although both regions contributed large numbers of species to Central America and the Caribbean (Fig. 9a). For molluscs, on the other hand, South American species dominated the exchanges between regions and many of these species, often *Pomacea* species, were introduced to North America or the Caribbean (Figs 9b, 10a). No mollusc species were recorded as moving into or out of Central America.

For vertebrates, the directions of species' movements also were variable. A disproportionate number of the fish species that moved between regions originated in North America, which also received the most fish. Most of these contributions were from either Central America or South America (Fig. 9c), but the patterns differed among genera. Both *Cichlasoma* (Fig. 10b) and *Pterygoplichthys* (Fig. 10c) moved into North America, but *Cichlasoma* species originated in Central America

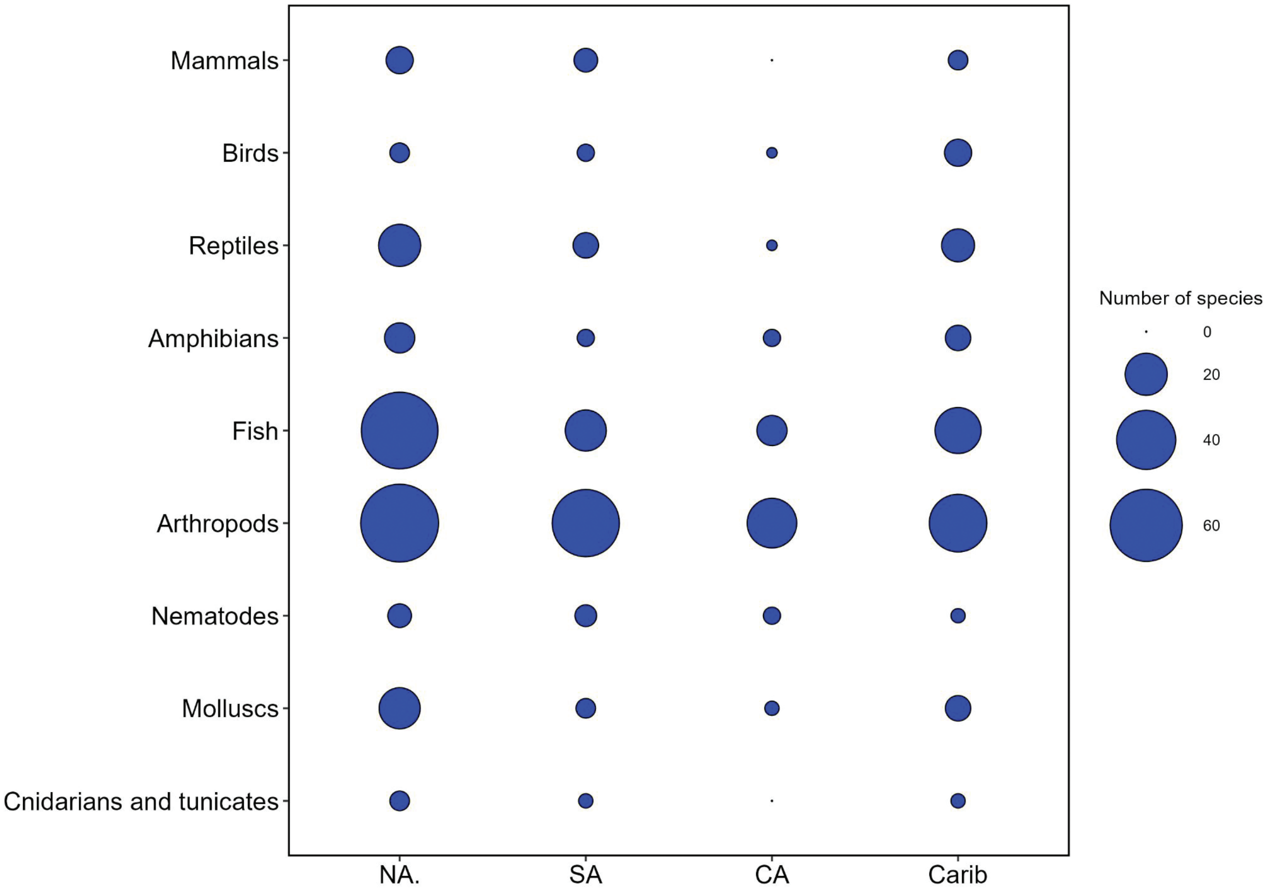

Figure 7. Invasion abundance of different animal taxa into each of the four regions of the Americas (North America - NA, South America - SA, Central America - CA, Caribbean - Carib).

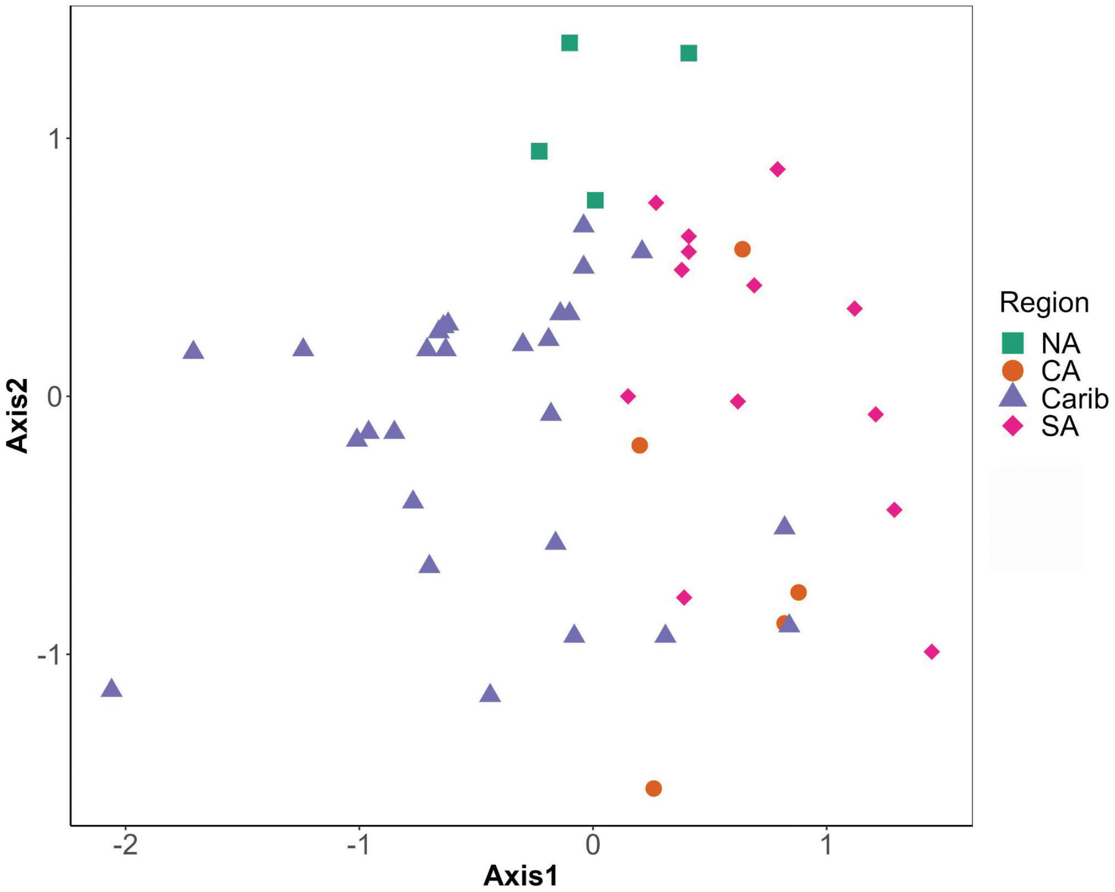

Figure 8. nMDS plot of the differences in taxon composition of invading animal species in different regions of the Americas (North America - NA, South America - SA, Central America - CA, Caribbean - Carib).

and *Pterygoplichthys* species originated in South America. These aquarium trade species were exchanged for North American *Lepomis* species (Fig. 10d), which invaded all three regions south of North America. The tiny *Poecilia* species (Fig. 10e) were exchanged in all possible directions.

In contrast, the largest number of amphibian and reptile species that moved between regions originated from Caribbean islands (Fig. 9d). Most of these species' movements were to other Caribbean islands, Central America, or South America. In particular, *Eleutherodactylus* tree frogs (Fig. 10f) moved from Cuba and Puerto Rico to other Caribbean islands or to the other three regions. The pattern was similar for *Anolis* lizards (Fig. 10g), but these species were exported from a greater diversity of Caribbean islands. Relatively few birds and mammals occurred in the database. The largest number of birds moved from South America to North America and the Caribbean, although species also moved between these two regions (Fig. 9E). Most of the mammals moved between North and South America, although a few species moved from South America into the Caribbean (Fig. 9F).

What vectors were important in the movement of species?

Vectors differed in the number of species that they transported, both for different regions (Chi-square: $X^2 = 70.8$, df = 21, $p = 2.63\times10^{-7}$) and for different taxa (Chi-square: $X^2 = 190.0$, df = 64, $p = 2.02\times10^{-14}$). For North America, the most important vector moving species into the region was food production. Although this

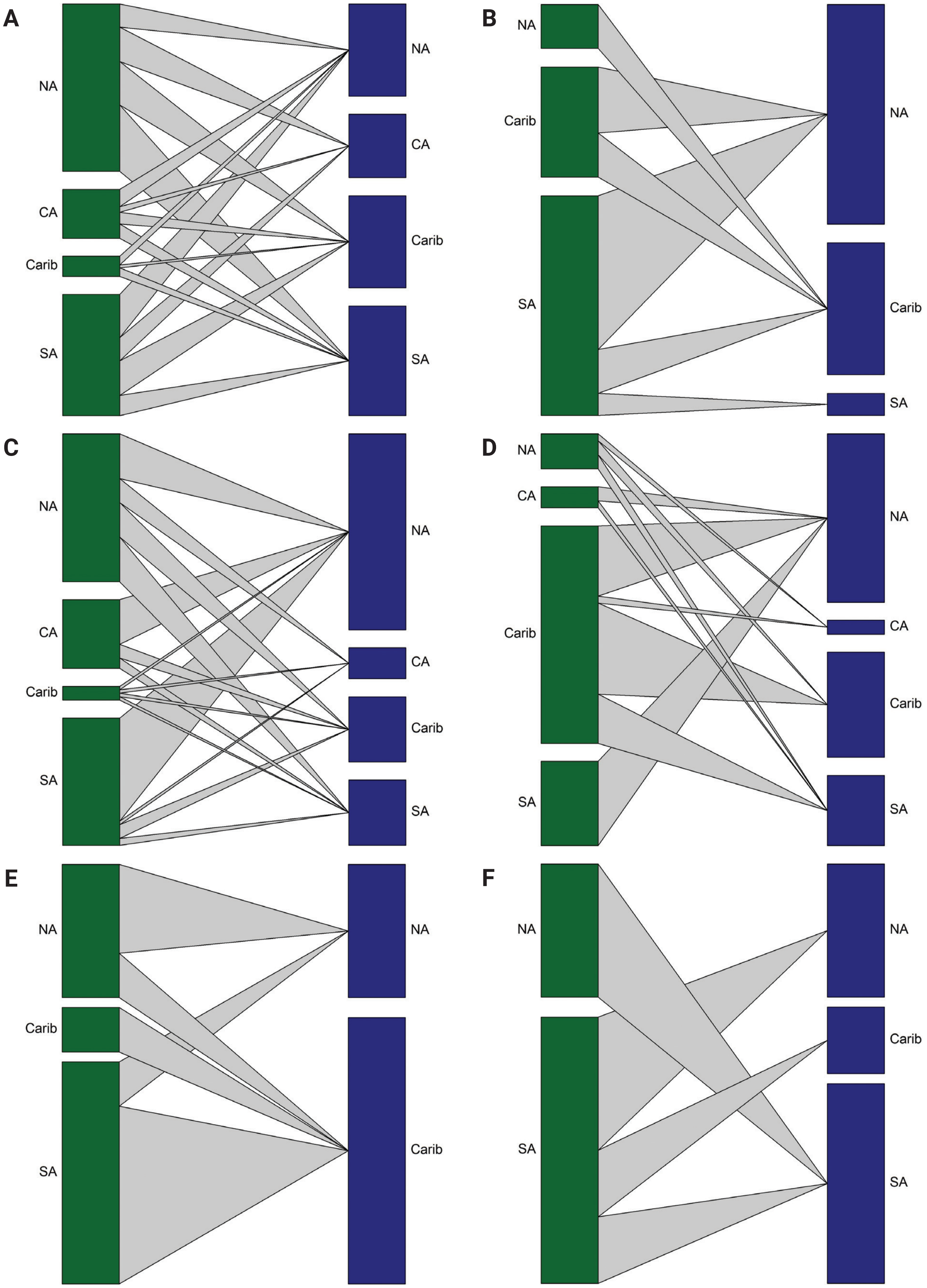

Figure 9. The recorded exchanges of **A** arthropods (n = 79) **B** molluscs (n = 19) **C** fish (n = 72) **D** reptiles and amphibians (n = 34) **E** birds (n = 7), and **F** mammals (n = 10) between regions of the Americas. Green bars show the regions that exported the taxa, whereas blue bars show the region that imported the taxa (North America - NA, South America - SA, Central America - CA, Caribbean - Carib).

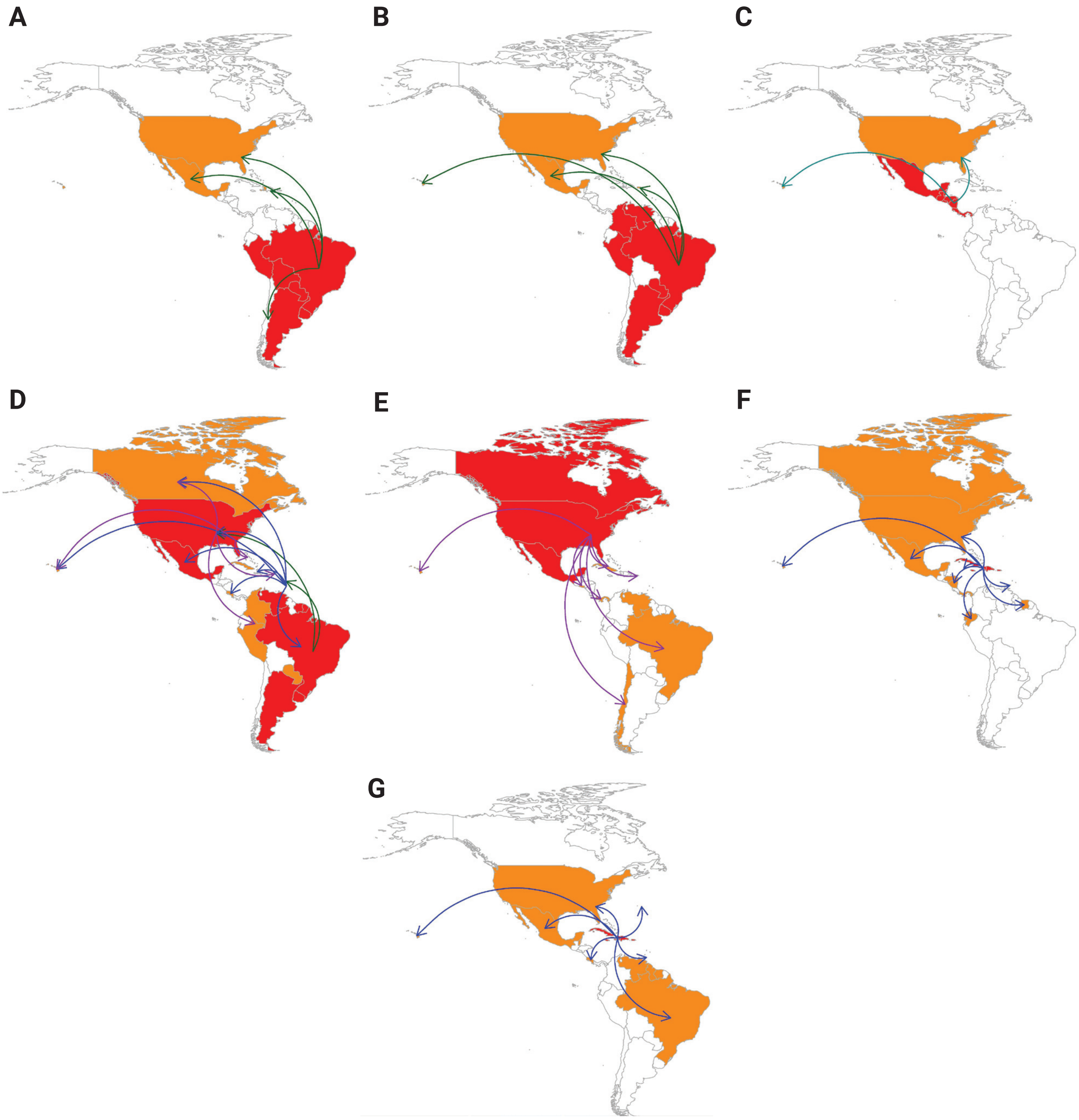

Figure 10. The American exchanges of animal genera that were represented by more than two species in the database. For molluscs, only one genus included more than two species: **A** *Pomacea* (n = 6). For fish, four genera included more than two species: **B** *Pterygoplichthys* (n = 3) **C** *Cichlasoma* (n = 6) **D** *Poecilia* (n = 3), and **E** *Lepomis* (n = 4). Amphibians and reptiles were each represented by one genus only: **F** *Eleutherodactylus* (n = 3) and **G** *Anolis* (n = 11). Areas colored red represent native ranges, whereas areas colored orange represent introduced ranges with arrows showing the direction of movement. Arrow color represents region of origin (green = South America, purple = North America, blue = Caribbean, teal = Central America).

vector also was important for species' movement into Central and South America, the pet and ornamental species trade moved more species into these regions. In the Caribbean, the pet and ornamental species trade also moved a lot of species, but many species also moved by hitchhiking (Fig. 11a).

The importance of different vectors also varied greatly among taxa (Fig. 11b). Food production and hitchhiking were particularly important for many invertebrates (arthropods, nematodes and other worms, and marine invertebrates), but only for

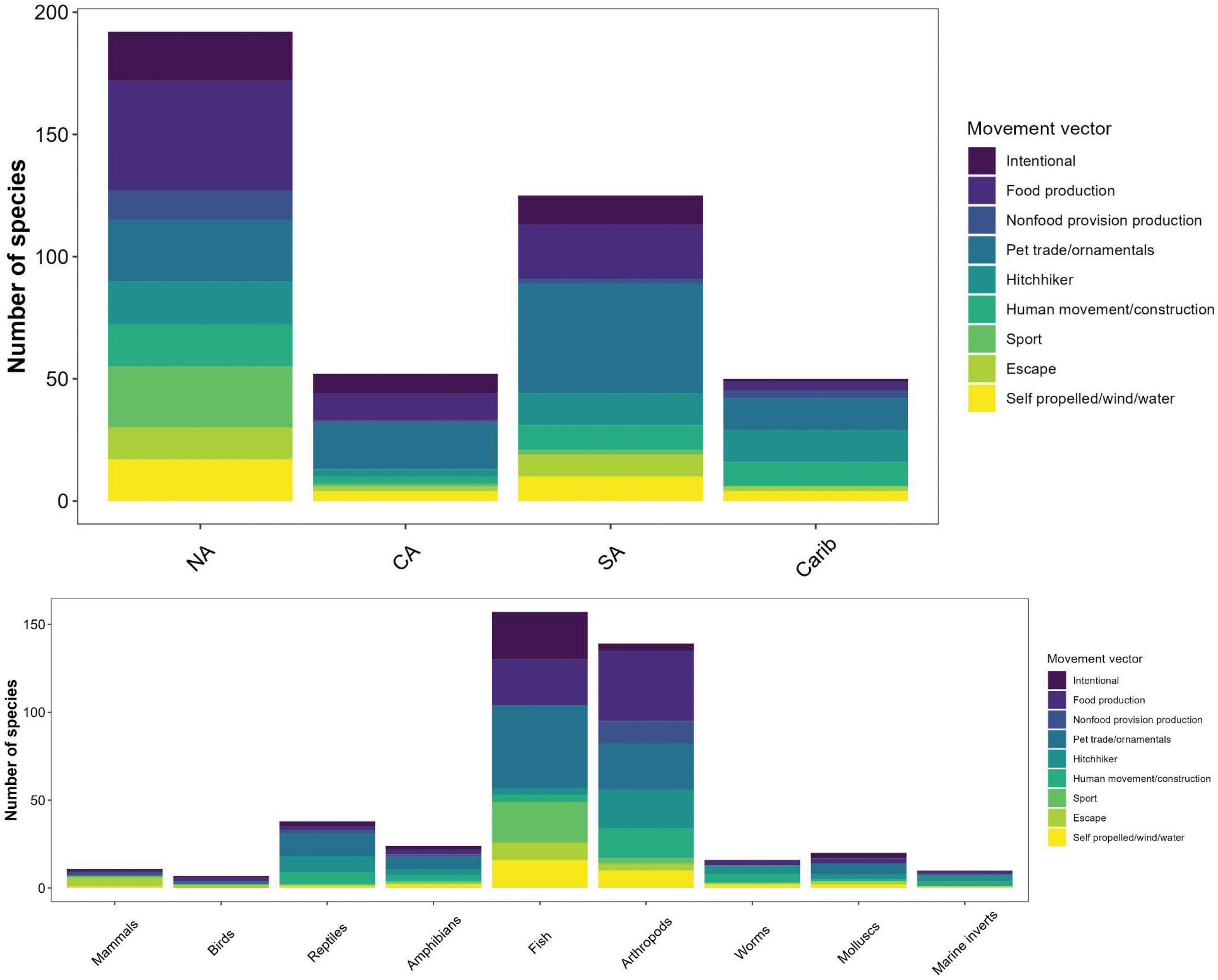

Figure 11. Importance of different transport vectors in moving animal species into the four regions of the Americas (a) and in moving different taxa among regions (b) (North America - NA, South America - SA, Central America - CA, Caribbean - Carib).

some vertebrates (some birds and fish). However, the pet or ornamental species trade was an important vector in movement for both invertebrates (arthropods and molluscs) and vertebrates (birds, reptiles, amphibians, and fish). Escape from confinement in ponds, gardens, or zoos also was an important vector for many vertebrates (mammals and birds), as was intentional release for ornament or sport (fish).

Did GDP predict species exports?

For all countries that reported GDP, this symbol of economic activity significantly predicted the number of native species that have been moved from one country to another within the Americas (Regression: $r^2 = 0.51$, $F_{1,39} = 42.25$, $p = 1.05\times10^{-7}$). Countries with a higher GDP exported more species (Fig. 12).

Discussion

This study suggests that species have been swapped extensively among countries in the western hemisphere, particularly between countries in close proximity (e.g., Cuba and Jamaica) or with strong trade ties (e.g., the US and Brazil)

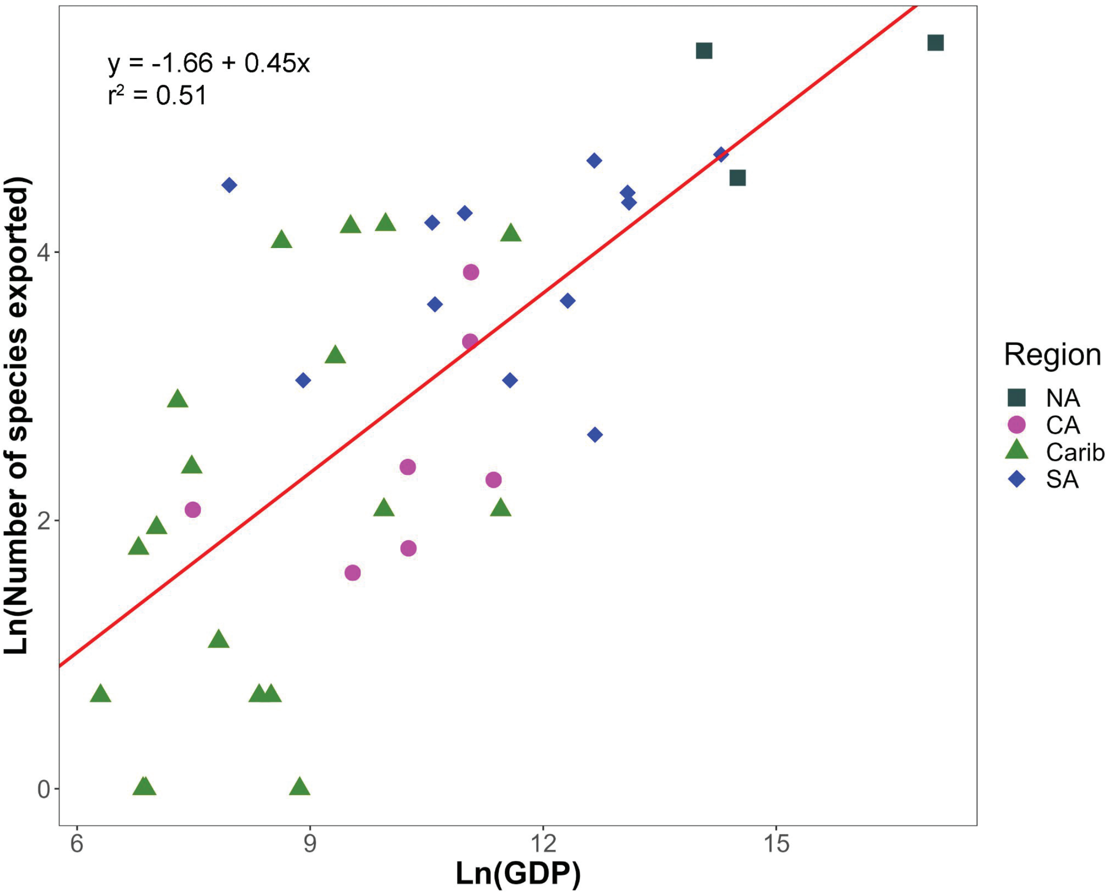

Figure 12. The relationship between countries' GDP and the number of animal species exports. Grey squares labeled "NA" represent North America, purple circles labeled "CA" represent Central America, green triangles labeled "C" represent the Caribbean, and blue diamonds labeled "SA" represent South America. The line is a regression line, with $r^2 = 0.51$.

(World Bank 2023), or both (e.g., the US and Cuba in the past; Deere 2017). Furthermore, it is highly likely that the colonizing species recorded in the CABI database are a fraction of the true problem and that the recorded colonization dates underestimate how long many of these species have been moving. Using archeological evidence, Kemp et al. (2020) recorded invasions dating back to the pre-Columbian era, long before most species' transport was recorded in the literature. Because some species may have moved prior to written records, some species that have been considered endemic in their current location may not be at all. For example, the Puerto Rican hutia, *Isolobodon portoricensis* (Allen 1916), originated in Hispaniola rather than Puerto Rico, but was imported for food in the pre-Columbian era (Rivera-Collazo 2015; Kemp et al. 2020). Missing or inaccurate records due to the antiquity of some introductions or to variation in record keeping efficiency may have contributed to the high variation in several of the analyses, such as the low r value in the nMDS analysis. Despite the limitations of the database, we can make a strong case for significant transplantation of species in the western hemisphere, including what could be considered reciprocal and perhaps repeated exchanges. For example, the US is now home to several Cuban herps (e.g., Cuban tree frogs, *Osteopilus septentrionalis* Duméril & Bibron, 1841, Cuban anoles, *Anolis sagrei* Duméril & Bibron, 1837, and northern curly-tailed lizards, *Leiocephalus carinatus* Gray, 1827), whereas Cuba hosts amphibian and fish species that are native to the US (American bullfrogs, *Lithobates catesbeianus* Shaw, 1802, bluegill sunfish, *Lepomis macrochirus* Rafinesque, 1819, and largemouth bass, *Micropterus salmoides* Lacépède, 1802). Some of these introductions may have occurred multiple times, possibly increasing the genetic diversity and persistence of the new populations (Garnas et al. 2016). According to the dataset, largemouth bass were introduced to Brazil in 1900–1924, to Cuba in 1928, and to several countries in the Caribbean (Dominican Republic, Puerto Rico), Central America (El Salvador, Guatemala, Honduras, Panama), and South America (Argentina) in the 1940–50s. Therefore, this particular species moved between the US and other regions of the Americas for decades and it is highly likely that exchanges continue between trading partners in the Americas, albeit perhaps more commonly with agricultural hitchhikers or ornamental species rather than species used for sport.

Why are the species moving?

While recognition of the problem is an important goal on its own, investigation of the vectors of transport point to possible avenues for reducing the problem. For North, Central, and South America, some of the most common exports were associated with food, sport, and ornamental trade, such as intentional transport for use in aquaculture/sport fishing/hunting or unintentional transport as hitchhikers with plants. On the other hand, for Central America, South America, and the Caribbean, transport of species as pets or for ornamental uses (or as ornamental hitchhikers) were the most common types of species movement. These vectors have been associated with exotic species' movement globally (Mack and Lonsdale 2001; Jeschke and Strayer 2006; Saul et al. 2017; Turbelin et al. 2017; Chan et al. 2019; Gippet and Bertelsmeier 2021) and the taxa associated with these vectors were predictable. For example, arthropods were commonly transported with food and with ornamental plants, whereas vertebrates, like fish, amphibians, reptiles, and birds, often were transported as pets or with ornamental plants. Furthermore, the problem of ornamental and pet transport has only increased with the development of online markets (Olden et al. 2021). Hitchhiking species may be traveling on other organisms or in packing material through either air or oceanic shipping (Early et al. 2015; Turner et al. 2021), but they also may be traveling with domestic air travel (Early et al. 2015; Turner et al. 2021), all of which are projected to increase over time (Tatem 2009; Sardain et al. 2019; Hulme 2021). As a result, gross domestic product (as a proxy for export activity) appears to be a good predictor of species exports to countries to which they are not native.

The effect of global trade and travel on species transport may be a long story. Essl et al. (2011) suggest that, in Europe at least, socioeconomic status in the early 1900s better predicts the establishment of many invasive species than current economic health, a phenomenon that they describe as "invasion debt". To establish a population, invasive species must arrive in the new area, but they also must colonize it, often with multiple waves of propagules. In the Caribbean, both the current economic status of the islands and historical trade may play a major role in the introduction and establishment of exotic species. For example, many smaller, less wealthy Caribbean islands have only one introduced gecko species, compared with larger, more economically well-off islands such as the Bahamas, which have six introduced gecko species (and 14 records of attempted introduction). Cuba, the largest island in the Caribbean, has eight introduced gecko species (with 30 records of introductions). All records of gecko introductions in Cuba occur prior to the US trade embargo, which began in 1962 and likely has had an impact on introductions through the strict trade sanctions (Perella and Behm 2020).

Why is this transport a problem?

Of the 25 biodiversity hotspots identified by Myers et al. (2000), sixteen are found in the tropics globally, with almost all tropical islands falling into one of the hotspots. Of these, eight fall into the four major regions of this study: North America, South America, Central America, and the Caribbean. Central America falls within the Mesoamerican hotspot and the Caribbean islands (and southern Florida) in the Caribbean hotspot; there is one additional hotspot in North America and five in South America (Myers et al. 2000). These hotspot regions are important not only for their overall biodiversity, but also for their high levels of endemism, especially on islands. Tropical rainforest ecosystems, in particular, have high plant and vertebrate endemism (Myers et al. 2000). Because of the restricted range of their endemic species, Caribbean islands and tropical rainforests are likely to be more vulnerable to the effects of exotic species (Bellard et al. 2017; Moser et al. 2018; Dueñas et al. 2021). The introduction of exotic species into these hotspots can negatively impact the biodiversity found there, threatening native species with habitat degradation, competition for resources, predation, novel parasites, and modified ecosystem properties (Vitousek et al. 1997; Mack et al. 2000; Mooney and Cleland 2001), although not all invasions produce negative effects (Gurevitch and Padilla 2004; Florencio et al. 2019). For example, Perella and Behm (2020) examined exotic gecko introductions in the Caribbean and found that introductions, both intentional and unintentional, have increased over time and that the range of the geographical origins of the invading species has increased. Once present, the exotic species that establish may have an advantage over native species, due to habitat competition and generalist lifestyles, allowing them to negatively impact native species and the ecosystem (Perella and Behm 2020).

The success and effect of invasions may depend on the condition of the habitat, including the level of disturbance and the presence of other exotic species (Florencio et al. 2019; Pyšek et al. 2020). For example, when comparing native and exotic reptile species on two Caribbean islands (St. Martin and St. Eustatius), Jesse et al. (2018) found that native species declined following a reduction in forested habitat, but both the abundance and richness of exotic species increased in human-impacted areas. Another example is the Cuban tree frog, *Osteopilus septentrionalis*, which presents a well-known example of the effects of an exotic species following its introduction to Florida. Initially introduced in 1951, the Cuban tree frog has many traits of successful exotic species; it has a short generation time and high fecundity, habitat flexibility, and can feed on a diversity of prey species (Meshaka 2001; Glorioso et al. 2012), resulting in a range expansion to cover most of the state (Schwartz 1952; Glorioso et al. 2012). This species' tadpoles may reduce native frog populations by competitively reducing native tadpole growth (Smith 2005), by directly preying on native frogs (Wyatt and Forys 2004), and by interfering with the soundscape of frog calls in Florida (Tennessen et al. 2013), but they also have impacted native populations through the introduction of non-native parasites. Of the nine parasitic species identified in Cuban tree frogs necropsied from Tampa, FL, at least one was from its native range, with several acquired parasites from Floridian fauna. However, the parasite native to Cuba (*Oswaldocruzia lenteixeirai* Perez Vigueras, 1938) also was recorded in native Florida herpetofauna, suggesting that it now also is an introduced species (Ortega et al. 2015). These non-native Cuban tree frogs also have been identified as possible intermediate hosts of *Angiostrongylus cantonensis* Chen 1935, the rat lungworm nematode parasite, after a frog was found with larvae in Volusia County, FL (Chase et al. 2022). These invasive frog hosts, especially ones that are so abundant in residential areas, could serve as carriers for transmission of the parasitic nematode. Given the wide range of potential effects of exotic species, from parasite transport to ecosystem alteration, some authors have likened the spread of exotic species to agents of global change (e.g., Vitousek et al. 1997; Mack et al. 2000; Ricciardi 2007).

Species invasions clearly are a world-wide problem, only increasing with global travel and transport (Hulme 2009; Sardain et al. 2019; Olden et al. 2021; Turner et al. 2021). The numbers of individuals and species documented in trade activity and travel are staggeringly high; Turner et al. (2021) documented almost two million insects from over 8,000 species transported through ports between the US, the UK, Europe, southeast Asia, and Oceania over a two-decade period. Some species were intercepted at ports hundreds of times. Although many studies have documented transport of species from distant countries and continents (e.g. Olden et al. 2021; Turner et al. 2021), relatively few have highlighted the reciprocal nature of species translocations. Ferus et al. (2015) analyzed the potential of reciprocal exchange of plant species with trade between Romania and Slovakia and concluded that this potential was high, although many of the potential invaders actually originated in North America. Turner et al. (2021) showed that the composition of border interceptions of potential invaders was most similar between pairs of geographically close countries, such as between Australia and New Zealand and between Japan and South Korea. Clearly, reductions in species transport from anywhere in the world are critical for protecting biodiversity globally, but perhaps this exchange between nearby trading partners is particularly frequent. Movement of species with trade and travel among near neighbors, such as in the western hemisphere, is likely an important contributor to the homogenization of the world's biodiversity (McKinney and Lockwood 1999; Olden and Poff 2003; Florencio et al. 2019). Furthermore, the threat of exotic species to the Neotropics, in particular, has been underestimated (Rodríguez 2001) and understudied (Florencio et al. 2019). Early et al. (2015) suggested that increases in air travel and land conversion for agriculture together increase the likelihood of species invasion in countries with lower economic development, potentially endangering biodiversity hotspots in Central and South America—and, undoubtedly, the Caribbean Islands as well. We hope that this study will help to increase awareness of the reciprocal nature of the problem in the Americas and the ability to prevent and respond to potential future invasive species introductions.

Acknowledgements

We would like to thank the multitudes of researchers contributing research to the exotic species literature and to the CABI datasets. We also would like to thank Janardan Mainali for work in starting up the project.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

No funding was reported.

Author contributions

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Data availability

Data used are available with open access from the Exotic Species Compendium in the CABI Digital Library (https://www.cabi.org/isc/about).

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A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 94: 311–331 (2024)
DOI: 10.3897/neobiota.94.123363

Research Article

Uprooting is a promising tool to control invasive giant ragweed and recover native diversity

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Abstract

Ambrosia trifida (giant ragweed) is an invasive species that causes habitat destruction and competitively excludes native plants in many parts of Europe and Asia. In this study, we evaluated the effects of selective cutting and uprooting on *A. trifida* and native plant diversity, as well as the effects of sowing the seeds of native annual, perennial and woody species after eradication. We hypothesised that: (i) selective uprooting will be more effective than cutting in controlling invasion by *A. trifida* because fewer propagules would be left behind, with no increase in the number of existing invasive propagules and (ii) sowing native seeds will increase invasion resistance and accelerate the recovery of native plant diversity. The eradication methods were applied in July 2022, seeds were sown in March 2023 and the response variables (i.e. importance values (%) of *A. trifida* and diversity index (H') of native species) were measured in September 2023. The importance values of *A. trifida* were lowest and diversity index of native species was highest in the uprooting treatment, supporting the first hypothesis. Sowing native seeds following invasion removal did not exert significant additional suppressive effects on invasion or increase native species diversity. These results reveal that selective uprooting is a promising tool to control *A. trifida* and to support the recovery of native diversity, while sowing native seeds does not improve the quality of restoration.

Key words: *Ambrosia trifida*, ecological restoration, eradication methods, diversity index, hand-pulling, native diversity, invasive plant management, selective cutting, selective uprooting

OPEN ACCESS

Academic editor: Graeme Bourdôt
Received: 18 March 2024
Accepted: 1 August 2024
Published: 23 August 2024

Citation: Byun C, Singh K, Hong SH, Lee J, Yoon TK, Kang H (2024) Uprooting is a promising tool to control invasive giant ragweed and recover native diversity. NeoBiota 94: 311–331. https://doi.org/10.3897/neobiota.94.123363

Introduction

Biological invasion is a major factor contributing to global ecological and socioeconomic instability (Simberloff et al. 2013; Roy et al. 2023). Invasive alien plants cause biodiversity loss through the competitive exclusion of native species, ultimately leading to the loss of valuable endemic species (Vilà et al. 2011; Jauregiberry et al. 2022; Adhikari et al. 2023). Invasive plants often disrupt ecosystem processes (Pearson et al. 2018), such as nutrient and water cycling (Vilà et al. 2011) and alter the soil composition (Castro-Díez et al. 2019). Several plant species in introduced areas produce allergens (Shackleton et al. 2016) and cause severe health issues. Shifts in biological integrity and natural ecosystem processes due to invasive plants affect the supply of ecosystem services, livelihoods and human well-being (Shackleton et al. 2018). Therefore, managing plant invasion is crucial for conserving biodiversity and sustaining natural ecosystems and human health. The management of invasive species includes the early identification of new invasive species, prevention of spread, eradication of existing invasive species and subsequent restoration initiatives (Pyšek and Richardson 2010).

Ambrosia trifida L., or giant ragweed, is an annual herb native to North America (Laforest et al. 2024). It is adapted to a wide range of habitats, including agricultural fields, roadsides and disturbed areas, contributing to its invasive success in Europe and East Asia (Abul-Fatih and Bazzaz 1979; Soltani et al. 2011; Chauvel et al. 2021). Its rapid growth, high seed production and tolerance to a variety of ecosystem disturbances and environmental conditions allow it to outcompete native plants and associated fauna (Regnier et al. 2016; Dong et al. 2020a; Chauvel et al. 2021; Wang et al. 2022; Xian et al. 2023; Chen et al. 2024). The plant was unintentionally introduced into the Republic of Korea in 1970, probably as a contaminant of agricultural equipment and/or crop seed. It has been considered an invasive species since 1999 (Park et al. 2012); initially, it was discovered near the demilitarised zone, in the central region of Korea (Lee et al. 2010). After it spread throughout the country, it was registered as an ecosystem-disturbing species (Lee et al. 2010; Yin et al. 2010; Kim 2017; Montagnani et al. 2017; Li et al. 2022). *Ambrosia trifida* has high reproductive ability and is difficult to remove once established. It grows quickly, attains heights of up to 5 m and forms high densities, greatly reducing the growth of native species and economic crops in cultivated areas. Pollen from flowers of *A. trifida* can also cause allergies (Ling et al. 2022). Therefore, the eradication of *A. trifida* and restoration of native diversity is of paramount socioeconomic and ecological importance.

A number of mechanical (physical), chemical and biological eradication methods for invasive plant species have been developed (Kettenring and Adams 2011; Weidlich et al. 2020), with variable outcomes in terms of invasion control (Pearson et al. 2016; Chenot et al. 2018; Courkamp et al. 2022) and recovery of native plant communities (Boxriker et al. 2022; Farmilo and Moxham 2023). The mechanical approach involves the physical removal of invasive species through cutting, mowing, uprooting, burning, mechanical harvest and other similar approaches. Except in cases where uprooting is applied, re-sprouting and habitat disturbance facilitate re-invasion and hinder the recovery of native communities (Schooler et al. 2010; Byun et al. 2020a; Nagy et al. 2022). Chemical application is the most frequently used method (Kettenring and Adams 2011) to eradicate specific plant species before and/or after germination. However, the application of herbicides, such as glyphosate, indaziflam, 2,4-2,4-dichlorophenoxyacetic acid and picloram, deters native vegetation and fauna (Carlson and Gorchov 2004; Robichaud and Rooney 2021; Donaldson and Germino 2022) and many nations banned their use to control weeds and invasive species (Peng et al. 2020; Pergl et al. 2020). Although the introduction of natural enemies, such as insects and pathogens (bacteria, fungi, viruses etc.), can hinder the germination, growth and spread of invasive plants (Clewley et al. 2012), this approach is logistically complex and not well investigated (David and Lake 2023; Shen et al. 2023). The mechanical method in which invasive species are uprooted is considered a new tool to control invasion (Kollmann et al. 2011) and to have multiple advantages compared with conventional methods, such as cutting because it does not leave any reproductive or regenerative parts of plants behind. This is important because this species can regrow from its roots or stem fragments after cutting or disturbance, which contributes to its resilience and ability to spread rapidly, making it a challenging invasive species to manage. However, selective uprooting can be labour-intensive and time-consuming and its efficiency is unclear, in part owing to the small number of relevant studies (Pickart et al. 1998a; Pickart et al. 1998b; Ussery and Krannitz 1998). Recent empirical studies, systematic reviews and meta-analyses clearly indicate that the use of control methods alone does not facilitate the recovery of native communities, suggesting that it is imperative to consider active restoration initiatives (Kettenring and Adams 2011; Adams et al. 2020; Singh and Byun 2023). For example, sowing native seeds can contribute to the recovery of native communities (Kettenring and Adams 2011).

Extensive research suggests that sowing native seeds after the removal of invasive species can establish native vegetation cover and reduce the probability that invasive species regrow and establish as a result of niche pre-emption and resource utilisation (Levine et al. 2004; Enloe et al. 2005; Sheley et al. 2006). Therefore, native seed addition following invasion control is essential for restoring native diversity (O'Donnell et al. 2016; Johnson et al. 2018). The presence of functionally similar and dissimilar species can provide clues about the potential of various native species to protect against invasion (Sheley and James 2010; Byun et al. 2013). However, Yannelli and colleagues reported that seed density (propagule pressure in ecological terms) is more effective than trait similarity with respect to invasion suppression (Yannelli et al. 2020). Therefore, it is critical to determine if sowing native seeds suppresses invasion and facilitates the recovery of native diversity, as well as the effects of species of different functional groups.

Ambrosia trifida is a noxious weed and its control has been a challenging task at introduced sites and in its native range. The suppression of *A. trifida* in agricultural fields by the application of various herbicides, growing cover crops and diversifying cropping systems is rarely successful (Kouame et al. 2023; Silva et al. 2023; Werle et al. 2023). Similarly, in natural ecosystems, such as grasslands, the impact of mechanical eradication methods on the spread of *A. trifida* varies and the approach is generally either ineffective or only slightly effective (Byun and Lee 2018; Park et al. 2020; Byun et al. 2020b; Byun 2023). Intense mowing management of *A. trifida* followed by sowing native seed suppressed invasion up to 77%; however, seeding did not amplify suppression or facilitate native recovery (Byun et al. 2020b). The competitive ability of *A. trifida* was not suppressed, even when grown with eight native species from three functional groups (Byun and Lee 2018). However, the effectiveness of selectively targeting only invasive species (*A. trifida* in this study) by cutting (removing aboveground plant parts) or uprooting (pulling out whole plant) has not been tested. In addition, the impact of sowing native seeds following the selective eradication of *A. trifida* on native diversity is not known.

This study, therefore, aimed to investigate the effectiveness of mechanical control, including selective uprooting and selective cutting, on the dominance of *A. trifida*. Although the eradication of invasive species can suppress their dominance to some extent, the recovery of native diversity requires additional assistance (e.g. through sowing seeds following invasive plant removal) (Enloe et al. 2005; Sheley et al. 2006; Kettenring and Adams 2011; Adams et al. 2020; Singh and Byun 2023). Sowing seeds of various functional groups can further protect against invasion through trait similarity (annuals), structural asymmetry (perennials) and diversity (three species). Therefore, we sowed seeds of nine native species from three functional groups (i.e. annual, perennial and woody species) after invasion removal to evaluate whether there are additional benefits in the suppression of

A. trifida invasion. We hypothesised that selective uprooting will be more effective than cutting in controlling invasion by *A. trifida*, because uprooting leaves fewer propagules behind and does not add to the number of existing propagules. We also hypothesised that sowing native seeds will protect against invasion and suppress *A. trifida* through limiting similarity with annuals and increasing diversity and will accelerate the recovery of native diversity.

Methods

Study site

Experimental plots were installed in July 2022 at two sites in Busan, Republic of Korea separated by 18 km. Site #1 was located at 1200-5 Daejeo 2-dong, Gangseo-gu (35°11'46"N, 128°58'02"E) and site #2 was located at 1211 Hadan-dong (35°05'29"N, 128°56'40"E) (Fig. 1). The sites represent a typical riparian habitat that was originally dominated by *A. trifida* (> 90% cover).

Removal of invasive *A. trifida* and sowing native seeds

We applied a split-plot design with main plots consisting of different eradication methods and subplots consisting of different seed mixtures for sowing. Based on the cover and distribution of *A. trifida*, two blocks at site 1 and four blocks at site 2 were established. Thus, a total of six blocks were prepared. Each block was 10 m × 10 m. Within each block at each site, three main plots measuring 2 m × 2 m were randomly developed and equidistant. All plots were placed 2 m inside the block and plots were situated with distance of 2 m from each other. Three plots represented three treatments (i.e. control (no action), selective cutting and selective uprooting). The layout of the main plots at the six blocks in two sites is shown in Suppl. material 1: fig. S2). Selective cutting and uprooting involved the removal of individuals of *A. trifida* only, with minimal disturbance to other vegetation. In the case of cutting, all *A. trifida* individuals were removed by manual cutting using secateurs (pruning shears) and all other plant individuals were left intact. Individuals of *A. trifida* were cut from the lowest part of the plant stem (right above the ground). For the uprooting treatment, the entire plant, including roots, was uprooted or pulled out from the plots. As *A. trifida* has a fibrous root system, manual uprooting was easy. Any disturbance to original native plant communities, such as touching them, was minimised. All removal treatments were applied on 20 July 2022 (i.e. shortly before blooming). Within each main plot of 2 m × 2 m, four subplots measuring 1 m × 1 m were prepared for four sub-treatments. Three subplots were sown with three different seed mixtures and the remaining unsown subplot was used as the control. In seed mixture 1 (SM1), the seeds of three annual species (*Lactuca indica*, *Elsholtzia splendens* and *Portulaca oleracea*) were sown at 200 viable seeds of each species per subplot (i.e. 600 seeds m^{-2} per subplot). A similar method was followed for sowing seed mixture 2 (SM2) including three non-woody perennials (*Phragmites australis*, *Pennisetum alopecuroides* and *Plantago asiatica*) and seed mixture 3 (SM3) with three woody perennials (*Lespedeza bicolor*, *L. juncea* and *Sorbaria sorbifolia*). Seeds in each mixture were broadcast very close to the ground. Each seeded subplot was watered with a 15 L plastic watering pot. Manual watering of each subplot was done only at this stage. The application of seed mixtures was completed between 3 and 4 March 2023.

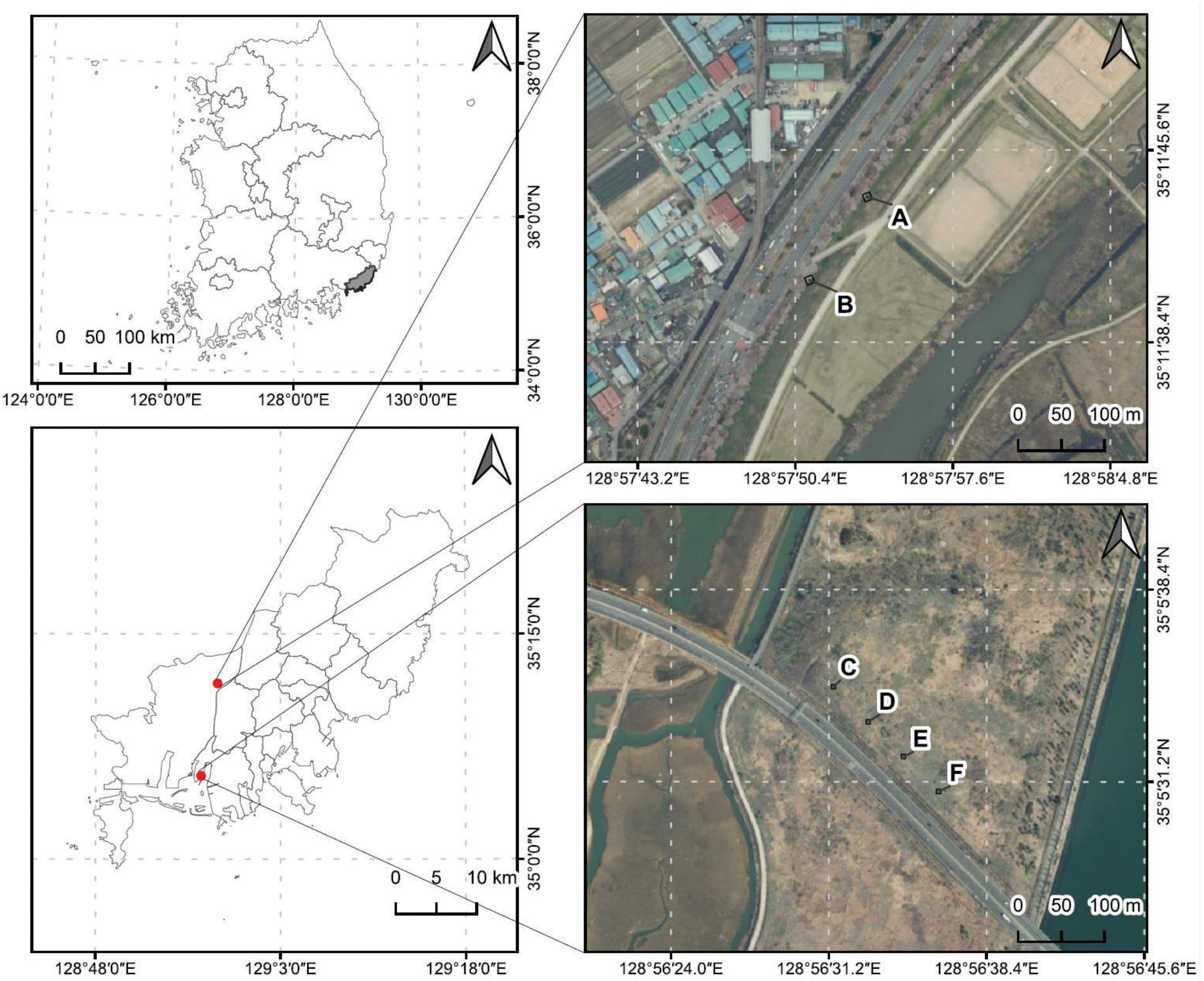

Figure 1. Map of the study sites and aerial view of blocks. Blocks A, B are located at site 1 and blocks C–F are located at site 2 in Busan, Republic of Korea.

Species selection and functional classification

Nine native species from three functional groups, annual (*Lactuca indica*, *Elsholtzia splendens* and *Portulaca oleracea*), non-woody perennials (*Phragmites australis*, *Pennisetum alopecuroides* and *Plantago asiatica*) and woody perennials (*Lespedeza bicolor*, *Lespedeza juncea* and *Sorbaria sorbifolia*) were identified for the current experiment. These native species were selected, based on their ability to suppress invasive plants in previous pot and field scale studies (Byun and Lee 2018; Byun et al. 2020b; Byun 2023), availability of their seeds in the market, their high germination rates, their distribution (native to S. Korea) and their ability to maximise functional diversity. The plant nomenclature used in this paper is the same as that used in the PLANT List (http://www.theplantlist.org), which lists accepted names and Flora of Korea Editorial Committee (2007).

Seed preparation

Seeds of native plants were purchased from authorised seed suppliers (in winter 2022). Seeds were obtained from multiple suppliers, because they could not be obtained from a single supplier. Seed suppliers included Aram Seeds (Seoul, Republic of Korea), Xplant (Seoul, Republic of Korea) and New Korea Farm (Seongnam, Republic of Korea) and others. Seed viability was standardised by applying the same number of viable seeds per species (600 seeds m^{-2}) to experimental units. To determine the percentage of viable seeds, a germination test was conducted in the laboratory. All seeds were cold-stratified (6 months) at 3 °C before the germination test, following standard methods (Lindig-Cisneros and Zedler 2001). Then, 100 seeds per species were placed on three Petri dishes with Whatman® No. 1 filter paper moistened with 6 ml of distilled water under fluorescent light. Only species with high germination rates (above 5%) were used for the field experiment.

Measurements and statistical analyses

In August 2023, the number of shoots, plant height and plant cover of all species (including *A. trifida*) in each treatment and control plot were measured. For the number of shoots, we counted all shoots of each species in each plot manually. To determine plant cover, the percentage of each species was estimated using reference frames representing 50% and 25% of the total plot area. The main response variable was the importance value index, calculated based on the abundance of *A. trifida*. The importance value index (IVI) is a crucial metric in ecological studies, particularly when assessing the performance and impact of an invasive species (Byun et al. 2020b). IVI integrates multiple factors to provide a comprehensive picture of a species' dominance and ecological role within a community. IVI values were calculated by summing the relative plant cover (%) and the relative shoot density (%). This holistic approach is essential for understanding how invasive species perform relative to native species. High IVI values for an invasive species indicate that it is not only widespread, but also occupies a significant portion of the community's resources. This can highlight an invasive species' potential to outcompete and displace native species, altering community structure and ecosystem functions (Kohli et al. 2004). Additional main variables were the Shannon–Wiener diversity index (H') of all species (Keylock 2005) in each quadrat and invasive seed yield. The raw diversity index datasheets are included in Suppl. materail 2. To test the re-invasion potential of *A. trifida*, seeds were harvested on 9 November 2023 (all individuals were fully mature) in each subplot (Ferreras and Galetto 2010; Leskovsek et al. 2012; Goplen et al. 2016). All seeds of only *A. trifida* within each quadrat were manually detached by hands. The seed yield was measured as an indicator of the potential for invasion in the future. Seeds were oven-dried for 48 hours at 80 °C before measuring their yields in each treatment. Given that invasive seed yield in subplots did not show any specific pattern, data are not presented here.

ANOVA was used to evaluate the effects of various treatments on response variables. A generalised linear mixed model (REML; *F*-test) was used to account for the random block design (Bolker et al. 2009). In the ANOVA analysis model, main factors of treatments (different eradication methods for Fig. 2. and different seed mixtures within each eradication method for Fig. 3) and the site factors were included along with the random block effect. The three main response variables were: (1) the importance of the value index, calculated, based on the abundance of *A. trifida*, (2) the Shannon–Wiener diversity index (H') of all species in each quadrat and (3) invasive seed yield (g, log-transformed).

The normality of residuals and homoscedasticity were evaluated, and the response variables were transformed when necessary. Amongst the main response variables,

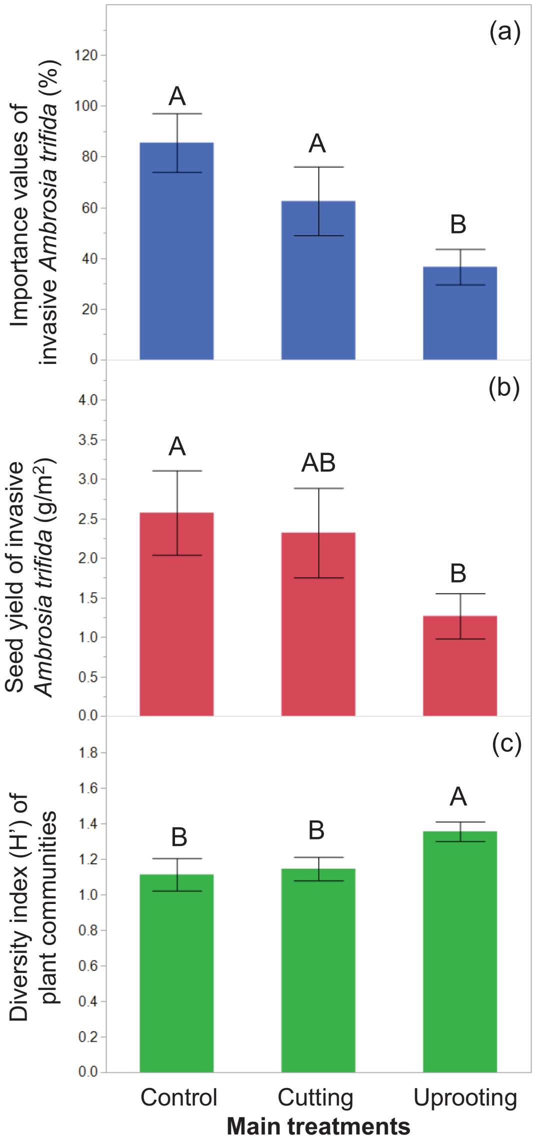

Figure 2. Effects of various eradication methods on *Ambrosia trifida* performance (importance values (**a**) and seed yield (**b**)) and plant community diversity (**c**). Error bars indicate standard errors. Bars with the same letters were not significantly different at $P < 0.05$.

only invasive seed yield (g) was log-transformed during analysis. When significant ($P < 0.05$) treatment effects were detected, *t*-tests were used to compare means of treatments. ANOVA and correlation analyses were conducted using JMP (SAS Institute Inc., Cary, NC, USA). Pearson correlation coefficients were calculated for *A. trifida* importance values and the diversity index (H') using data for 2023 in JMP.

Results

Response of invasive species *A. trifida* and native diversity to control measures

The effects of different control measures on the performance of *A. trifida* (Fig. 2a, b) and diversity of native plant communities (Fig. 2c), as well as the effects of sowing native seeds on invasion (Fig. 3a) and native diversity (Fig. 3b) were determined.

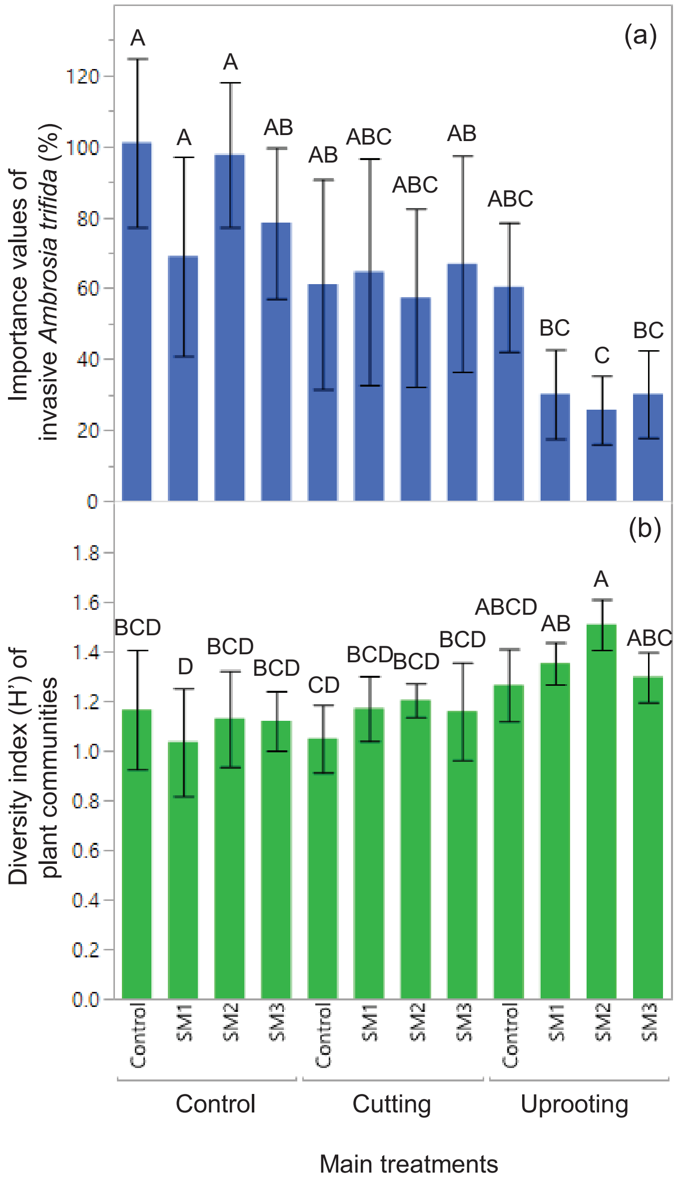

Figure 3. Effects of sub-treatments (sowing native seeds) within main treatments (eradication methods) on the importance values of *Ambrosia trifida* (**a**) and diversity of native plant communities (**b**). Error bars represent standard errors. Bars with the same letters were not significantly different. SM1 stands for seed mixture 1 (annuals), SM2 for non-woody perennials, SM3 for woody perennials.

The main eradication method had significant effects on abundance of *A. trifida* ($F_{2,50}$ = 9.5624; P = 0.0003) and the effect of the site factor was also significant ($F_{1,3}$ = 21.8288; P = 0.0167). The importance values for *A. trifida* were significantly lower in uprooting treatments than in control and cutting treatments (Fig. 2a). In addition, site 2 had much more abundant *A. trifida* populations than site 1 (as site factor). The seed yield of *A. trifida* was marginally affected by the control method ($F_{2,31}$ = 3.2787; P = 0.0511) (Fig. 2b). Similarly, the main eradication method had significant effects on native diversity ($F_{2,53}$ = 8.9271; P = 0.0005), but the effect of the site factor was not significant ($F_{1,3}$ = 7.2109; P = 0.0747). Uprooting significantly increased native diversity; however, cutting did not improve diversity over that in the control (Fig. 2c).

Response of *A. trifida* and native diversity to sowing native seeds following invasion control

Sowing native seeds did not have significant effects on the *A. trifida* importance value ($F_{9,41}$ = 0.7458; P = 0.6653), while the main eradication treatments had significant effects ($F_{2,41}$ = 9.2215; P = 0.0005) and the effect of the site factor was also significant ($F_{1,3}$ = 21.5360; P = 0.0168) in a nested experimental design. The importance values of *A. trifida* were much lower in the subplots with uprooting than in the control (no seed added) (Fig. 3a). Sowing native seeds did not influence the diversity index ($F_{9,44}$ = 0.7998; P = 0.6184), despite significant effects of eradication ($F_{2,44}$ = 8.6236; P = 0.0007) and the effect of the site factor was not significant ($F_{1,3}$ = 7.2109; P = 0.0747) in a nested experimental design. The diversity index was much higher in the subplots with uprooting than in the control (Fig. 3b).

Variations in plant cover of native species after eradication, but before sowing native species and after sowing seeds, were also observed (Table 1). Only four (one of each woody and annual and two of perennials) out of nine sown species were established. One of the most abundant native species was a woody species (*L. bicolor* Turcz.) (Table 1). However, this species was already observed in the community before sowing native seeds. The annual herb *L. indica* L. amongst the sown species was absent in the existing native vegetation and showed a many-fold increase in its cover (Table 1). There was little success in the establishment of the perennial grass *P. australis*. Three native annual herbs, *Acalypha australis*, *Commelina communis* and *Persicaria lapathifolia*, one annual legume, *Glycine soja* and one perennial herb, *Stellaria aquatica*, had appeared after eradiation of *A. trifida* and sowing native species. The establishment of these species after restoration initiatives, regardless of whether or not seeds of these plants were included in the seed mixture, is likely due to a decrease in competition for resources in the absence of *A. trifida*, a strong competitor, facilitative effects of companion native species and differences in the growth season of these species. *Humulus japonicus*, a perennial invasive climber, appeared immediately after the eradication of *A. trifida*, but its abundance decreased slightly after sowing native seeds. Another invasive annual herb, *Lactuca scariola*, was present before restoration initiatives began, but disappeared afterwards. One non-native annual herb, *Bidens pilosa*, a non-target species, also disappeared after eradication of *A. trifida* and sowing native species (Table 1). A significant negative correlation was observed between importance values of *A. trifida* and diversity index (Fig. 4).

Discussion

When invasive plants are partially removed, the effects on re-invasion can vary depending on the mode of reproduction of the invasive species, such as sexually (through seeds) and asexually (through rhizomes), as well as the persistence of the seed bank. For instance, re-sprouting from roots, rhizomes and plant stubs occurs in various invasive species (e.g. *Cyperus rotundus*, *Lantana camara*, *Phragmites australis* and *Rosa rugosa*), facilitating re-invasion after cutting or mowing aboveground plant parts. However, as observed in the current study on *A. trifida* (Fig. 2a), selective uprooting can significantly reduce the dominance of such invasive species. Similar results were found in a study of the sand dune invasive species *Rosa rugosa* (an invasive shrub in north-western

Table 1. Plant species and their cover before sowing native seeds (August 2022) and after sowing native seeds (September 2023). Species names in bold font were the sown species. PC, plant cover (%).

Species names	Growth habit	Native or not	Invasiveness	Sown species	PC 2022 (%)	PC 2023 (%)
Lespedeza bicolor Turcz.[#]	**Perennial legume**	**Native**	**–**	**Sown**	**23.00**	**37.25**
Humulus japonicus Siebold & Zucc.	Perennial climber	Native	Invasive	–	46.12	36.80
Pueraria lobata Maesen S. M. Almeida ex Sanjappa & Predeep	Perennial vines	Native	–	–	28.37	34.00
Melothria japonica L.	Annual climber	Native	–	–	10.67	33.22
Lactuca indica L.[#]	**Annual herb**	**Native**	**–**	**Sown**	**0**	**21.83**
Rubus parvifolius L.	Perennial shrub	Native	–	–	20.00	15.63
Acalypha australis L.	Annual herb	Native	–	–	0	14.50
Achyranthes bidentata var. *japonica* (Miq.) Nakai	Annual herb	Native	–	–	16.70	14.18
Pennisetum alopecuroides (L.) Spreng.[#]	**Perennial grass**	**Native**	**–**	**Sown**	**17.00**	**13.33**
Commelina communis L.	Annual herb	Native	–	–	0	11.49
Artemisia indica Willd.	Annual herb	Native	–	–	2.50	10.75
Persicaria perfoliate (L.) H.Gross	Annual climbing	Native	–	–	7.00	7.57
Paederia foetida L.	Perennial herb	Native	–	–	18.5	7.20
Setaria viridis (L.) P. Beauv.	Perennial grass	Native	–	–	7.00	6.04
Cocculus trilobus (Thunb.) DC.	Climbing shrub	Native	–	–	8.25	5.50
Equisetum arvense L.	Perennial herb	Native	–	–	3.00	5.50
Stachys japonica L.	Perennial herb	Native	–	–	7.00	5.17
Phragmites australis (Cav.) Trin. ex Steud.[#]	**Perennial grass**	**Native**	**–**	**Sown**	**0**	**4.75**
Glycine soja Siebold & Zucc.	Annual legume	Native	–	–	0	4.14
Artemisia lancea Van.	Perennial	Native	–	–	8.75	3.73
Digitaria ciliaris (Retz.) Koeler	Annual grass	Native	–	–	0.67	3.65
Persicaria lapathifolia (L.) Delarbre	Annual herb	Native	–	–	0	3
Stellaria aquatica (L.) Scop	Perennial herb	Native	–	–	0	1.25
Lactuca scariola L.	Annual herb	Non-native	Invasive	–	21.00	0
Fallopia dumetorum (L.) Holub	Annual climber	Native	–	–	1.00	0
Amphicarpaea bracteata edgeworthii Benth.	Annual climber	Native	–	–	6.67	0
Bidens pilosa L.	Annual herb	Non-native	–	–	20.00	0

[#] Seed mixtures of native species. Five sown native species (two annuals: *E. splendens* and *P. oleracea*; one non-woody perennial: *P. asiatica*; and two woody perennials: *L. juncea* and *S. sorbifolia*) did not become established.

Europe with negative effects on coastal biodiversity) (Kollmann et al. 2011), in which no re-sprouting was detected 2 months after uprooting. However, in the current study, uprooting reduced the abundance of *A. trifida* after 1 year. Mechanical control, including uprooting, cutting and ploughing, is an effective measure to eradicate *Ambrosia* species (Gerber et al. 2011). Generally, habitat disturbance caused by the mechanical removal of invasive plants facilitates re-invasion and hinders the recovery of native diversity. For instance, ploughing can significantly disrupt the soil environment, while mowing down all species can eliminate valuable native plants that resist *A. trifida* invasion. Selective uprooting, on the other hand, minimally impacts the habitat, preserving native vegetation and facilitating the eradication of invasive species *A. trifida*, ultimately benefitting native diversity. This expectation is supported by our results (Fig. 2c) demonstrating a significantly higher diversity of plant communities in uprooting plots than in control or cutting plots. The insignificant suppressive effect of

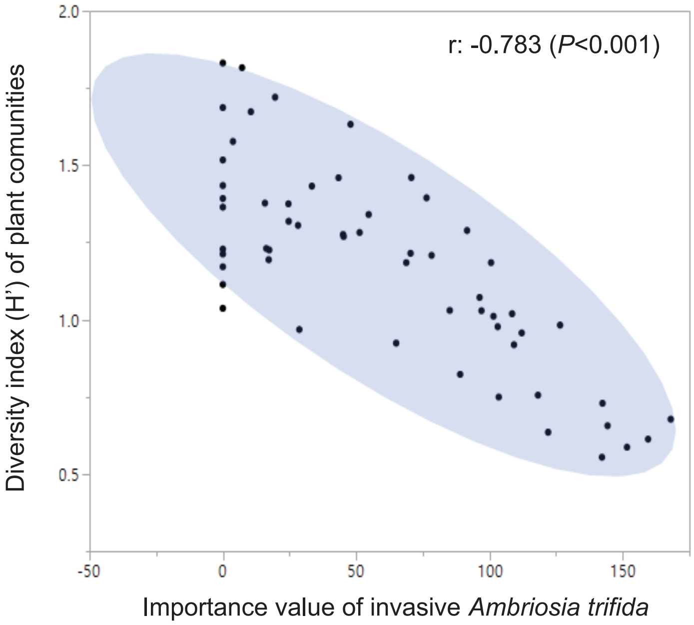

Figure 4. Relationship between the importance values of *Ambrosia trifida* and diversity index (H') of plant communities.

selective cutting in the current study is contrary to results of an earlier study in which a 77% reduction in *A. trifida* importance values was reported after cutting all plant species (Byun et al. 2020b). The lack of a significant reduction in *A. trifida* abundance in the current study might be explained by a difference in the extent of the disturbance (Fox 1979; Byun et al. 2020b). Although the selective nature of cutting in this study was not destructive to the habitat and other vegetation, re-sprouting from *A. trifida* plant stubs and belowground plant parts did not support an increase in native plant diversity in this treatment (Fig. 2c). This indicates that *A. trifida* has strong potential to regrow and flower from remaining plant parts at the removal site. In a previous study, after clipping aboveground stems of *A. artemisiifolia* four times in a growing season, > 67% of individuals survived and, amongst these, > 97% flowered (Patracchini et al. 2011). Our findings on the seed yield of *A. trifida* in cutting and uprooting treatments (Fig. 2b) were consistent with these earlier results.

The addition of native seeds following invasion control is an effective strategy for controlling re-invasion and increasing biodiversity (Bucharova and Krahulec 2020); however, this approach has not been investigated extensively (Kettenring and Adams 2011; Singh and Byun 2023) and the results of various studies have been contradictory (Patracchini et al. 2011; Byun and Lee 2018; Byun et al. 2020b; Dong et al. 2020a; Wang et al. 2022). The responses of invasive species and native diversity to sowing native seeds following invasion control can be neutral, positive (facilitation) or negative (suppression) depending on various factors, including the identity of invasive species, removal strategy (Flory and Clay 2009; Kollmann et al. 2011; Cutting and Hough-Goldstein 2013), diversity, density and features of seeds (Falk et al. 2013) and ecological conditions (Reinhardt Adams and Galatowitsch 2008). The responses of *A. trifida* invasive species to annual seed mix (SM1) were insignificant, but negative in control plots and significant, but positive in the cutting plots (Fig. 3). The suppressive effect of annuals on *A. trifida* abundance might be explained by the concept of limiting trait similarity (Yannelli et al. 2017), suggesting that interspecific similarity (annuals in this case) leads to the competitive exclusion of invasive species (Macarthur and Levins 1967). Given that cutting did not reduce the abundance of *A. trifida* (Fig. 2) or increase diversity index (Fig. 3a), annuals might have facilitated further invasion in the cutting plots. A consistent increase in invasive seeds and decrease in native seeds at invaded sites can also contribute to *A. trifida* invasion and inhibit diversity recovery. A decrease of up to 83% in native seeds was reported within 8 years of *A. trifida* invasion (Wang et al. 2022). We detected the suppression of *A. trifida* invasive species in response to sowing native seeds in selective uprooting plots; however, the magnitude of the effect was similar for seed mixtures including annual and woody species (Fig. 3a). The perennial seed mix following uprooting had a greater suppressive effect on *A. trifida* invasion than those of both annual and woody seed mixes. Thus, the eradication of *A. trifida* by uprooting and follow-up restoration by sowing native seeds can be an effective strategy to protect against its invasion. The lower seed yield of *A. trifida* in uprooting plots supports this assumption.

The variations in plant cover of native vegetation between 2022 and 2023 can be attributed to the combined effects of invasive species removal, sowing of native species, differences in growth habits, improved environmental conditions and interspecific interactions. The eradication of *A. trifida* may have reduced competition for resources, such as light, water and nutrients and, thereby, allowed other species to flourish. The sowing mixtures of native species would be expected to directly increase the presence and cover of these species. This is evident from the appearance of species that were absent in 2022, such as *L. indica* and *P. australis* in 2023. Annual species such as *L. indica* and *A. australis* can quickly colonise and cover ground within a single growing season. Perennials, on the other hand, might show more substantial growth over several years. This explains why some annual species were completely absent in 2022 and appeared in 2023 after sowing, while some perennials maintained or slightly increased their cover. For example, *M. japonica* increased its cover from 10.67% to 33.22%, indicating a competitive advantage or favourable conditions for this species post-eradication. Likewise, the increase in cover of *L. bicolor* from 23.00% to 37.25%, might be due to reduced competition and to its being a sown species.

While native seed sowing suppressed *A. trifida* invasion to different extents in each treatment, it did not impact the recovery of native diversity significantly. Sowing native seeds following invasion removal has been reported not to be a promising strategy for increasing native plant biodiversity, as reported in recent studies of *A. trifida* (Bucharova and Krahulec 2020; Byun et al. 2020b; Nagy et al. 2022) and other invasive plant species (Dickson and Busby 2009; Cutting and Hough-Goldstein 2013; Tarsa et al. 2022). In addition, there were no differences amongst seeds of different functional groups with respect to native diversity recovery, regardless of the method employed for *A. trifida* invasion eradication (Fig. 3c). A recent greenhouse study also reported that the diverse native species of different functional groups does not suppress *A. trifida* (Byun and Lee 2018). However, the early arrival of native species strongly contained *A. trifida* invasion. There are multiple explanations for the lack of recovery of native diversity after seed addition: (1) Field conditions may not be favourable for seed germination; (2) An important factor in restoring native diversity using native seeds is appropriate seed density. Considering the harsh conditions and low germination rate, 600 seeds m^{-2} may not be a sufficient density (Burton et al. 2006; Reinhardt Adams and Galatowitsch 2008; Byun and Lee 2018; Shaw et al. 2020; Byun et al. 2020b; Tarsa et al. 2022); however, insignificant recovery with a high seed density has also been reported (Dickson and Busby 2009); (3) A narrow niche range of some of the native species did not support their dominance in current distinct habitat conditions (Pulliam 2000); (4) Seeds of perennials, such as *P. australis* and *P. alopecuroides*, were hairy (Suppl. material 1: fig. S1) and hardly encountered the ground or soil directly during sowing; it is, therefore, possible that these species could not grow and contribute to native diversity; (5) Commercial sources of seeds, generally agronomically propagated (Höfner et al. 2022), may contribute to low adaptability to targeted local conditions (Bucharova et al. 2019); (6) Sowing seeds of one functional group in each plot might not exert strong effects on diversity. More diversified functional groups or types of native seeds in seed mixtures are needed to better assess whether seeding native species counteracts invasive plants and restores native biodiversity; (7) *A. trifida* may be too difficult to control via native species (Byun and Lee 2018). Given that uprooting reduced invasion substantially, consistently uprooting *A. trifida* for a longer period, at least 3 years (soil seed-bank densities decreased by > 99% in 2 years (Dong et al. 2020b)), in a larger area to limit the arrival of invasive seeds and adding native seeds can facilitate the recovery of native diversity. We did not test the role of functional diversity; therefore, further studies should evaluate whether sowing seeds of all functional groups in combination restores native diversity.

Limitations of study

A major limitation of this study was relatively short monitoring time (1 year or less) after restoration. We think that longer monitoring would have yielded better results. The short monitoring time may explain, at least partly, why sowing native seeds did not bring any additional benefit to the control of, or resistance to, *A. trifida* invasion. The seed mixtures of three functional groups of native plant species were employed: annuals, non-woody perennials and woody perennials, because we wanted to determine which functional group was most effective in providing biotic resistance to invasion in the year following eradication of *A. trifida*. Annuals were expected to perform better as they are usually fast-growing and become established in the first year after eradication. This is also expected, based on the limiting similarity hypothesis (*A. trifida* is also an annual plant species). However, we did not find any difference in biotic resistance to invasion between the functional groups of seeds; in fact, there was no difference between sowing and not sowing seeds. We only monitored plots soon after eradication because we considered one year as the critical window for invasive species re-invasion. If invasive species are not controlled within this short time frame, then it will be difficult to stop re-establishment of the invasive species afterwards. As we were acutely aware of the limited timeframe of this study, we ended up measuring the invasive seed yield as an indicator for potential future re-invasion after one year of monitoring.

One of the critical aspects of restoring native species using native seeds is the seed density. For instance, 600 pure live seeds (after considering germination rates per species) m^{-2} per subplot were sown. Originally, this density was considered sufficient in the initial experimental design, but under actual heterogeneous field conditions, many different factors can influence seeding efficiency. For example, the characteristics of experimental sites might not match the ecological niches of the restorative native species. In addition, it is also likely that seed density is reduced by their ingestion by some animals, such as birds, in the Spring. Considering these field limitations, we now consider that 10-fold higher seed density would have been required to obtain meaningful and significant results; in fact, this density was recommended by a seed-based restoration workshop at a conference of the Society of Ecological Restoration (SER).

Implications for practice

The findings of this study have strong implications for the management of invasive plants and recovery of native plant diversity: (1) Cutting to eradicate plant invasion can result in wasted effort and resources, particularly if the targeted species can regrow or re-sprout from remaining plant parts. In the current study, cutting was selective and resulted in minimal disturbances of native vegetation. However, invasion was suppressed to only a small extent with insignificant differences between cutting plots and the control plot. Complete and destructive cutting of the entire vegetation may further increase invasion by reducing native plant diversity; (2) Selective uprooting is a promising tool for invasive plant management. Complete removal of invasive species from invaded communities and ecosystems will reduce competition pressure on native species for space, light and nutrients and form invasive propagules (roots, rhizomes, seeds etc.) and increase the performance (germination, establishment and diversity) of native communities; (3) Sowing seeds of diverse species following removal of the invasive species is critical for the rapid recovery of native diversity; (4) Compared with selective uprooting, mowing of all species is not an effective strategy for the management of invasive plants because it does not leave any native species to resist re-invasion; (5) Although this study was conducted solely at two field sites within the Republic of Korea, our findings can readily be extrapolated to other countries. This generalisability stems from the underlying ecological principles uncovered, namely, the importance of leaving no propagule behind for achieving effective eradication outcomes. This fundamental principle is relevant, irrespective of geographic context, making it applicable across diverse regions; (6) Lastly, it is important to consider the potential environmental or ecological side effects of selective uprooting. For instance, hand-pulling to uproot all invasive plants can slightly disturb soil composition, potentially impacting soil microorganism communities and the legacy effects of soil on biogeochemical processes. Therefore, selective uprooting must be executed with meticulous care to minimise disturbances to the soil surface and other native species.

Conclusions

This study concludes that selective uprooting is a more effective tool than cutting for suppressing *A. trifida* invasion and increasing the diversity of native plant communities. The findings of this study support the expectation that uprooting of invasive species before flowering with minimum habitat disturbances can immediately reduce competition for remaining native species and concurrently can increase native diversity in the next growing season due to decreases in the number of seeds of *A. trifida*, the invasive species. Suppression of plant invasion further increased after sowing native seeds; however, this was only valid if the invasive plant was eradicated by uprooting. Therefore, sowing seeds to restore native diversity at sites where *A. trifida* invasion has been eradicated by cutting may result in the waste of native seeds, time and other resources. Sowing native seeds after removal of *A. trifida* by cutting and uprooting facilitated recovery of native diversity; however, uprooting followed by sowing native plants was more effective.

Acknowledgements

We thank Dr. Ho Choi and Dr. Norul Sobuj for their assistance with the fieldwork. We also thank Dr. Minwoo Oh for drawing the study site map in QGIS. We thank Eulsukdo Ecological Park Nakdonggang Estuary Eco Center for field cooperation.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2022R1A2C1003504). This study was supported by a joint research project from the Ministry of Environment, Republic of Korea (Project number: 2021002270004).

Author contributions

CB conceptualised and designed the study; CB, KS and JL collected data, CB analysed and constructed graphs and tables; CB, KS and JL wrote the manuscript; SHH, TKY and HK reviewed and edited the manuscript; CB and SHH engaged in project collaboration.

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Data availability

Dataset was shared in the open access file directories of Figshare. https://doi.org/10.6084/m9.figshare.26425879.

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Supplementary material 1

Supplementary information

Authors: Chaeho Byun

Data type: docx

Explanation note: **fig. S1.** Sown species: Photographs of native seeds used in this study. **fig. S2.** Experimental layout (blocks) in two sites.

Link: https://doi.org/10.3897/neobiota.94.123363.suppl1

Supplementary material 2

Diversity index raw datasheets

Authors: Chaeho Byun

Data type: xlsx

Link: https://doi.org/10.3897/neobiota.94.123363.suppl2