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Nuisance species in lake constance revealed through eDNA

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Abstract Biological invasions are a global threat to biodiversity especially for aquatic resources. The distribution of alien species is associated with human activities; therefore, exotic species tend to accumulate near big urban areas through different invasion vectors such as ballast water, hull fouling, aquarium and pet releases. The Rhine River region is one of the most economically important in Europe. Around 60 million people live in the river basin that is connected with other large European rivers via the Rhine–Main–Danube shipping canal. The Alpine Rhine flows to Lake Constance, which is the second largest subalpine lake in Europe.

Here, eDNA metabarcoding was employed to inventory aquatic species from water samples in six riverine and four lake localities within Lake Constance region. A 313 bp fragment within cytochrome c oxidase subunit I gene was PCR amplified using generalist primers for metazoan and sequenced with MiSeq High-Throughput Sequencing platform. Seven invertebrate invasive species and the invasive fish *Oncorhynchus mykiss* were detected from eDNA. Species-specific primers were employed to confirm metabarcoded species. Most of the invasive species detected in this study correspond to samples from areas around lake ports, followed by other lake and degraded downstream river areas. Samples taken upstream of Lake Constance were free of invertebrate aliens. To establish common regulation and management actions regarding aquatic invasions in the three countries that share Lake Constance is recommended.

Keywords Metabarcoding · High-throughput sequencing · Specific-primers · Rhine river · Non-indigenous species

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Introduction

Biological invasions are a global threat to biodiversity especially for aquatic resources (Chown et al. 2015). Most translocations of aquatic organisms are derived from human activities (Leprieur et al. 2008), and the

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factor that best explains the number of biological invasions in a region is often the density of the human population nearby (e.g. Pyšek et al. 2010; Spear et al. 2013). Alien species have been introduced into rivers worldwide for recreational fishing, aquaculture or derived from aquarium trade (Havel et al. 2015; Duggan et al. 2010). Concomitantly, global transport is facilitating the spread of many species out of their native distribution through fouling and ballast water (Alonso and Castro-Díez 2008; Molnar et al. 2008; Thomaz et al. 2014). In addition, the increase of temperature due to climate change may benefit the dispersion of some invasive species in northern regions; examples are the spread of Ponto-caspian zebra mussel (*Dreissena polymorpha*) in the Baltic Sea (Holopainen et al. 2016), or the expansion of the Asiatic clam *Corbicula fluminea* in northern areas (Gollasch and Nehring 2006; Crespo et al. 2015).

In Europe, UK, France, and Germany are donors of alien species to northern countries and are among the main gateways of alien species introduction in freshwater ecosystems (Hulme et al. 2008). At least 13 species from North America were introduced to Germany and distributed to the Netherlands, Denmark, Hungary and Poland. Most of the exotic species established in Germany are causing adverse ecological effects (García-Berthou et al. 2005). The distribution of alien species is highly associated with human activities (Wolter and Röhr 2010; Spear et al. 2013).

The Rhine River is the most important river in Germany from the economic point of view. From its 1250 km length, 825 km of the river are navigable from the port of Rotterdam on the North Sea coast to Basel in Switzerland. Around 60 million of people live in the river basin and the river supplies drinking water for more than 30 million people (Plum and Schulte-Wülwer-Leidig 2014). Moreover, it is connected with nearly all large rivers in southern, central and Eastern Europe. Together with the Danube, Rhine River is the most invaded river in Europe (Leuven et al. 2009). The fact that both rivers are connected via the Rhine–Main–Danube shipping canal since 1992, might facilitate the entrance of aquatic alien species. At least 26 alien species reported in German waters can be directly related with this canal, for example, the arrival of the amphipod *Dikerogammarus villosus* in the Rhine basin (Gollasch and Nehring 2006). This pathway is the main vector for recent invaders in Germany and Austria, especially species from Ponto-Caspian region (Rabitsch et al. 2013). The number of non-indigenous macroinvertebrate species in the Rhine River increased over the period from 1800 to 2005, from one to more than 13 species per decade. The rapid dispersion of exotic species is highly facilitated by shipping activities and the interconnection of river basins (Leuven et al. 2009).

In addition, the Rhine river has several hydrological power plants along its way from Lake Constance to Basel (e.g. in the upper part of the river, High Rhine). There are twelve in-stream barriers due to hydropower plants (N'Guyen et al. 2016), which altered the river flow and whose cooling waters can become suitable habitat for invasive species, as has happened with the gobies in this region (Kalchhauser et al. 2013) or with the invasive mussel *Mytilopsis leucophaeata* in southern Bothnian Sea, Sweden (Florin et al. 2013). The reservoirs have been associated with a higher number of exotic species introductions (Clavero et al. 2004; Johnson et al. 2005). Havel et al. (2015) suggested that once an exotic species is established in a lake, it could easily colonize nearby lakes and rivers.

In the Rhine basin, Lake Constance is the second largest subalpine lake in Europe. It is situated at the northern fringe of the European Alps and is shared among Germany, Switzerland and Austria. It is the main reservoir of Rhine River. The lake itself is an important drinking water source for southwestern Germany and economically important for recreational and commercial fisheries and for tourism (N'Guyen et al. 2016). Twenty-nine fish species occur in the lake of which only a few are commercially exploited: two lake whitefish (*Coregonus clupeiformis* and *C. lavaretus*); perch (*Perca fluviatilis);* European eel (*Anguilla anguilla*); brown trout (*Salmo trutta*); pike (*Esox lucius*); Arctic charr (*Salvelinus alpinus*) and pike perch (*Sander lucioperca*) (Eckmann and Rosch 1998). The Lake Constance population of *Salmo trutta* was almost extirpated in the 1950s due to dam construction in the alpine Rhine, but thanks to protective measures, they have made a significant return (Ruhlé 1996). The lake was the home of the considered extinct species of trout *Salvelinus profundus*, as well as of the Lake Constance whitefish (*Coregonus gutturosus*) (Freyhof and Kottelat 2008). Among other factors, the extinction of the former fish species in the lake might be associated with the introduction of exotic species, because exotic invasive

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species are often a cause of animal extinctions (Clavero and García-Berthou 2005).

Since the Rhine basin and Lake Constance could serve as a reservoir and point of entry of invasive species that could rapidly spread all over Europe, prevention and early detection of new alien species is highly recommended. However, the management of aquatic biota in this region does not seem to be efficient because there are decentralized political structures in the surrounding countries Austria, Germany and Switzerland (Essl et al. 2011). This situation is far from ideal especially when the river acts as border between Germany and Switzerland in the High Rhine region. In the current European regulation EU No 1143/2014 of 22 October 2014 on Invasive Alien Species (http://ec.europa.eu/environment/nature/invasivealien/index_en.htm) the list of invasive alien species includes 26 animals, amongst them ten species that inhabit freshwater ecosystems: the crab *Eriocheir sinesis*; the bullfrog *Lithobates catesbeianus*; the crayfishes *Orconectes limosus*, *O. virilis*, *Pacifastacus leniusculus*, *Procambarus clarkii* and *P. fallax f. virginalis*; the fishes *Perccottus glenii* and *Pseudorasbora parva*; the slider *Trachemys scripta.* There is not a common list of invasive species for Switzerland, Austria and Germany (Wittenberg et al. 2005; Gollasch and Nehring 2006; Nehring et al. 2010). When searching the three countries in EASIN database (European Alien Species Information Network, https://easin.jrc.ec.europa.eu/), the list of invasive species in each region/country is different with only a few species in common.

Prevention and early detection of new invasions are recommended to control dispersion of invasive alien species (Thomaz et al. 2014). In the last few years, the development of environmental DNA (eDNA) techniques has become a promising tool to early detect and survey alien species in aquatic ecosystems (Goldberg et al. 2015). There are numerous examples of the use of eDNA to successfully detect invasive species (e.g. Ficetola et al. 2008; Ardura et al. 2015; Clusa et al. 2016). In this study we applied eDNA Metabarcoding for the detection of nuisance species in the Rhine basin. This technique is based on high throughput sequencing of DNA barcodes on eDNA coupled with bioinformatics analysis of the sequences to compare them with databases and identify the species present in the sample. It has been employed for species inventories in ports (e.g. Borrell et al. 2017), rivers (e.g. Deiner et al. 2016; Fernandez et al. 2018) and lakes (e.g. Bista et al. 2017).

The main objective of the present study was to assess, by applying metabarcoding and species-specific primers on eDNA from water samples, the presence of alien species in the Rhine region. The results will be employed to inferring hotspots of nuisance and non-indigenous species (NIS) in the basin so informing for future management actions.

Materials and methods

Study area

The Rhine River is divided in six sections: the Alpine Rhine and the Lake Constance; the high Rhine from Lower Lake Constance (LLC) to Basel, where many barriers are, including a 23 m waterfall situated 30 km downstream the lake (Rheinfall) and many hydrological power plant dams; the Upper Rhine that extends from Basel to Bingen; the Middle Rhine; the Lower Rhine from Bonn to Lobith and the Delta Rhine in the Netherlands (Leuven et al. 2009). The alpine part of the Rhine River flows into the lake in the southeast (near Bregenz) and flows out near Stein am Rhein in the LLC. The Rhine River is the primary artery of one of the most important economic regions of Europe. It has a total length of about 1250 km, a drainage area of circa 185,260 km^2 and an average discharge of about 2300 $m^3\ s^{-1}$ (Rabitsch et al. 2013). Lake Constance is 63 km long, and at its widest point expands nearly 14 km. It covers approximately 571 km^2 and is 395 m above sea level. The greatest depth is 252 m in the middle of the eastern part. It consists of two basins: the deep Upper Lake Constance (ULC) and Lower Lake Constance (LLC), which is smaller (Jeppesen et al. 2012). Daily, car ferries link Romanshorn to Friedrichshafen as well as Constance to Meersburg (Gergs and Rothhaupt 2015) in the Upper Lake Constance.

Between October and November 2017, ten sampling points were visited in the region: from the Alpine Rhine (R0) to the Upper Rhine downstream Basel (R5), including the main ports areas of Lake Constance (Table 1, Fig. 1). The following features of the sampling sites were considered: degree of modification of the river bottom, since artificial substrates may be preferred by some invasive species (e.g. Wasson et al. 2005; Tyrrell and Byers 2007); and number of

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Table 1 Sampling points both from Rhine River and Lake Constance

Sample	Watershed	Country	Location	Human Inhabitants	Coordinates	Port	Appearance	Current
R0	Rhine River	Switzerland	Reichenau (Tamins), alpine Rhine River	3200	46.82453 N, 9.41161E	No	Sandy soil, few stones	+
R1	Rhine River	Austria	Alter Rhein	5890	47.45600 N, 9.64358E	No	Small stones and channeled	+
LP1	Constance Lake	Germany	Friedrichshafen port	42,470	47.650778 N, 9.483804E	Large port, ferry	Large blocks of concrete and stones, deep (~ 2 m)	–
LP2	Constance Lake	Germany	Constance port	84,440	47.68337 N, 9.21094E	Large port, ferry	Small stones with a lot of moss, *Corbicula sp* shells	–
L3	Constance Lake	Germany	Reichenau Insel	3300	47.68671 N, 9.06711E	Marina, sailing school	Small stones	–
L4	Constance Lake	Germany	Radolfzell am Bodensee	31,200	47.73523 N, 8.96839E	Marina, yatch club	Small stones	–
R2	Rhine River	Switzerland	Diessenhofen	3630	47.69024 N, 8.75222E	Small river port	Sandy soil, many small stones, bivalve shells	+
R3	Rhine River	Switzerland	Below Rheinfall	36,580	47.67615 N, 8.61020E	No	Sandy soil, many small stones, bivalve shells	+ +
R4	Rhine River	Germany	Stein	13,920	47.55143 N, 7.95023E	No	Large stones and algae, deep (~ 2 m)	+ + +
R5	Rhine River	Germany	Breisach am Rhein	16,000	48.04345 N, 7.57271E	River port	Large stones and algae, deep (~ 2 m)	+ + +

Coordinates, location in the basin, number of inhabitants in the nearby area, Visual appearance of the substrate and current speed observed during water sampling are shown

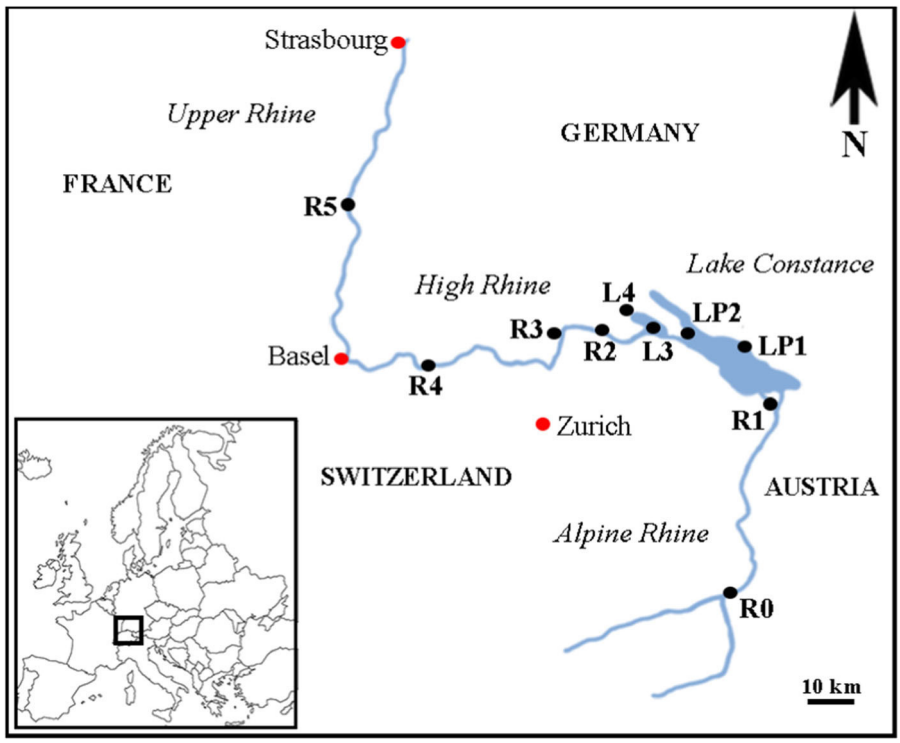

Fig. 1 Map showing the sampling points. The region is divided in four sections from downstream to upstream: Upper Rhine, High Rhine, Lake Constance, and Alpine Rhine. All sampling points are indicated with a black circle

human inhabitants in the 10 km nearby the sampling point for the known relationship between biological invasions and human population density (e.g. Spear et al. 2013).

Sample collection

Three 1L water replicates per sampling point were collected in sterile bottles. The samples collection was done in different days and always from downstream to upstream. Additionally, water bottles were kept in the cooler (blanks) and used as field controls. All the personal equipment was cleaned with 50% bleach between points and new gloves and sterile bottles were used in each point, in order to avoid contamination between sampling points. In every sampling point, water was collected approximately 30 cm below the surface, since recent DNA is located in the surface whereas in the sediments old eDNA can be accumulated and preserved long time at low temperatures even when the source of DNA has disappeared (Turner et al. 2015; Goldberg et al. 2016).

All the samples were immediately transported to the laboratory, stored at 4 °C and immediately filtrated using an Acetate cellulose membrane (Fisher Scientific) of 0.22 µm pore size and a filter holder. Filtration took place inside a laminar flow cabinet previously treated with UV light to avoid any contamination. The filter holder was dismantled, cleaned with 50% bleach, rinsed with distilled water and treated with UV for 20 min before use and between samples. A negative control consisting of 1L of milliQ water filtrated between two real samples was included in all the analysis. Filters were stored at − 20 °C until extraction.

All the collection, filtration, extraction and analysis process were done following the recommendations from Goldberg et al. (2016) to avoid any cross contamination in the different steps.

Environmental DNA (eDNA) extraction

DNA from 1L water samples was extracted with the PowerWater® DNA Isolation Kit (Mobio laboratories) following the manufacturer's protocol. Every

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replicate from each sampling point was extracted separately in time, therefore, all the analysis and extraction from the same water sample was done in different weeks, minimizing the possibility of contamination. In addition, the whole extraction process was done inside the laminar flow cabinet. Additionally, two negative controls were included in each extraction and in all posterior PCRs amplifications; consisting of a negative control for filtration (sterile water) and a negative control for extraction which consisted in a clean membrane. All the pre-PCR steps were done inside the laminar flow cabinet after 20 min of UV light decontamination, and the post-PCR steps were done in a separate laboratory unit.

Inhibitors test

The presence of PCR inhibitors in eDNA samples might represent a serious problem due to the fact that it could be wrongly identified as a false negative (Thomsen and Willerslev 2015). Thus, to control for the presence of inhibitors in the samples, DNA from the fish species *Gambusia holbrooki* was spiked to one replicate from each of the sampling sites at two different concentrations similar to the experiment done by Clusa et al. (2016). For the high concentration test, 1 µl of *Gambusia* DNA from 10 ng/mL was spiked to 5 µl of the eDNA; and for the low concentration assay 1 µl of *Gambusia* DNA from 10 pg/mL, near the detection limit of the specific primers, was added to 5 µl of eDNAHigh quality DNA samples obtained from fish tissue were added outside the cabinet, in the last minute when all the tubes with eDNA samples were closed inside the PCR machine. The amplification reaction was performed in a total volume of 20 µl, including Green GoTaq®Buffer 1X, 1 mM $MgCl_2$, 0.25 mM dNTPs, 1 µM of each primer, 0.65 U of DNA Taq polymerase (Promega) and 5 µl of template DNA. PCR conditions were the following: an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 68 °C for 1 min, extension at 72 °C for 2 min and a final extension step at 72° for 7 min. PCR products were visualized in 2% agarose gels with 2.5 µL of SimplySafe™.

In order to discard false negatives due to excessive DNA degradation or other reasons, the cytochrome c oxidase subunit I (COI) gene was amplified from eDNA with generalist primers (Geller et al. 2013) in all the samples.

Metabarcoding library preparation

One replicate water sample was used in the HTS analysis to obtain a global view of biodiversity in the sample. The other two replicates were employed for amplification of species-specific primers and checking inhibition.

The target of the barcoding assay was a fragment of 313 bp from the COI gene, using the generalist primers mlCOIIintF and jgHCO2198 for metazoan described by Leray et al. (2013) and adapted to Illumina platform. The protocol used was the one described for Illumina platforms (Illumina), which consisted in two sequential PCRs. The first PCR amplification was performed using general primers with a barcode and a tag and the second PCR using primers with the tag and adapters for Illumina (Table S1). After each sequential PCR, the amplified product was purified with HighPrepTM PCR beads (MagBio Genomics, Maryland). For the first PCR, we used the 515F and 806R primers with a universal 5′ tail, for DNA amplification of a fragment of COI gene (313 bp). Briefly, 2 ng of eDNA were used as template for the first PCR (2 min at 98 °C, 10 amplification cycles consisting of 15 s at 98 °C, 20 s at 55 °C and 20 s at 72 °C and a final elongation at 72 °C for 2 min) and the purified PCR amplicons were the template for the second PCR (2 min at 98 °C, 20 amplification cycles consisting of 15 s at 98 °C, 20 s at 67 °C and 20 s at 72 °C followed by a final elongation at 72 °C for 2 min) using primers including sequencing barcodes as well as the Illumina adapter sequences (Table S1). Both PCRs were performed in 25 µl reaction volumes and amplifyed with the Q5 High-Fidelity polymerase (New England Biolabs, MA). After purification, DNA concentrations were measured and specificity of amplification was checked for all samples using gel electrophoresis. Negative controls for filtration and extraction were used in the PCR, where no quantifiable DNA was detected using a Qubit v2.0 Fluorometer (Thermo Fisher Scientific, Massachusetts), so they were not processed further.

The quality of the pooled libraries was assessed using a Bioanalyzer 2100 (Agilent Technologies, Germany). The genomic libraries were pair-end

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sequenced (2 × 250 bp) on MiSeq platform at TUFTS genomic centre in USA.

Sequence processing

The Fastq files were split by barcodes, allowing obtaining all the sequences from each sample. All Fastq files were checked in the FastQC version 0.11.3 visor.

A small subset of 5000 reads was used to adjust the pipeline settings to later analyze the rest of the samples. Different merge (e.g. minimum overlapping: j:100, j:80 or j:115) and assignment (identity and e-value: -i and -e parameters) settings were tested using QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso et al. 2010). At least five sequences from all the species in the resulting OTU table were manually checked to confirm the suitable performance of the assignment (data not shown). Merged pair-end files were obtained using the script *join_paired_ends* (Aronesty 2011) included in QIIME (Caporaso et al. 2010), with a minimum overlap of 100 bp (j = 100) and a maximum error of 15% (p = 15), being the parameters selected based on the consistency of the number of species recovered and on previous experience with different datasets (e.g. Fernández et al. 2019). After that, the sequences were left- and right- trimmed using PrinSeq version 0.20.4 (Schmieder and Edwards 2011) to remove primer sequences. Sequences were filtered by length and quality, allowing a maximum length of 340 bp and a minimum of 230 bp and sequences with a mean quality score lower than 25 were removed.

To create a reference taxonomic database, an exhaustive search for "mitochondrial COI gene" sequences was performed in the NCBI website in June 2017. All the cytochrome c oxidase subunit I sequences available were downloaded with the script *entrez qiime.py* (Baker 2016). After that, blast assignments were performed using the script *assign taxonomy* from QIIME (Caporaso et al. 2010) using as database the file generated with all the COI sequences downloaded from GenBank. The assignment was done using a 97% of identity and an e-value of 10^{-50}. Finally, the OTU table was obtained using the script for python *fromTaxassignment2Otutable*. The pipeline is similar to the one used by Galal-Khallaf et al. (2016). Singletons were eliminated from the OTU table for further downstream analysis.

Validation using species-specific primers

One fish and two mollusc invasive species were chosen to double check the Metabarcoding results from independent genetic markers and methodology. Three-spine stickleback (*Gasterosteus aculeatus*) DNA was detected using the specific primers designed by Thomsen et al. (2012). This species is native to the region and known to inhabit the Lake Constance and the Rhine River. New Zealand mudsnail *Potamopyrgus antipodarum* was detected using the primers described in Clusa et al. (2016), and *Corbicula fluminea* using Clusa et al. (2017) primers. Primer sequences and PCR amplification conditions are described in the Table S2. All PCR amplicons were visualized in 2% agarose gel with DNA Stain clear G (SERVA). The species-specific primers were used in two out of the three samples taken from each site. To consider a sample as positive or negative, the two samples had to be positive or negative, respectively. The third sample of each point was reserved for running an extra PCR with specific primers to confirm the presence of the species in case of doubtful results (when only one of the samples was positive). Every positive PCR band was purified with Zymoclean™ Gel recovery kit and sequenced with ABI 3130 sequencer to confirm the species identity using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against NCBI GenBank Nucleotide database.

Estimations of environmental quality

Global diversity (Shannon index) was estimated from the OTU table with the script *alpha diversity* from QIIME (Caporaso et al. 2010), using the reads of all the species after removing duplicated species and sequences which assignments with BLAST correspond to entries catalogued as "Environmental samples" in GenBank. However, since there is little consensus on the extent to which proportions of reads generated corresponds to the original proportions of species in a community (Lamb et al. 2019), this index should be considered only a rough proxy of real diversity. A better diversity estimate was calculated using the number of species, instead of the reads, of each phylum as a variable.

Six variables were measured from HTS data and used to take into consideration the environmental status of the sampling sites. Due to the known

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correlation between habitat degradation and invasive species, which does not implying causality (Didham et al. 2005), the presence of two types of nuisance species was used as proxies of bad ecological status: harmful algae (HABs) and exotic invasive species. Therefore, the number and proportion of each type of species were the variables. It is worth noting that the mere presence of invasive species does not necessarily indicates degraded habitat and other indicators would be necessary for environmental quality assessment. Variables associated with good ecological status were: the number of native fish species as an ecosystem service, and the number of EPT (Ephemeroptera, Plecoptera and Trichoptera insects) species as world-wide indicators of good water quality (e.g. Lenat 1988; Masese and Raburu 2017; Ab Hamid and Md Rawi 2017). Two additional diversity estimates were the number of other native invertebrate species, and the number of other algae species (non-HABs).

The list of exotic species was taken from the Invasive Species Compendium (CABI 2019, https://www.cabi.org/isc, accessed on September 2019); the species contributing to Lake Constance fisheries from Eckmann and Röchs (1998); the list of reference of HAB species was the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae (http://www.marinespecies.org/hab/, accessed in August 2019; Moestrup et al. 2009 onwards). To calculate the proportion of invasive species in the samples, only sequences from aquatic metazoans were taken into account, excluding sequences from human and avian DNA, as well as fungi and protists.

Statistical analyses

Non-metric multidimensional scaling (nMDS) analysis was performed for visualizing the differences among samples, using the following six variables: EPT, Native Fish, HABs, NIS, HABs, non-HABs and other native invertebrates. The minimum spanning tree among samples was calculated from Manhattan pairwise similarity indices using 9999 bootstrapping and visualized by Scatter plot.

Pairwise correlations between the biotic indicators and proxies were performed using linear Pearson´s r after checking for normality using Jarque–Bera tests and Monte Carlo simulations, using PAST software version Past3.dmg (Hammer et al. 2001). False discovery rate (FDR) adjustment for multiple comparisons was carried out in R (R Core Team 2020) using "psych" library.

Results

The amplification of the COI gene with universal primers confirmed the presence of good quality DNA in all eDNA samples. The spike test to discard the presence of inhibitors in the samples was successful, obtaining positive PCR amplifications in all eDNA samples with both high or low concentration of *Gambusia* DNA, discarding the presence of inhibitors in the samples.

From each sample a minimum of 500,000 raw reads were obtained. After merging and filtration steps the 76.80 ± 11.1% of sequences remained from the raw dataset of reads. A 12.6 ± 7.6% of the raw reads were assigned to a reference barcode with the 97% of identity. The sample with least sequences assigned was R5 with only the 2.6% of raw reads, whereas the sample with the highest number of sequences assigned was L4 (29.1% of raw reads) (Table S3).

In number of sequences, the taxon most amplified from the HTS analysis was Porifera (more than 50% of the sequences) followed by Arthropods (25.85%), and Protista (11.32%); only 1.87% correspond to molluscs and 0.42% to chordate species (Fig. 2A). The taxonomic profile varied in the different samples, for example in R0 81.3% of the sequences corresponded to arthropods, in R1 the 81% corresponded to Protista or in R3 12.2% were molluscs (Fig. 2B). Shannon's diversity index, taking into account all the sequences from all the taxa identified by HTS, showed that the highest value was found in samples R1 (3.9), R5 (3.8), and R4 (3.6) (Table S5).Considering the number of species per metazoan phylum, the samples were also clearly different (Fig. 3), although not as much as in the number of sequences for which they differed principally in Protista (Fig. 2B). River samples were richer in arthropods while lake samples were richer, in general, in mollusc species –absent from the river samples taken upstream of Lake Constance.

From the assigned sequences eight NIS were detected (Table 2A) including two arthropods, one fish, one cnidarian and four molluscs: The killer shrimp *Dikerogammarus villosus*, the Caspian slender shrimp *Limnomysis benedeni*, the rainbow trout *Oncorhynchus mykiss*, the freshwater jellyfish

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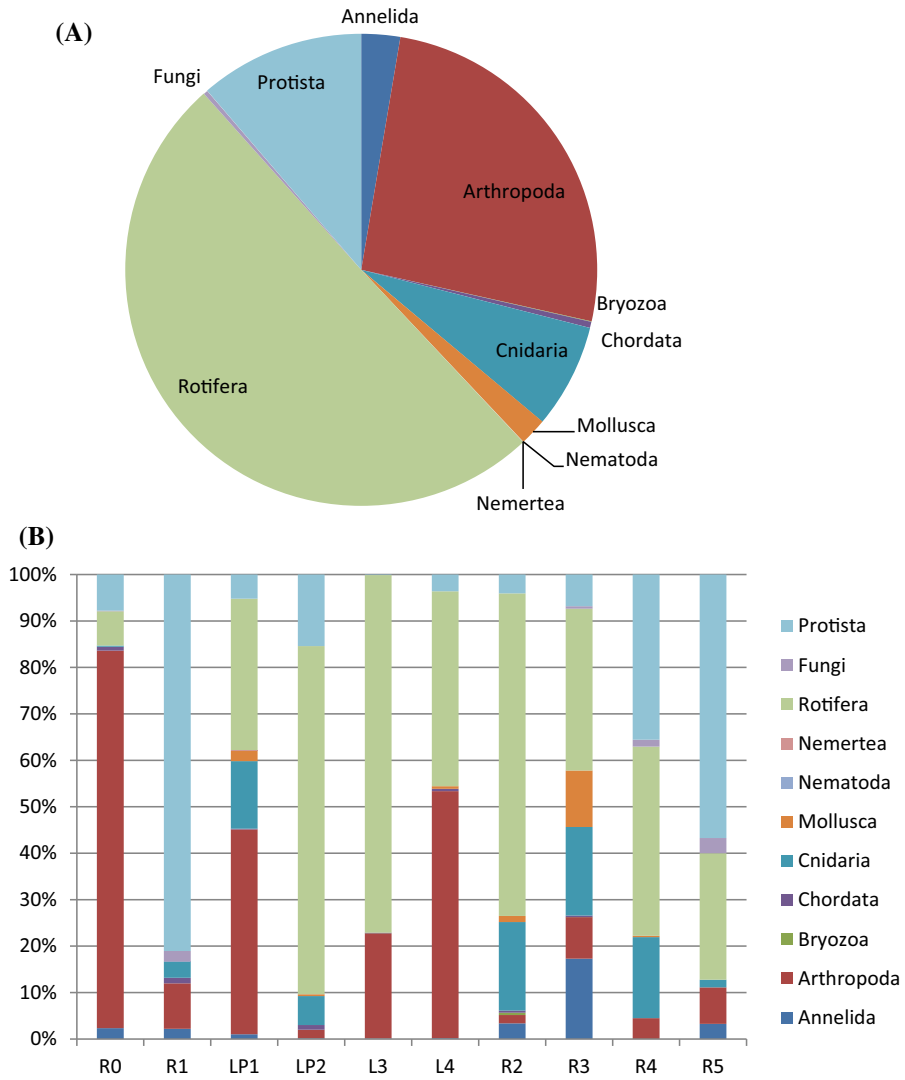

Fig. 2 Sequences from HTS analysis by taxon. **a** Percentage of sequences of each taxon obtained from the HTS analysis. **b** Percentage of sequences from each taxon in each sampling site

Craspedacusta sowerbyi, the zebra mussel *Dreissena polymorpha,* the Asiatic clam *Corbicula fluminea,* the New Zealand mudsnail *Potamopyrgus antipodarum* and the freshwater pulmonate snail *Physella acuta.* On the other hand, DNA of four species of Dinoflagellates catalogued as harmful algae (HAB) represented by more than one sequence was found in the dataset: *Alexandrium catenella*, *A. ostenfeldii*, *A. tamarense* and *Karlodinium veneficum* (Table 2B). All of them occurred in the lake, principally in lake ports, while only one sequence of *A. tamarense* was found in the upstream river point closer to the lake (R1). It is worth noting that the mere detection of harmful algae does not implicate its bloom.

Three species were double-checked from species-specific primers on the eDNA samples where they were found from Metabarcoding. All the positive amplifications were sequenced and the species confirmed by Blast in the NCBI webpage. All the assigments are available in Table S4 The results were totally coincident and confirmed the presence of DNA of those species in the samples analyzed (Supplementary Table S4. The native fish *Gasterosteus aculeatus* was detected in the samples LP2 and L3, the invasive *Potamopyrgus antipodarum* appeared in the lake and

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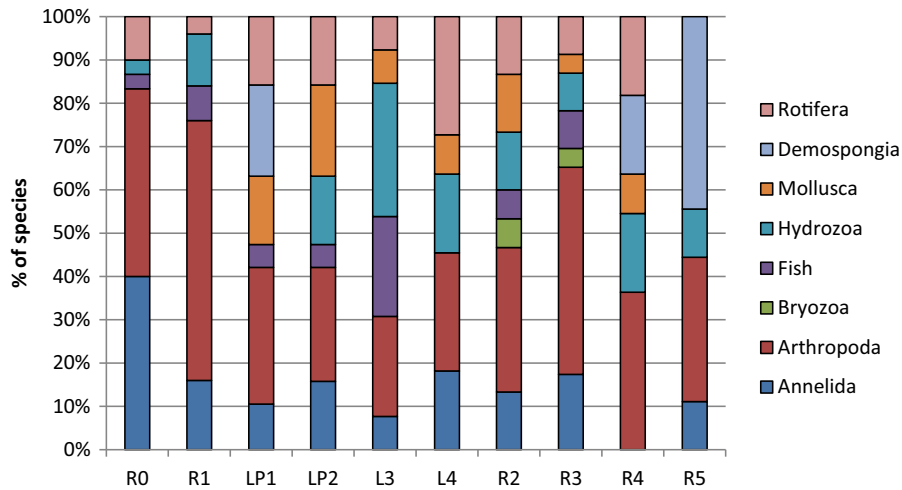

Fig. 3 Proportion of species of each taxonomic group in the four lake and six river samples analyzed

in the first river sample downstream, and *Corbicula fluminea* in the lake port where it was found from metabarcoding. The fact that these species were independently detected with species-specific primers on the same water samples (eDNA), allowed to discard these species to be false positives in the metabarcoding, and confirmed the robustness of our metabarcoding results. For *P. antipodarum* the haplotype found in eDNA samples was the European haplotype *t* described by Städler et al. (2005) also found by Clusa et al. (2016) in Nora River in Northern Spain.

In general, lake samples contained more invasive species and HABs than the river samples analyzed in this study (Table 3). In contrast, traces of EPT DNA were not found from lake samples. The highest number of native Metazoans was found upstream of Lake Constance, while on the other hand the phylogenetic diversity was lower in that upstream area than in the lake and downstream (Table 3), due to the absence of sponges, molluscs and bryozoans. Considering all the species present, the samples obtained within the lake and the first sample downstream were the most diverse (for Metazoans). DNA from eight fish species native to the region was found: *Abramis brama*, *Barbus barbus*, *Coregonus lavaretus*, *Cottus gobio*, *Esox lucius*, *Gasterosteus aculeatus*, *Salmo trutta*, and *Squalius cephalus*. Their distribution suggested a clear basin zonation for the fish community. *Cottus gobio* and *Salmo trutta* were found upstream the lake, and *Barbus barbus* and *Squalius cephalus* from river locations downstream. The other four species were found only from lake samples.

The difference among sampling sites was evident in the nMDS. The Shepard plot, with stress of 0.108 and $r^2 = 0.855$ and 0.007 for axis 1 and axis 2, respectively (Fig. S1), showed most points aligned along the diagonal. The scatter plot showed the sites arranged by basin sections: lake samples connected together in the spanning tree, with the two ports very close to each other (Fig. 4), next to the four downstream river samples, and finally upstream river samples R0 and R1 located farther. R0 and LP1 as the least and most disturbed samples respectively were located in opposite extremes of the minimum spanning tree.

From our results, the localities with a lower environmental quality were the two larger lake ports (LP1 and LP2), with six and eight nuisance species, respectively, followed by the lake point L3 with five, then the other sampling sites with two nuisance species and the uppermost point R0 with none (Table 3). Thus, the lake ports could be considered hotspots of nuisance species.

Regarding the relationships between the biotic indicators of environmental quality considered, only one of them were significantly correlated after FDR correction (Table 4): there was a negative correlation between EPT and HABs ($r = -0.769$, 4 *d.f.*, $P = 0.009$). This correlation is expected since EPT is considered a positive indicator of environmental health and HAB is often correlated with habitat degradation. On the other hand, the effect of substrate artificiality was not clear—only two sites R1 and LP1 had artificial substrate. Noteworthy, a significant correlation was found between the number of human

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Table 2 Nuisance species found from eDNA in the Rhine basin around Lake Constance. A) Non-indigenous invasive metazoans; common name, geographical origin, most likely introduction pathway, link to references within the Invasive Species Compendium CABI (2019), accessed on September 2019. B) Algae species listed in IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae (Moestrup et al. 2009), reported harmful effects elsewhere, link in the reference list (accessed on September 2019). The number of sampling points within each sector where the species was found is given: U, LP, L and D are upstream, large port in the lake, lake, and downstream, respectively

A

Species	Common name	Native range	Distribution from eDNA in the studied area				Introduction pathway	Reference
			U	LP	L	D		
Dikerogammarus villosus	Killer shrimp	Ponto-Caspian	0	1	0	2	Interbasin transfers	https://www.cabi.org/isc/datasheet/108309#tosummaryOfInvasiveness
Limnomysis benedeni	Danube mysid	Ponto-Caspian	0	1	1	0	Interbasin transfers	https://www.cabi.org/isc/datasheet/108853
Craspedacusta sowerbyi	Freshwater jellyfish	China	0	1	1	2	Transported with aquatic plants	https://www.cabi.org/isc/abstract/20153351153
Corbicula fluminea	Asiatic clam	S & E Asia	0	1	0	0	Ship, ballast water	https://www.cabi.org/isc/datasheet/88200
Dreissena polymorpha	Zebra mussel	Ponto-Caspian	0	1	1	1	Artificial waterways, shipping	https://www.cabi.org/isc/datasheet/85295
Potamopyrgus antipodarum	New Zealand mudsnail	New Zealand	0	2	0	1	Recreational vessels, ballast water, aquatic trade	https://www.cabi.org/isc/datasheet/43672
Physella acuta	Freshwater snail	North America	0	1	0	0	Shipping, aquarium trade	https://www.cabi.org/isc/datasheet/116316
Oncorhynchus mykiss	Rainbow trout	North America, North Asia	1	0	0	0	Stocking, fish farming	https://www.cabi.org/isc/datasheet/71813

B

Species	Harmful effects	Distribution from eDNA in the studied area				Reference
		U	LP	L	D	
Alexandrium catenella	paralytic shellfish poisoning and fish mortality	0	1	1	0	Sakamoto et al. (1992) Mar. Ecol. Prog. Ser. 89, 229–235 Mackenzie (2014). Harmful Algae. 39, 161–164
Alexandrium ostenfeldii	paralytic shellfish poisoning toxins and spirolides	0	2	0	0	Hansen et al. (1992). J. Phycol. 28: 597–603 Salgado et al. (2015) Toxicon, 103, 85–98
Alexandrium tamarense	paralytic shellfish poisoning	1	2	1	0	Asakawa et al. (1995) Toxicon 33, 691–697
Karlodinium veneficum	broad-spectrum lytic effect on membranes	0	1	2	0	Bachvaroff et al. (2008). Harmful Algae 7, 473–484

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Table 3 Values of HTS-based biotic variables obtained in the ten sampling sites within Rhine River basin

	Upstream R0	Upstream R1	Port LP1	Port LP2	Lake L3	Lake L4	Downstream R2	Downstream R3	Downstream R4	Downstream R5
HABs	0	1	3	3	3	1	0	0	0	0
NIS	0	1	3	5	2	1	2	2	2	0
EPT	2	1	0	0	0	0	2	3	1	1
Native fish	1	1	1	1	2	0	1	2	0	0
Other native invertebrates	27	22	16	13	8	10	10	16	8	8
Other algae	7	6	2	4	2	0	3	3	4	2
Metazoan Shannon	1.186	1.185	1.667	1.709	1.631	1.547	1.802	1.567	1.516	1.215
Metazoan Simpson	0.64	0.592	0.792	0.809	0.781	0.777	0.809	0.715	0.76	0.667

The diversity indices Shannon and Simpson (1-D) calculated based on the number of species per Metazoan phylum are presented. HABs, NIS and EPT are harmful algae, non-indigenous species and Ephemeroptera-Plecoptera-Trichoptera, respectively

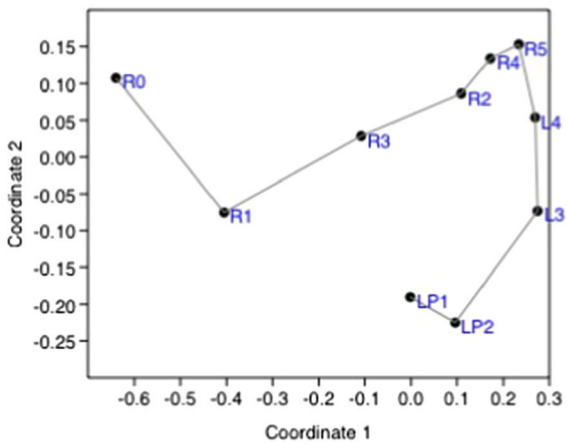

Fig. 4 Non-metric multidimensional scaling analysis of the biotic indicators and proxies. Scatter plot showing the minimum spanning tree constructed from Manhattan pairwise similarity indices is shown

inhabitants (population) and the number of NIS (r = 0.784, 8 d.f., P = 0.007). The number of human inhabitants was not significantly correlated with any of the other community features considered (Table 4).

Discussion

The results of the present study have revealed hotspots of nuisance species associated with large lake ports in Lake Constance. The accumulation of NIS in lake ports can be explained by the fact that ships facilitate the spread of exotic species, and the higher water temperature due to sheltered conditions in ports, can increase the survival of these exotics species (Strayer 2010; Gollasch and Nehring 2006). In our case study, the daily ferries crossing the lake may contribute to this transport (Gergs and Rothhaupt 2015). The HABs were used here as proxies of bad water quality, and were negatively correlated with the EPT—indicators of good water quality-, and positively correlated with NIS. All together, the results emphasize the role of ports as disturbed areas and shelters of nuisance species (Seebens et al. 2013). Moreover, we found a significant correlation between the number of NIS and the surrounding population density at a regional level. The same pattern has been found at both large (Pyšek et al. 2010) and regional (Spear et al. 2013) scales in other studies.

From the technical side, this case study illustrates the utility of eDNA to detect invasive aquatic species using next generation sequencing methods, as found in recent studies (Rius et al. 2015; Borrell et al. 2017). Except in one sample (R5), where only the 2.6% of the raw reads were taxonomically assigned with the strict criteria employed here, the proportion of assigned reads was around 12% for all the samples (Table S3). These values are similar to other HTS studies (Deiner et al. 2016), indicating that the overall molecular and data treatment procedures were generally good.

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Table 4 Pairwise correlations between the main HTS-based high and low quality environmental proxies employed in this study and the human population size (see Table 1) near the sampling sites

	HABs	NIS	EPT	Native fish	Population
HABs	–	0.034	*0.009*	0.335	0.129
NIS	0.670	–	0.311	0.424	*0.007*
EPT	− *0.769*	− 0.357	–	0.424	0.347
Native fish	0.341	0.286	0.286	–	0.964
Population	0.513	*0.784*	− 0.333	0.017	–

Pearson's r and their *P*-value are shown below and above the diagonal, respectively. Significant values after False Discovery Rate (FDR) corrections are shown in bold italics

Focusing on the invasive species, our study builds upon some of the invasions occurring in the Rhine basin. Regarding the invasive clam *Corbicula fluminea*, the first record of this clam in the Rhine River was in 1985 in the lower Rhine region in Netherlands. After that, it was recorded in Basel ten years later (1995), and it was later found in Rheinfelden (22 km upstream Basel) in 2003, but not any further. Thus, there was no record of the presence of this clam between Rheinfelden and Lake Constance (Schmidlin and Baur 2007). The first detection of this species in Lake Constance was in 2003 in a sandy shallow-water near Bregenz (Werner and Mörtl 2004). Our study locates the species in the Upper Lake Constance near Constance port (LP2). *Corbicula* larvae and small individuals can travel attached to avian feet or feathers and might be transported over large physical barriers (Schmidlin and Baur 2007). The singular conditions found in the ports, relatively sheltered and stable water level, might favor the survival of this species, since it has been shown that low temperatures and water level decreases produce massive mortality in *C. fluminea* (Werner and Rothhaupt 2008). Moreover, it has been suggested that climate change may benefit warm-water invaders (Rahel and Olden 2008; Chown et al. 2015). In this region, the average water temperature increased 0.22 °C per decade between 1965 and 2009 (Jeppesen et al. 2012); this change might explain the expansion of *C. fluminea* in the lake from Bregenz in 2003 (Werner and Mörtl 2004) to the other side of the lake (Constance) in 2017.

We also found the New Zealand mudsnail *Potamopyrgus antipodarum* in lake ports and downstream localities. This organism is able to travel through animal vectors (Alonso and Castro-Díez 2008), but our results strongly suggest it is transported associated with ships. In previous studies, Gergs and Rothhaupt (2015) found only two *P. antipodarum* individuals in 2005, none in 2006 and three in 2007 in Lake Constance. Therefore, our results suggest the species has expanded in the last decade. Unlike *C. fluminea*, *P. antipodarum* is able to survive winter conditions, since it tolerates water temperatures from 0 to 28 °C and even resists short times of desiccation (Moffitt and James 2012; Alonso and Castro-Díez 2012). Further surveillance together with rapid response would be convenient in order to control its spread.

It is worth to mention that we found relatively few fish species in our study: only eight native and one NIS (*O. mykiss*). Using eDNA, species may remain undetected due to the sampling strategy (Comtet et al. 2015). Here, sampling was performed at the shore of the river and the lake around 1-m depth. The sampling strategy was the same as that used in Ebro River (Clusa and Garcia–Vazquez 2018), which, like the Rhine River, is a big river with high flow and rapid current speed. Indeed, it is possible that the DNA of some species, especially for those fish swimming far from the shore, was at very low concentration and remained undetected. For a more detailed species inventory based on eDNA, samples should also be obtained from many points inside the lake and the stream, and at different depths, ensuring a good coverage of the habitats surveyed, but this was beyond the scope of this work.

The lack of detection of a species from HTS could be also due to the primer bias; the primers used to build HTS libraries might have different affinity for the species present in the samples (Deagle et al. 2014). The COI gene primers employed in our study

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amplified a high proportion of arthropods and Porifera species, similar to the results of Leray et al. (2013) and Deiner et al. (2016). Data processing may also produce some false negatives (Thomsen and Willerslev 2015), and better reference databases are needed since the scarcity of references from many taxonomic groups has been pointed out as the main limitation to assign HTS sequences (Comtet et al. 2015; Goldberg et al. 2016). False negatives can also result from failures in the sequencing process (Kelly et al. 2014; Thomsen and Willerslev 2015), in the PCR conditions (Ushio et al. 2017; Pochon et al. 2013) or even in the amount of DNA released by the different species of the environment (Minamoto et al. 2017). The use of several samples to build HTS libraries and several genes as metabarcodes is recommended to diminish the errors mentioned (Kelly et al. 2014; Shaw et al. 2016). This is a limitation of our study, based only on one sample per point and one metabarcode. However, and despite this flaw, the results allowed to detect eight NIS and to confirm the presence of *P. antipodarum* and *C. fluminea* in Lake Constance. Surely more replicates and metabarcodes will give a better global vision of the real biodiversity of the Rhine basin.

The studied zones of the Rhine basin contain many dams, and this feature may have implications on the diversity patterns observed. Dams may prevent the arrival of exotic species as many authors have described (Fausch et al. 2006; McLaughlin et al. 2007), for instance, Dana et al. (2011) stopped the expansion of the invasive crayfish *Procambarus clarkii* in a Mediterranean stream by constructing small dams. Dams may also work as refuges for imperiled native species (Beatty et al. 2017). But, at the same time, they block the migration route of diadromous species and can cause the decrease of diversity and abundance upstream (Nislow et al. 2011; Limburg and Waldman 2009; Britton et al. 2011). Despite the presence of barriers in the High Rhine region (12 hydropower dams and a 23-m waterfall) many species have colonized Lake Constance by unknown routes (Eckmann et al. 2008). In our case study, lake samples contained a higher proportion of NIS than downstream samples, therefore, the role of dams for preventing biological invasions is not clear here. Conversely, the presence of dams altered water temperatures and flow regimes in High Rhine and generated a suitable environment for the invasive goby *Neogobius melanostomus* (Kalchhauser et al. 2013). In the case of *Physella acuta* and *C. fluminea* in the lake, their possible original introduction could be aquarium releases (Schmidlin and Baur 2007). Moreover, recreational activities can aid in the dispersion of invasive species in this basin; for example, in the High Rhine region the river and lake are crossed by recreational boats that could work as a transport for exotic species, such as round gobies (N'Guyen et al. 2016) as well as for exotic invertebrates attached to the boat hull or in bilge water to other water bodies in the region (Ricciardi 2015; De Ventura et al. 2016).

The upstream location R0 was the only sampling point where the native brown trout (*Salmo trutta*) was found, no NIS was detected, and the diversity index was as low as in R1 (Table 3). It is becoming more evident that invasive species tend to accumulate in degraded areas near human populations (Havel et al. 2015; Johnson et al. 2008; Spear et al. 2013), therefore, the absence of NIS in R0 –with low population density nearby- could be explained from a relatively lower anthropogenic influence.

Conclusions

The ports and sites near big urban areas were identified as potential hotspots of NIS in the region, therefore, better management measures should take into account the surveillance of these areas to avoid the spread of the invasive species already established in the region, and also the surveillance of recreational boats would be advisable. They could spread these NIS to other water bodies nearby, especially in this region with high number of tourists in summer who visit multiples lakes over a short period of time. Prevention measures have to be focused on human behaviour; educational efforts should reduce intentional releases. Additionally, stricter regulations of ornamental species and aquaculture would be desirable in order to reduce contamination of stocks and pet releases. Any garden pond or aquarium might represent a potential threat especially when global warming is causing the increase of winter water temperatures which would promote the establishment of ornamental species. Finally, it is highly advisable to establish a common regulation and management actions by all the countries implied in the region.

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Data availability The data that support the findings of this study is provided as Supplementary Material.

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