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Advances in the use of molecular tools in ecological and biodiversity assessment of aquatic ecosystems

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ABSTRACT

Advances in the use of molecular tools in ecological and biodiversity assessment of aquatic ecosystems

Conservation and sustainable management of aquatic ecosystems is a priority in environmental programs worldwide. However, these aims are highly dependent on the efficiency, accuracy and cost of existent methods for the detection of keystone species and monitoring of biological communities. Rapid advances in eDNA, barcoding and metabarcoding promoted by high-throughput sequencing technologies are generating millions of sequences in a fast way, with a promising cost reduction, and overcoming some difficulties of the traditional taxonomic approaches. This paper provides an updated broad perspective of the current developments in this dynamic field presented in the special session (SS) "The use of molecular tools in ecological and biodiversity assessment of aquatic ecosystems" of the XIX Congress of the Iberian Association of Limnology (AIL2018), held in Coimbra, Portugal.

Developments presented are mainly focused on the Iberian Peninsula (Portugal and Spain, including Atlantic Macaronesian islands) but include studies in France, Germany, Finland, Russia (Siberia) and South America. The networks within which these researchers are involved are yet even broader, profiting from existing molecular facilities, and traditional taxonomic expertise, which can be viewed as a characteristic of this new research area. It was evident in the SS that the use of molecular tools is widespread, being used to study a diversity of aquatic systems, from rivers' headwaters to estuaries and coastal lagoons, and volcanic, mountain and frozen lakes to hot springs. The organisms targeted are likewise varied and include fish, macroinvertebrates, meiofauna, microalgae such as diatoms and dinoflagellates, other protists, fungi, and bacteria (cyanobacteria and other). Some studies address the whole biodiversity (i.e., all species present independently of the taxonomic group) from environmental samples of water, biofilms and preservative solution from field samples (e.g., ethanol from macroinvertebrate samples). Great advances were acknowledged in the special session, namely in the use of metabarcoding for detecting hidden biodiversity, juvenile stages, low-abundance species, non-indigenous species and toxicity potential, and ultimately for ecological monitoring of diatoms and invertebrates. Yet, several drawbacks were highlighted and need further work, which include: taxonomic gaps in the reference databases (including gaps at species level and on intraspecific variability) or absence of public databases (e.g. for meiofauna), still high sequencing costs, the need of a substantial bioinformatics effort, difficulties in establishing the amount of environmental sample necessary for a good DNA extraction and the need for testing different genetic markers to obtain accurate results.

Key words: eDNA, metabarcoding, conservation, ecological quality, species detection, rivers, lakes, thermal springs, estuaries, lagoons

RESUMO

Avanços no uso de ferramentas moleculares na avaliação ecológica e biodiversidade dos ecossistemas aquáticos

A conservação e gestão sustentável dos ecossistemas aquáticos é uma prioridade nos programas ambientais em todo o mundo. No entanto, esses objetivos são altamente dependentes da eficiência, precisão e custo dos métodos existentes para detectar espécies e monitorizar comunidades biológicas. Avanços recentes no que respeita ao ADN ambiental e 'barcoding' e 'metabarcoding', promovidos por tecnologias de sequenciação designadas 'high-throughput sequencing', têm gerado milhões de sequências de forma rápida, com uma promissora redução de custos num futuro próximo, e superando algumas dificuldades das abordagens taxonómicas tradicionais. Este artigo vem fornecer uma perspetiva atualizada e abrangente dos desenvolvimentos neste campo que foram apresentados na sessão especial (SE) "O uso de ferramentas moleculares na avaliação ecológica e da biodiversidade dos ecossistemas aquáticos", no XIX Congresso da Associação Ibérica de Limnologia (AIL2018) realizado em Coimbra, Portugal.

Os desenvolvimentos apresentados centram-se principalmente na Península Ibérica (Portugal e Espanha, incluindo as ilhas atlânticas), mas também em França, Alemanha, Finlândia e Rússia (Sibéria). No entanto, as redes em que estes investigadores estão envolvidos são ainda mais amplas, aproveitando as infraestruturas moleculares e o conhecimento taxonómico existentes. Ficou claro na SE que o uso de ferramentas moleculares está disseminado, sendo usado numa diversidade de sistemas aquáticos, desde as cabeceiras dos rios aos estuários e lagoas costeiras, e desde lagos vulcânicos, de montanha e congelados, a fontes

termais. Os organismos estudados são também variados e incluem peixes, macroinvertebrados, meiofauna, microalgas tal como diatomáceas e dinoflagelados, outros protistas, fungos e bactérias (cianobactérias e outros). Alguns estudos abordam toda a biodiversidade a partir de amostras ambientais de água, biofilmes e solução conservante. Grandes avanços foram reconhecidos na sessão especial, nomeadamente no uso de 'metabarcoding' para a deteção de biodiversidade críptica, estádios juvenis, espécies de reduzida abundância, espécies não nativas, do potencial de toxicidade e, finalmente, para a monitorização ecológica de diatomáceas e invertebrados. No entanto, dificuldades também foram assinaladas, que necessitarão de mais investimento futuro, e que incluem: lacunas taxonómicas das bibliotecas de referência (incluindo ao nível da espécie e da intra-variabilidade de espécies), ausência de bibliotecas públicas (por exemplo, para meiofauna), altos custos de sequenciação, a necessidade de um esforço substancial de bioinformática, dificuldades em estabelecer a quantidade de amostra ambiental necessária para uma boa extração de DNA e a necessidade de testar diferentes marcadores genéticos para obter resultados precisos.

Palavras chave: *eDNA, metabarcoding, conservação, qualidade ecológica, detecção de espécies, rios, lagos, fontes termais, estuários, lagoas*

INTRODUCTION

Biological diversity means the variability among living organisms from all sources including terrestrial, marine and other aquatic ecosystems and ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems (Wilcox, 1984). Biodiversity reflects the ecosystem's health and resilience to withstand and recover from a variety of disturbances. Therefore, it is essential to discover and understand the biodiversity present in a certain study area, which is a challenging task. Most of the traditional approaches for assessing biodiversity, where species are identified based on their morphological characters, are time-consuming, expensive and require high taxonomic expertise (Leese *et al.*, 2016). On the other hand, rapid assessment based on an estimation of the abundance and distribution of target species through

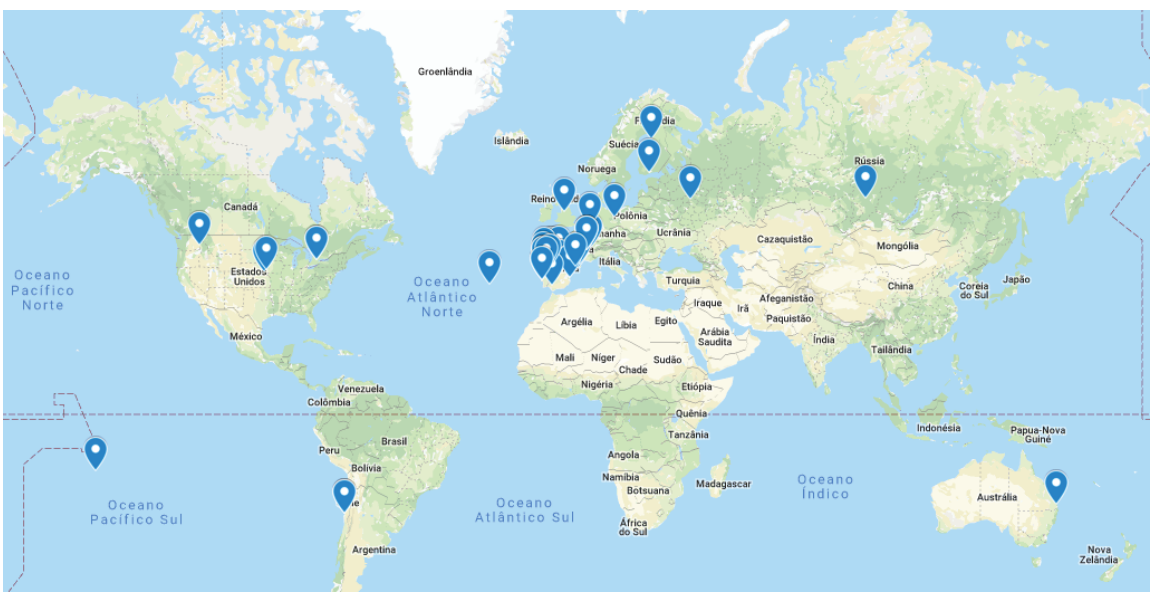

Figure 1. Distribution of the contributors to the special session "The use of molecular tools in ecological and biodiversity assessment of aquatic ecosystems" of the XIX Congress of the Iberian Association of Limnology (AIL2018) in the World. Image produced in Google Maps (2019). *Distribuição dos autores da sessão especial "O uso das ferramentas moleculares na avaliação ecológica e biodiversidade dos ecossistemas aquáticos", do XIX Congresso da Associação Ibérica de Limnologia (AIL2018) no mundo. Imagem produzida no Google Maps (2019).*

molecular tools may be conducted in a short time more cheaply and easily (Minchin *et al.*, 2016). For instance, using species-specific DNA markers, the presence of one target species from water samples can be detected using PCR and simple electrophoresis in agarose gel. This is an efficient and convenient approach when the target species is known because it is a reproducible, fast and a cost-efficient method (Ardura *et al.*, 2015a; Clusa *et al.*, 2016; Devloo-Delva *et al.*, 2016; Ardura *et al.*, AIL2018).

The special session "The use of molecular tools in ecological and biodiversity assessment of aquatic ecosystems" of AIL2018 (XIX Iberian Association of Limnology meeting in Coimbra, Portugal, June 2018) aimed to present and discuss recent studies undertaken in the Iberian Peninsula, other European countries and South America, in order to promote knowledge exchange and envisage on future research directions in this area. The authors represented 13 countries and 46 institutions (including research institutions, official agencies and companies), which highlights the fast development of this area around the world and the importance of broad networks in the advancement of this particular research field (Fig. 1).

The different ways of using molecular approaches in the context of ecological and biodiversity assessment in aquatic ecosystems highlighted in the studies presented in the SS (Table 1) were synthesized in the section "Perspectives on the use of molecular tools." From those studies, we extracted the main contributions for the area (section "Main findings"), as well as the main problems and gaps identified by the researchers (section "Main drawbacks") and ended with general inferences and future research directions (section "Conclusions").

PERSPECTIVES ON THE USE OF MOLECULAR TOOLS

I. Improvement of biodiversity detection and biological quality monitoring with molecular tools

Biodiversity

Molecular tools are particularly useful to assess the diversity of concealed communities, allowing a more accurate species detection and distribution in a specific ecosystem. This is the case of the meiofauna, which comprises organisms between 30-1000 μm (Higgins & Thiel, 1988). Due to their small size, morphotaxonomic inventories can largely fail to identify accurately (Alves *et al.*, 2015). Various taxonomic meiofaunal groups of an estuary in the North of Portugal have been detected by a target region (Fais *et al.*, AIL2018). Phytoplankton and general microeukaryotic plankton dynamics under the formation of ice-and-snow cover were studied in a Siberian mountain lake through molecular techniques (Díaz-Quijano *et al.*, AIL2018).

Other examples of detection of small organisms are the microalgae dinoflagellates or diatoms, which have additionally high morphological similarities and lack of unique characteristics between different species (Lin *et al.*, 2009). The eDNA (environmental DNA) analysis has been used in French coastal lagoons to detect a set of signal species using mitochondrial cytochrome oxidase I gene (COI), such as, 21 genera of Dinoflagellates and 9 genera of diatoms, including *Chaetoceros* and *Nitzschia* involved in harmful algal blooms (HABs); and invasive invertebrate species (barnacles, copepods, polychaeta and ascidians), some of them being pollution indicators (*Polydora cornuta*, *Ficopomatus enigmaticus* and *Hydroides elegans*) (Ardura *et al.*, AIL2018).

Ecological impact of algal toxicity is also being investigated through molecular tools (Cordeiro *et al.*, AIL2018). Toxins are transferred along the food chain, from different microalgae (mainly Dinoflagellates, Cyanobacteria, and Diatoms) and HABs can be responsible for massive fish mortality (Thangaraja *et al.*, 2007), while the presence of toxins in fish or shellfish can cause severe human diseases (e.g., diarrheic shellfish poisoning). In the Azorean archipelago (Portugal), the potential for cyanotoxin production was assessed in thermal environments and freshwater lakes, which are common in these volcanic islands. The confirmation of cyanobacteria's DNA and potential risk of cyanotoxin production in the eDNA samples (Cordeiro *et al.*, AIL2018), revealed to be an efficient method for monitoring these ecosystems and help to prevent threats to public and environmental health (Pear-

son & Neilan, 2008; Salmaso *et al.*, 2017).

Genetic tools have been increasingly used for studying invasions, because it allows species identification (e.g. Ardura *et al.*, 2010; Ardura & Planes, 2017), determination of the region of origin (Ardura *et al.*, 2013) and time of initial incursion of non-indigenous species (Hilbish *et al.*, 2000; Rius *et al.*, 2014; Teske *et al.*, 2014). This is especially important as the number of introduced species has been increasing during the last decades, in freshwater ecosystems (Elvira & Almodóvar, 2001; Anastácio *et al.*, 2018). One example is the minnow species (*Phoxinus* genus), a freshwater fish that has been used as live bait since the 1900s. Individuals were sampled in the Douro basin (Portugal) and morphologically identified as *Phoxinus bigerri*, a common minnow in the Iberian Peninsula. Nevertheless, barcoding showed that the population caught closer to the Atlantic Ocean is phylogenetically closer to *Phoxinus phoxinus* from Charente river in France, confirming for the first time the presence of this species in the Douro basin (Garcia-Raventós *et al.*, AIL2018).

Apart from the tools used for single and mixed-organism samples, other sources of DNA have been explored for faster biodiversity assessment such as, DNA from sediment samples, water or sample preservation liquids (e.g., Aylagas *et al.*, 2016; Deiner *et al.*, 2017; Hajibabaei *et al.*, 2012). These approaches avoid the traditional sampling protocols that require a large investment in human resources with many specialists studying different biological elements. In these cases, DNA is extracted directly from environmental samples (e.g., water) followed by high-throughput sequencing (HTS) metabarcoding. Taking into account previous results of DNA extraction directly from the water (Ardura *et al.*, 2015a; Zaiko *et al.*, 2015; Ardura & Planes, 2017) a HTS tool was developed to obtain a baseline of biodiversity from 10 different coastal lagoons (Ardura *et al.*, AIL2018).

Alternatively, Martins and collaborators (CIBIO/InBIO University of Porto, Aqualogus company and Polytechnic Institute of Bragança) are exploring the option of DNA metabarcoding from preservative ethanol of freshwater macroinvertebrate samples (Martins *et al.*, AIL2018; Martins *et al.*, 2019). This approach requires following the Water Framework Directive (WFD; European Union 2000) sampling protocols but avoids the sorting step of separating animals in a sample from vegetation, sediment and litter, which is very time-consuming. The authors are examining the performance of different laboratory procedures on species detection based on the preservative liquid, and compared taxa recovery with the conventional morphological method. More than half of the taxa found in ethanol were macroinvertebrates targeted by WFD, while the remaining percentage was identified as, e.g., bacteria, Stramenopiles, terrestrial invertebrates, amphibians and fishes (Martins *et al.*, AIL2018; Martins *et al.*, 2019).

Biological quality monitoring

The use of molecular tools in biological quality monitoring is becoming more and more realistic and several studies highlighted its potential (e.g., Filipe *et al.*, 2018; Filipe *et al.*, AIL2018). Comparison between morphology and metabarcoding-based approaches to determine species composition at estuarine sites indicated that species richness, one of the metrics frequently used in bioassessment, would be considerably underestimated if only morphological methods were used (Lobo *et al.*, 2017a).

In the ecological quality assessment of rivers, diatoms are one of the obligatory elements, according to the WFD. Thus, a considerable effort has been made to develop diatom metabarcoding and optimize different stages of the process (choice of primers, Kermarrec *et al.*, 2014; diatom barcode database, Rimet *et al.*, 2016; DNA extraction, Vasselon *et al.*, 2017a; quantification bias, Vasselon *et al.*, 2018; bioinformatics treatment, Coissac *et al.*, 2012). In France, diatom metabarcoding has been applied successfully at small (80 samples, Vasselon *et al.*, 2017b; Rivera *et al.*, 2020) and larger monitoring networks (447 samples). In rivers of Central Portugal, the comparison between the Portuguese official monitoring index for diatoms (IPS – *Indice de Polluosensibilité Spécifique*), calculated based on morphological identification data and on Operational Taxonomic Units (OTUs) converted into species data, showed a high correlation

Table 1. Biological groups, water bodies and barcode genes assessed in studies presented in the special session "The use of molecular tools in ecological and biodiversity assessment of aquatic ecosystems" of the XIX Congress of the Iberian Association of Limnology (AIL2018). *Grupos biológicos, massas de água e barcodes analisados nos estudos apresentados na sessão especial "O uso das ferramentas moleculares na avaliação ecológica e biodiversidade dos ecossistemas aquáticos", do XIX Congresso da Associação Ibérica de Limnologia (AIL2018).*

Biological group	**Type of water body/location**	**Barcode gene**	**Reference**
Total biodiversity – eDNA (water)	Coastal lagoons of Gulf of Lyon - France	COI, 18S	Ardura *et al.,* AIL2018
Total biodiversity – eDNA (water, sediment)	Rivers and estuaries – Pas, Asón, Miera rivers (Cantabria), Douro, Ebro	COI, 18S, 16S	Sainz-Bariáin *et al.,* AIL2018
Fish	Rivers – Douro catchment	12S – MiFish region	Filipe *et al.*, AIL2018
Fish (non-indigenenous species) – *Phoxinus phoxinus*	Rivers - Douro catchment	COI, Cytb	Garcia-Raventós *et al*., AIL2018
Macroinvertebrates and eDNA (ethanol)	Rivers – Tua (Douro catchment)	COI	Martins *et al.,* AIL2018
Macroinvertebrates	Rivers – Spain (Mediterranean rivers), Finland and Germany	16S	Pujante *et al.,* AIL2018
Macroinvertebrates and eDNA (water)	Rivers – Lobregat, (Mediterranean river, Catalonia)	COI	Múrria *et al.,* AIL2018
Diatoms	Rivers – central Portugal	*rbcL*	Mortágua *et al.,* AIL2018; Mortágua *et al.,* 2019
Diatoms	Lakes – Bourget, France	*rcbL*	Rivera *et al.,* 2018; Rivera *et al.* AIL2018
Biofilms (bacteria, fungi, microalgae)	Rivers (mesocosms)	16S, 18S	Calapez *et al., AIL2018*; Calapez *et al.,* 2019
Phytoplankton	Mountain lake - Oiskoe, Siberia	18S	Díaz-Quijano *et al*., AIL2018
Algae (toxicity, Cyanobacteria)	Thermal waters and freshwater lakes – Azores islands	16S and sxtA, sxtI, sxtH, sxtG for saxitoxinas, anaC, anaF for anatoxina, and mcyC, mcyD, mcyE, mcyG for microcistina	Cordeiro *et al*., AIL2018
Meiofauna (sediment)	Estuary – Lima river, Portugal	COI, 18S	Fais *et al.,* AIL2018

(Mortágua *et al.*, AIL2018). Besides, more than half (ca. 56 %) of the samples shared the same water quality class either using the conventional or the molecular approach. These results show the potential for adaptation of present taxonomic indices to molecular data, as it was concluded in studies in Mayotte island, France (Vasselon *et al.*, 2017b) and in the UK (Kelly *et al.*, 2018).

The benthic invertebrates are another compulsory quality element of the WFD. In Portugal, five sites sampled in Tua river (Douro basin) were classified to the same quality status through both morphological identification and ethanol-based DNA metabarcoding (Martins *et al.*, AIL2018; Martins *et al.*, 2019) when applying the Iberian Biological Monitoring Working Party (IBMWP) index with presence/absence data, at family level (Alba-Tercedor *et al.*, 2002). However, only about half of the species identified by metabarcoding were detected by morphology, whereas the former missed about 20 % of the species identified morphologically, corresponding to taxa with a low frequency (< 5 individuals).

In Valencia, the Laboratorios Tecnológicos de Levante (Pujante *et al.*, AIL2018) in the context of the European project BIOWAT-KIT (DNA-based kit for biodiversity assessments and biomonitoring of European water bodies), are developing and validating a genomic tool for the identification and assessment of diversity of benthic invertebrate communities in Europe, with the aim of improving and facilitating the bioassessment. An audit (made by taxonomists) to an official European freshwater monitoring program, based on macroinvertebrate samples, revealed that 29-30 % of the specimens had been overlooked by the primary taxonomists (Haase *et al.*, 2010). For 16 % of the samples, these discrepancies led to different final ecological assessment and demonstrated the need for adequate quality control and auditing in freshwater monitoring. Múrria and collaborators (University of Barcelona, Spain and Salford, Manchester, UK) used metabarcoding techniques to compare the estimates of the ecological status using traditional morpho-taxonomy against high-throughput DNA sequencing of: 1) bulk sampling (after sorting individuals from multi-habitat Surber samples), 2) eDNA (water samples) and 3) invertebrate drift sampling (intervals of 1 hour). Results showed that while the traditional and bulk sampling approaches detected essentially riverine species, the eDNA also captured terrestrial associated fauna (Múrria *et al.*, AIL2018).

Development of indices based on molecular information for the monitoring of aquatic ecosystems (i.e., ecological status or conservation status) is the purpose of the work developed at the University of Cantabria. Yet here, the main goal is a global assessment of water bodies through eDNA from water and sediment (Sainz-Bariáin *et al.*, AIL2018). Additionally, the study of bacterial diversity and primary producers through metagenomics is aimed, which could give complementary information on ecosystem functions (e.g., organic matter degradation or primary production under different conditions).

Molecular analysis constitutes, in addition, a simpler way of analysing the impact of anthropogenic and natural alterations in complex communities composed of microorganisms. A study in mesocosms run by Calapez and collaborators (Universities of Aveiro and Coimbra, Portugal) analysed stream biofilm responses to multiple-stressors typical of Mediterranean streams and found biofilm community shifts induced by flow stagnation, organic loads and grazing activity. Specifically, the OTUs determination helped to investigate how biofilm microbial communities' proportions changed under the different stressor combinations more quickly. The interaction of those three stressors altered algae, fungi and bacteria diversity proportions within the biofilm, with a synergistic effect on fungal diversity, while algae and bacteria had an antagonistic response to stressors' interaction (Calapez *et al.*, AIL2018).

II. Molecular analysis in aquatic water bodies

Different aquatic systems have been studied through molecular techniques by the teams present in the SS: rivers and streams, lakes, thermal waters and estuaries and coastal lagoons.

Rivers and streams

Rivers of NW Iberian Peninsula (Portugal and Spain) have been studied under the FRESHING

project (Next-generation biomonitoring: freshwater bioassessment and species conservation improved with metagenomics) by CIBIO/InBIO, covering up to 150 sampling sites (Filipe *et al.*, AIL2018). Each site was sampled using conventional methods along with water sampling from different microhabitats in order to maximize the detection of several taxa present in the water body through eDNA. However, results shown in the special session focused on freshwater fish. In central Portugal, the studies of the University of Coimbra and Aveiro and partners from INRA Thonon, France, include 88 sites located in the catchments of rivers Vouga, Mondego and Lis in a total area of 11 215 km^2. These sites were sampled for algae and macroinvertebrates, but present results report to diatoms only (Mortágua *et al.*, AIL2018; Mortágua *et al.*, 2019).

In the BIOWAT-KIT project, three rivers from each country (Spain, Finland and Germany) have been selected to test a genomic tool across different European regions covering a variety of climatic and geomorphological conditions. In Spain, the rivers are typically Mediterranean with different characteristics: Júcar is a calcareous mountain river; Mijares is a low-mountain river with high mineralization; while the Turia river is a low altitude river (Pujante *et al.*, AIL2018). Another Mediterranean river from Catalonia, the Llobregat (156 km in length), was studied by Múrria and collaborators, which covers a gradient of pollution and anthropogenic impact. This is a well-studied river (Munné & Prat, 2004, 2011), which includes a pollution gradient from pristine headwater reach, through site located downstream of a big reservoir or salt mining, to urban and agricultural landscapes at lowlands. A sampling of macroinvertebrates was done in 5 sites along the river (Múrria *et al.*, AIL2018).

In Cantabria, two rivers, Pas and Asón (Spain), with temperate hyper-oceanic climate with sub-Mediterranean characteristics were studied with molecular tools to compare diversity under pristine and polluted conditions. In addition, water and biofilm samples were recently collected from 96 river sites belonging to the Douro, Ebro and Cantabrian basins (Spain). These sites were sampled to determine the total biodiversity from microorganisms to vertebrates and are currently being identified (Sainz-Bariáin *et al.*, AIL2018).

Lakes

The studies presented in the SS addressed a wide diversity of freshwater lakes. The Azores archipelago (Portugal) located in the North Atlantic Ocean is composed of nine islands, which are very important and unique in terms of biodiversity, climate, volcanic activity and geomorphology (Antunes & Rodrigues, 2011). Fifteen freshwater lakes from the Archipelago of the Azores in São Miguel, Pico, Flores and Corvo islands were studied to investigate cyanotoxin production potential.

In France, diatom metabarcoding has been applied to assess the structure of diatom community and the ecological status of the littoral zone of Lake Bourget (deepest French lake). The structure of the assemblages based on the morphological (taxa lists) and molecular (OTUs lists) identification of diatoms were well correlated. However, the ecological status of the lake varied between these two methods since floristic inventories differed significantly (Rivera *et al.*, 2018; Rivera *et al.*, AIL2018). The main reason for this discrepancy was the incompleteness of the diatom reference database (Diat.barcode, formerly called R-Syst::diatom in Rimet *et al.*, 2016).

In Cantabria, five mountain lakes were sampled for molecular analysis of environmental samples (water and sediment). The first is located at ca. 1870 m of altitude in the Liordes Valley, a unique ecosystem in the Picos de Europa massif, located in a glacial-karst depression surrounded by calcareous walls. The Lloroza lakes (ca. 1800 m of altitude) are small lagoons of karstic nature located in *Picos de Europa* National Park in the Cantabria province. Finally, the Enol and Ercina (at ca. 110m of altitude) are two glacial lakes forming the Covadonga lakes located within the *Picos de Europa* National Park in the Asturias province. These samples are still being processed (Sainz-Bariáin *et al.*, AIL2018).

In Siberia, the Oiskoe mountain lake is being studied with phytoplankton samples through metabarcoding from a conservation perspective (Diaz-de-Quijano *et al.*, AIL2018). Located in the Ergaki Natural Park, West Sayan Mountains, is a

poorly studied area due to its extreme climate with a wide range of annual temperatures (-41 °C to +32 °C). The lake is surrounded by a mosaic landscape of bogs, sparse taiga forest, scree and alpine tundra and biodiversity has particular adaptations to these conditions (Anishchenko *et al.*, 2015). However, human activities, namely tourism and global warming in South Siberia and Central Asia, are the present threats to these ecosystems.

Thermal waters

In São Miguel island, in the Azorean archipelago, environmental samples were collected from 21 thermal sites, including hot springs, thermal pools and ponds, thermal streams and hydrothermal vents, with temperatures ranging from 28 °C to over 90 °C (Cordeiro *et al.*, AIL2018). Cyanobacteria were isolated from these samples and deposited in BACA-Banco de Algas e Cianobactérias dos Açores (Universidade dos Açores), which is part of REBECA (Red de excelencia en biotecnología azul (algas) de la región de la Macaronesia). From the 40 strains isolated, 24 strains and environmental samples were targeted for cyanotoxin production potential through conventional PCR. Preliminary results show that none of the studied cyanobacteria strains have cyanotoxin production potential (Cordeiro *et al.*, AIL2018).

Estuaries and coastal lagoons

Finally, studies have been undertaken in estuaries and coastal lagoons. A proof-of-concept study (Lobo *et al.*, 2017b) on the application of DNA metabarcoding for monitoring estuarine macrozoobenthic communities has been conducted in the Sado estuary (SW Portugal). The metabarcoding approach was able to discriminate macrozoobenthic communities among sampling sites successfully and provided biotic index levels comparable to the morphology-based approach (Lobo *et al.*, 2017b). Up north, in river Lima (NW Portugal), the estuarine area has become an important Portuguese harbour, used for commercial navigation and fishing activities and is subjected to constant dredging as well as the input of agricultural run-off and urban and industrial sewage (Sousa *et al.*, 2007). The University of Minho (Portugal) team is monitoring meiofauna communities of this estuary through metabarcoding, annually, whose preliminary results were presented at the AIL conference (Fais *et al.*, 2018, AIL2018).

In Cantabria, five sediment and five water samples were taken from 3 estuaries (Pas, Miera, and Asón) characterized by large intertidal surfaces and dominated by the tidal dynamic, making them well-mixed estuaries. This coast is subjected to various anthropogenic pressures. These sites have been sampled to determine general biodiversity through molecular analysis.

The team from the University of Oviedo (Spain) has been using metabarcoding (eDNA) to determine the biodiversity and detect particular organisms in the coastal lagoons of Gulf of Lyon, in the French Mediterranean coast (Ardura *et al.*, AIL2018). Ten lagoons were analysed: Berre, Beaduc, Bages-Sigean, La Palme, Leucate, Mejean, Prevost, Thau, Vic and Canet. These ecosystems provide habitat for many species, nursery areas and feeding grounds for marine and estuarine fish (Perez-Ruzafa *et al.*, 2011). They support important fisheries and allow for intensive aquaculture exploitation (Cataudella *et al.*, 2015). Despite most of them being under protection, they still suffer from several threats derived from human activities such as pollution, eutrophication, climate change and introduction of non-native species (≈100 non-indigenous species were identified; Reizipoulou *et al.*, 1996; Chapman, 2012).

III. Selection of adequate barcode genes for each group of organisms

The selection of barcode genes varies with the target taxonomic group studied and the focus of the studies. The researchers took different options in the studies presented in the SS:

COI

The DNA barcode region elected most frequently for the identification of individualized specimens is a fragment of the mitochondrial COI gene (Herbert *et al.*, 2003). The Cytochrome c (COI) is an amino acid sequence that is highly conserved

in eukaryotes, differing by only a few residues. There are robust universal primers for it that recover most animal phyla, and thousands of reference sequences are available in public databases such as BOLD and GenBank (Ratnasingham & Hebert, 2013; Hebert *et al.*, 2013). However, the high variability in the third position of the COI codons makes it difficult to design universal primers for metabarcoding DNA studies (Ficetola *et al.*, 2010). For fish identification, most used barcode markers in DNA reference collections are the COI and cytochrome b (Cytb) genes, other mitochondrial genes, which can confirm taxonomic identification at the species level. However, some studies are showing that COI might not be the best option for assessing and monitoring freshwater fish diversity using environmental DNA from water because this marker might not contain suitably conserved regions (e.g., Deagle *et al.*, 2014). Instead, the potential of using the MiFish region from the ribosomal 12S is under consideration (Miya *et al.*, 2015; Filipe *et al.*, AIL2018).

For Iberian freshwater macroinvertebrates, public repositories for COI DNA barcodes cover 35 % of the taxa (3348 morphospecies) (Múrria *et al.*, AIL2018, Múrria *et al.*, 2020). However, this coverage is highly variable across taxonomic groups. For instance, Odonata (79 species, 54.43 %), Hemiptera (81 species, 54.32 %), Mollusca (65 species, 53.85 %), Trichoptera (390 species, 50.77 %) and Crustacea (10 species, 50.5 %) were the best-represented groups, whereas Diptera (1693 species, 23.21 %), and Plecoptera (135 species, 31.11 %) were the less barcoded orders. Portuguese invertebrate communities sampled were also processed for metabarcoding using a small COI fragment (313bp) by Martins and collaborators in CIBIO (AIL2018; Martins *et al.*, 2019). The HTS data were identified against the invertebrate collection of the InBIO Barcoding Initiative (at CIBIO-UP) that includes hundreds of specimens of macroinvertebrate taxa from northeast Portugal.

Macroinvertebrates and marine fish have been the target of comprehensive DNA barcoding campaigns across multiple coastal ecosystems in continental Portugal. The primary marker was the COI, occasionally supplemented by other markers (e.g., Borges *et al.*, 2012). In the Lima estuary, DNA from meiofauna communities was extracted from intertidal sediments. In this case, the target genes were the COI and 18S ribosomal RNA (18S rDNA) gene. MiSeq amplicon sequences were processed in mothur (version 1.39.5, Schloss *et al.*, 2009) by using appropriate bioinformatic procedures; while the taxonomy of the processed sequences were assessed by blasting against the full ntNCBI database (Fais *et al.*, AIL2018). This database was chosen due to the lack of adequate reference sequences in better-known databases, such as BOLD (Ratnasingham & Hebert, 2007) and Silva (Pruesse *et al.*, 2007). In the French coastal lagoons, the invertebrate communities were as well analysed from eDNA with COI marker.

18S, rbcL and 16S

In the project BIOWAT-KIT a preliminary evaluation of different genomic regions using publicly available sequence data was carried out in order to identify the best-suited DNA barcode marker for the identification of 141 families of invertebrates belonging to four different phyla (Platyhelminthes, Annelida, Mollusca, and Arthropoda). Several primer pairs have been designed, including a degenerate primer pair and a cocktail of group-specific primers, which will presumably amplify all the target invertebrate taxa present in freshwater samples. Based on the results, the mitochondrial 16S gene was selected for the DNA metabarcoding analysis of freshwater invertebrate communities within this project, since it combines both conserved regions suitable for primer design, and variable regions with good taxonomic resolution at the family level (and potentially, also at the genus or species level) (Pujante *et al.*, AIL2018).

In Thonon (France), the INRA team targeted several genes for diatoms (18S, COI, *rbcL*) (Kermarrec *et al.*, 2013). While COI is found in mitochondrial DNA of eukaryotic organisms, the 18S is part of the ribosomal RNA of eukaryotes and the ribulose-1,5-bisphosphate carboxylase/oxygenase (*rcbL*) is present in plants chloroplasts. The *rcbL* showed to be the most suitable barcode for biomonitoring purposes with diatoms (Ker-

marrec *et al.*, 2013; Kermarrec *et al.*, 2014; Pawlowski *et al.*, 2016). Thus, DNA metabarcoding of periphytic diatom community samples from Portuguese and French rivers included a step for DNA extraction using commercial kit NucleoSpin® Soil and a second step for DNA sequencing with MiSeq system (Illumina) using *rbcL* plastid gene (312 bp barcode) (Mortágua *et al.*, AIL2018, Mortágua *et al.*, 2019, Rivera *et al.*, 2018; Rivera *et al.*, 2020). Sample sequences obtained from metabarcoding were then analysed using the software mothur (version 1.39.5, Schloss *et al.*, 2009). Taxonomic assignment of OTUs was based on the Diat.barcode library (available at: https://www6.inra.fr/carrtel-collection_eng/Barcoding-database). In French lagoons, the process was similar, but the DNA extraction was done with the kit Power Water DNA Isolation MOBIO® and sample sequences obtained from metabarcoding were then analysed using the software QIIME (https://qiime2.org).

In Azorean lakes and thermal springs, DNA was extracted up to 24h after sample collection, according to the gram-negative bacteria protocol of PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), followed by amplification of genes targeting 16S rDNA and cyanotoxins (Microcystin, Saxitoxin and Anatoxin-a) using conventional PCR and electrophoresis protocols (Cordeiro *et al.*, AIL2018). All protocols used were modified from existing ones available in the scientific literature (Ouahid *et al.*, 2005; Ballot *et al.*, 2010; Ledreux *et al.*, 2010; Rantala-Ylinen *et al.*, 2011; Casero *et al.*, 2014).

Biofilms from central Portugal and their response to multiple stressors in mesocosms were assessed through their OTUs composition in a study by Calapez *et al.* (AIL2018). DNA was extracted from a portion of the biofilm using PowerSoil® DNA Isolation Kit (Mobio Laboratories Inc., Carlsbad, CA, USA), followed by a PCR to amplify rDNA genes for each studied biofilm community, using a Taq DNA polymerase. The bacterial V3 region of 16S was amplified with the primer pair 338F-GC and 518R, the ITS1 region of rDNA of fungi using the primer pair ITS1F-GC and ITS2 and the 18S gene of eukarya by using the primer pair Euk1A and Euk516r-GC. Then a Denaturing Gradient Gel Electrophoresis (DGGE) was run for each community, conducted in a DCode system (Bio-Rad, Hercules, CA, USA). DGGE images were converted, normalized, and analysed with the software BioNumerics 7.6 (Applied Maths, Sint-Martens-Latem, Belgium) to obtain the relative abundances according to gel band intensity (OTUs).

In the Russian lake Oiskoe, planktonic microeukaryotes were assessed before and after ice-and-snow cover formation (Díaz-Quijano *et al.*, AIL2018). The focus was set on phytoplankton and general protists, but other eukaryotic actors of the microbial loop, such as ciliates and fungi were assessed as well. General eukaryote primer pair targeting the V4 region of the small subunit 18S rRNA gene was used (Balzano *et al.*, 2015). This is a modification of Stoeck's primer pairs (Stoeck *et al.*, 2010), with an extra degenerate nucleotide position, which allows haptophytes to be targeted.

Total biodiversity (from microorganisms to vertebrates) has also been addressed in projects developed in Cantabria, with the addition of 16S and 18S primers for prokaryotes and eukaryotes (Bact02 and Euka02 primers, respectively), besides COI for macroinvertebrates (Sainz-Bariáin *et al.*, AIL2018).

IV. New database entries

Continuous incorporation of data from new or updated biological surveys is essential to develop a good species database (Olenin *et al.*, 2016). Many of the studies presented in the SS originated important new barcode data that fed different databases.

Fish and invertebrates

For marine life, core COI reference databases for the most prominent groups of Portuguese and Iberian fish and macroinvertebrates were made publicly available on BOLD systems. Regarding fish, in addition to the Portuguese marine ichthyofauna (Costa *et al.*, 2012), reference databases have been generated for the Mediterranean (Landi *et al.*, 2014), the North Sea and British Isles species (Knebelsberger *et al.*, 2014). A published compilation for all European marine

fish species is available as well (Oliveira *et al.*, 2016). For freshwater fish species, the reference database for European species is almost complete concerning standard DNA barcodes (COI) and public data can be found in GenBank and BOLD databases. However, there is only very limited 12S sequence data available that can be used as a reference to taxonomically annotate eDNA derived OTUs. Among the invertebrates there are published databases and other scattered DNA barcode contributions available for annelids, namely Polychaeta (Lobo *et al.*, 2016; Ravara *et al.*, 2017), for molluscs (Gastropoda: Borges *et al.*, 2016; bivalve woodborers: Borges *et al.*, 2012), and for crustaceans (e.g. Amphipoda; Lobo *et al.*, 2017b).

Meiofauna

Concerning meiofauna, to the best of our knowledge, there are no specific databases. Yet, Tang and collaborators (2012) gathered a total of 12 000 sequences (generated and retrieved from GenBank) across 55 meiofaunal datasets comprising 3 taxonomic ranks (15 species complexes, 26 genera, and 14 higher taxa above the genus level, including orders, classes, and phyla), using either 18S or COI markers.

Diatoms

For diatoms, Diat.barcode library (formely called R-Syst::diatom in Rimet *et al.*, 2016) (https://www6.inra.fr/carrtel-collection_eng/Barcoding-database) and was used in the studies presented at AIL2018 conducted in Portugal by Mortágua *et al.* (AIL 2018; 2019) and in France by Rivera *et al.* (AIL 2018). This database is open access and contains 18S and *rbcL* barcodes. In addition, R-Syst::diatom provides information concerning morphological diatom features (e.g., biovolumes, chloroplasts, etc.), ecological features (taxa preference to pollution) and life-forms (mobility, colony-type). The database is uploaded and curated every six months. The sequences obtained in the Russian study are not attributed to any taxocenose-specific database but should be made available to the builders of a cryophyllic diatom and green algae ribosomic RNA database at the Helmholtz Centre for Polar and Marine Research in Potsdam, Germany (shuang@awi.de).

V. Multidisciplinary international networks

The metagenomics is an area where extended networks tend to be formed in order to easily tackle all the fields involved, encompassing fieldwork and sample collection to laboratory procedures, taxonomic expertise and molecular analyses. This need is clear in the global distribution of authors of the SS (Fig. 1).

The University of Minho team (Portugal) has integrated the Consortium for the Barcode of Life (CBOL) from early stages and later the International Barcode of Life (iBOL) and in collaboration with the *Museu Nacional de História Natural e Ciência*, *Instituto Português do Mar e da Atmosfera*, the Portuguese Institute of Malacology, the research institutes IMAR, CIIMAR and CNC/Biocant, and the Universities of Guelph (Canada), Bangor (UK) and Vigo (Spain) works to build core reference databases for marine life.

The teams from the Universities of Aveiro and Coimbra (Portugal) have been working with INRA at Thonon-les-Bains (France) in the laboratorial treatment of periphytic biofilms, from extraction, amplification, sequencing of DNA and bioinformatic analyses. MARE team is also collaborating with CIBIO (Portugal) for the assessment of freshwater invertebrate communities and biological quality through DNA. For the FRESHING project (CIBIO/InBIO, Portugal) the laboratory procedures and the HTS (MiSeq v2, 2x250bp PE) were performed in CIBIO-UP (Portugal) while fieldwork have been done in collaboration with the company Aqualogus and the taxonomical identification at Instituto Politécnico de Bragança (Portugal). These teams, like those from Universities of Minho, Coimbra and Aveiro (Portugal), Cantabria and Barcelona (Spain), are part of the larger network of the European COST action DNAqua-Net, which among other tasks are tackling problems such as an adaptation of currently used biotic indices for metabarcoding data.

Samples from the Cantabrian coast (Spain), Gulf of Lion (South France), Polynesian ports and Spanish rivers are being processed in molec-

ular facilities of the University of Oviedo. DNA sequencing will be done at the Massive Sequencing Service Unit from the IBBTEC (CSIC - *Universidad de Cantabria* – Sodercan). The University of Barcelona team is currently collaborating with the University of Salford (UK) and University of Tromsø (Norway) for sequencing facilities and bioinformatics. In the Azores, all molecular laboratory work is conducted in the laboratories of the University of Azores (UAc) and CIBIO. The cyanobacteria cultures were established and maintained in BACA-Banco de Algas e Cianobactérias dos Açores (UAc), which is part of the REBECA network. The team works on this topic with the Ecotoxicology team from CIIMAR, University of Porto. In Russia, the molecular facility used was the Laboratory of Experimental Hydroecology, at the Biophysics Institute (Siberian branch of the Russian Academy of Sciences). Sequencing (Illumina MiSeq) was performed in three facilities: Konstantin V. Krutovsky lab, at the Sukachev Institute of Forest; the Centre for Collective Use of the Institute of Bioorganic Chemistry, Novosibirsk, Russia; and the company Evrogen (Moscow).

MAIN FINDINGS

The SS showed several interesting results at the technical level but also new insights for the ecology and conservation of aquatic systems.

Technical aspects

It was found that the choice of the markers to target particular primer pair can considerably influence the metabarcoding-based analyses output. For estuarine meiofaunal. up to 85 % of the species constituting a mock community were detected by using a combination of 3 primer pairs targeting the COI region, while only 30 to 60 % were recovered by using any primer set alone (Hollatz *et al.*, 2017; Fais *et al.*, AIL218). Also, the amount of starting material from the sample for eDNA extraction is critical for a comprehensive assessment of meiofaunal communities in estuarine ecosystems.

The use of preservative ethanol from field samples seems to be a promising solution for macroinvertebrate biodiversity assessment, with faster processing of samples in the lab for DNA metabarcoding. However, the results are sensitive to various laboratory procedures, namely DNA extraction methods and/or the storage and collection timing of preservative ethanol (Martins *et al.*, AIL2018).

Ecology and conservation

Molecular analyses in aquatic ecosystems brought not only new information but also new questions. DNA barcoding studies on Portuguese marine life have been revealing numerous cases of comparatively high intra-specific divergences, suggesting the existence of considerable hidden diversity and putative cryptic species across diverse marine taxa, including fish and major groups of invertebrates (Costa *et al.*, 2012, Landi *et al.*, 2014, Lobo *et al.*, 2016, 2017a, Oliveira *et al.*, 2016). These findings suggest that populations of marine organisms may be much more structured than previously thought, calling for a continuous effort on the description of the hidden diversity and further completion of the reference databases. In order to improve the efficiency of amplification of COI barcodes from marine macrozoobenthos, Lobo *et al.* (2013) developed a new pair of degenerate primers with a broad scope of amplification success across a phylogenetically diverse range of marine metazoan taxa.

In the very first study based on molecular data of freshwater diatom communities in Portugal, the total number of diatom taxa identified was 125 from 88 river samples which corresponded to about 41 % of the number of taxa identified by using the light microscope (Mortágua *et al.*, AIL2018; Mortágua *et al.*, 2019). These results, somewhat unexpected, were in accordance with results registered in studies performed in other countries (Vasselon *et al.*, 2017b; Rivera *et al.*, 2018 and Keck *et al.*, 2018). A possible explanation might be the high number of unassigned reads, which is a consequence of the incompleteness of the reference database.

The molecular approach was also found important in the detection of new introductions of fishes and tracking introduction histories, which can be relevant for designing proper management plans. It is the case of the species *P. phoxinus* that

was recorded for the first time in the Douro Basin. This species can be easily misidentified as other species from the same genus when using only morphological identifications in the field.

The eDNA and metabarcoding approaches were found efficient to obtain accurate baseline information to be used in conservation planning and ongoing management of coastal lagoons in the south of France. Despite their different status of conservation within Natural Parks, Reserves or Natura 2000 Network, they are already contaminated with non-indigenous species, some of them already described as invasive species.

New records of cyanobacteria species presence were detected in the Azores through molecular analyses (Cordeiro *et al.*, AIL2018). In addition, some of the sampled lakes cyanotoxins production potential was confirmed, mainly associated with eutrophication and anthropogenic effects, which shows the potential of molecular tools for monitoring cyanotoxin risk in aquatic systems.

In Russia, a unique dataset of early winter lake water microbial communities was produced as winter dynamics are usually out of the scope of limnological studies in Siberia, due to the harsh fieldwork conditions (Diaz-de-Quijano *et al.*, AIL2018). The Cryptomycota clade LKM11, which was previously found in ice-covered lakes of Antarctica (Rojas-Jimenez *et al.*, 2017), represented up to 6-10 % of the reads in intermediate and deep layers of the water column of the ice-covered Oiskoe lake. Metabarcoding of microbial but also macroscopic communities enabled an easier calculation of phylogenetic diversity metrics, and testing hypotheses on the ecological mechanisms governing community assemblages.

MAJOR DRAWBACKS

Different technical drawbacks were signalized in the SS, in spite of the potential advantages of molecular approaches in biodiversity and ecological assessment of aquatic ecosystems.

Taxonomic gaps

In the SS it was often referred to the existence of taxonomic gaps in the reference databases when considering local fauna. One example is the study in the Lima estuary and in the Tua river in Portugal with benthic invertebrates, where a fair number of OTUs could not be assigned to phylum or other lower taxonomic rank due to the primers used for targeting the COI region (Mortágua *et al.*, 2019). A similar issue was reported for the diatoms as previously referred, in spite of the large database and diatom cultures existing in Thonon-les-Bains, INRA, with a high number of unassigned reads (67 %). The increase in the number of diatom barcodes in reference databases will allow for a complete study of diversity, namely in what concerns to rare taxa. In some cases, databases are not sufficient for assigning species and they must be assigned at genus level; in these cases, previous taxonomic work is necessary. In the French coastal lagoons, only ca. 10 % of reads obtained were identified to the species level and those that could not be described to the species level had multiple best BLAST hits or the best BLAST hit had no species-level information available. In addition, local databases covering intra-specific variability are important, especially when geographical barriers can lead to high intra-specific variability (e.g., Douro River Basin).

Extraction of eDNA

Protocols need further adjustments and should be adapted to the environments and types of samples (e.g., biofilms scrapings or preservative liquid of bulk samples instead of water). eDNA extraction was the biggest setback. This was found through the development of the work with cyanobacteria as they have a wide range of morphological characteristics, like mucilage sheaths (Codd *et al.*, 2017), that makes DNA extraction more complicated. Different methods were tested to improve cell lysis, like sonication, enzymatic lysis and readjustments of temperature and incubation time (Kim *et al.*, 2009). Similar results were found using ethanol from the preservation of macroinvertebrate samples where different DNA extraction methods retrieved different species diversity across time.

Amount of environmental sample

The amount of sample needed for good DNA extraction can be harder to determine since it

depends not only on the type of sample (e.g., water, sediment) but also on the study site. For example, in eutrophic lakes, there is a higher abundance and diversity of phytoplankton, while in thermal springs there is lower abundance and diversity of phytoplankton (Cordeiro *et al.*, AIL2018). Preliminary research employing metabarcoding on eDNA extracted from sediments at an estuarine site in the North of Portugal revealed that more OTUs assigned to meiofauna were recovered by using higher amounts of sediment samples (Fais *et al.*, AIL2018).

Genetic markers

Different genetic markers and bioinformatics pipelines must be considered to obtain the most accurate results. For fish, it is hard to find a single nuclear marker with enough resolution to delimit closely related species (Filipe *et al.*, AIL2018). Despite the appropriateness of COI and CytB markers for the majority of the species, some genera such as *Achondrostoma* or *Cobitis* can represent a bigger problem to identify the specimens taxonomically to species-level.

Cost of sequencing

The cost of HTS is still significantly high and highly variable, which limits their present use in large monitoring programs. Especially in Russia, the purchase of reagents and materials from western countries might take up to 6 months and cost up to twice their price in the West, which makes it difficult to match financing and project calendars, when it comes to using metabarcoding in a particular project.

CONCLUSIONS

Studies presented in AIL2018 meeting enhanced the importance and applicability of molecular techniques in environmental studies, towards fast and significant information acquisition. This information can be used in biodiversity and ecological quality assessments, conservation and management of aquatic water bodies.

During the SS, it became clear that molecular tools, and particularly the metabarcoding approach, could provide fine-scale taxonomical resolution data, contribute to detect new invasions and allow for unveiling hidden biodiversity resulting from low-abundance, small sizes and poor-developmental stages.

Yet, a lot of work and investment is still needed before molecular tools can be used routinely in monitoring programs, namely in the completion of databases, optimization and standardization of both laboratory and field protocols, in automation in sample handling and bioinformatics analyses and ultimately in reducing analyses costs. Moreover, considering the adaptation to the WFD, which requires reaching a quality status that could actually replace the existing ones based on taxonomy, it is necessary to establish new reference values for different types of rivers and other water bodies (Feio *et al.*, 2014) or check existing ones with molecular data, and establish clear responses to disturbance gradients (Filipe *et al.*, 2018). This however, might soon become a reality for diatoms, macroinvertebrates and fish. The relatively well-developed taxonomy and autoecology of diatoms make them an ideal case to compare genetic, morphological and ecological determination of species. On the other hand, by the use of primer pairs that target a phylogenetic range wider than diatoms, (e.g., targeting eukaryotes) studies could include a wider spectrum of autoecologies with more power to inform about the ecological state of aquatic ecosystems.

Despite most studies presented, in the special session being from Europe, the perspectives, main findings and drawbacks are likely to be common to other geographic areas across the globe. Therefore, we expect this review to be useful to other researchers across the world, dealing with molecular tools for ecological and biodiversity assessment of aquatic ecosystems.

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