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

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73 **ABSTRACT**

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

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

87 Developments presented are mainly focused on the Iberian Peninsula (Portugal and
88 Spain, including Atlantic Macaronesian islands) but include studies in France,
89 Germany, Finland, Russia (Siberia) and South America. The networks within which
90 these researchers are involved are yet even broader, profiting from existing molecular
91 facilities, and traditional taxonomic expertise, which can be viewed as a characteristic
92 of this new research area. It was evident in the SS that the use of molecular tools is
93 widespread, being used to study a diversity of aquatic systems, from rivers'
94 headwaters to estuaries and coastal lagoons, and volcanic, mountain and frozen lakes
95 to hot springs. The organisms targeted are likewise varied and include fish,
96 macroinvertebrates, meiofauna, microalgae such as diatoms and dinoflagellates, other
97 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 the 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 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

194 highlights the fast development of this area around the world and the importance of
195 broad networks in the advancement of this particular research field (Fig.1).

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

203

204 **PERSPECTIVES ON THE USE OF MOLECULAR TOOLS**

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

207 *Biodiversity*

208 Molecular tools are particularly useful to assess the diversity of concealed
209 communities, allowing a more accurate species detection and distribution in a specific
210 ecosystem. This is the case of the meiofauna, which comprises organisms between
211 30-1000 µm (Higgins & Thiel, 1988). Due to their small size, morphotaxonomic
212 inventories can largely fail to identify accurately (Alves *et al.*, 2015). Various
213 taxonomic meiofaunal groups of an estuary in the North of Portugal have been
214 detected by a target region (Fais *et al.*, AIL2018). Phytoplankton and general
215 microeukaryotic plankton dynamics under the formation of ice-and-snow cover were
216 studied in a Siberian mountain lake through molecular techniques (Díaz-Quijano *et al.*,
217 AIL2018).

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

227 Ecological impact of algal toxicity is also being investigated through molecular tools
228 (Cordeiro *et al*., AIL2018). Toxins are transferred along the food chain, from different
229 microalgae (mainly Dinoflagellates, Cyanobacteria, and Diatoms) and HABs can be
230 responsible for massive fish mortality (Thangaraja *et al.*, 2007), while the presence of
231 toxins in fish or shellfish can cause severe human diseases (e.g., diarrheic shellfish
232 poisoning). In the Azorean archipelago (Portugal), the potential for cyanotoxin
233 production was assessed in thermal environments and freshwater lakes, which are
234 common in these volcanic islands. The confirmation of cyanobacteria's DNA and
235 potential risk of cyanotoxin production in the eDNA samples (Cordeiro *et al*.,
236 AIL2018), revealed to be an efficient method for monitoring these ecosystems and
237 help to prevent threats to public and environmental health (Pearson & Neilan, 2008;
238 Salmaso *et al*., 2017).

239 Genetic tools have been increasingly used for studying invasions, because it allows
240 species identification (e.g. Ardura *et al.*, 2010; Ardura & Planes, 2017), determination
241 of the region of origin (Ardura *et al*., 2013) and time of initial incursion of non-
242 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). This approach requires following the Water Framework Directive (WFD; European Union 2000) sampling protocols but avoids

268 the sorting step of separating animals in a sample from vegetation, sediment and litter,
269 which is very time-consuming. The authors are examining the performance of
270 different laboratory procedures on species detection based on the preservative liquid,
271 and compared taxa recovery with the conventional morphological method. More than
272 half of the taxa found in ethanol were macroinvertebrates targeted by WFD, while the
273 remaining percentage was identified as, e.g., bacteria, Stramenopiles, terrestrial
274 invertebrates, amphibians and fishes (Martins et al. AIL2018).

275 *Biological quality monitoring*

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

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

high correlation (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) 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

318 Salford, Manchester, UK) used metabarcoding techniques to compare the estimates of
319 the ecological status using traditional morpho-taxonomy against high-throughput
320 DNA sequencing of: 1) bulk sampling (after sorting individuals from multi-habitat
321 Surber samples), 2) eDNA (water samples) and 3) invertebrate drift sampling
322 (intervals of 1 hour). Results showed that while the traditional and bulk sampling
323 approaches detected essentially riverine species, the eDNA also captured terrestrial
324 associated fauna (Múrria et al., AIL2018).

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

333 Molecular analysis constitutes, in addition, a simpler way of analysing the impact of
334 anthropogenic and natural alterations in complex communities composed of
335 microorganisms. A study in mesocosms run by Calapez and collaborators
336 (Universities of Aveiro and Coimbra, Portugal) analysed stream biofilm responses to
337 multiple-stressors typical of Mediterranean streams and found biofilm community
338 shifts induced by flow stagnation, organic loads and grazing activity. Specifically, the
339 OTUs determination helped to investigate how biofilm microbial communities'
340 proportions changed under the different stressor combinations more quickly. The
341 interaction of those three stressors altered algae, fungi and bacteria diversity
342 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

367 mountain river; Mijares is a low-mountain river with high mineralization; while the
368 Turia river is a low altitude river (Pujante *et al.,* AIL2018). Another Mediterranean
369 river from Catalonia, the Llobregat (156 km in length), was studied by Múrria and
370 collaborators, which covers a gradient of pollution and anthropogenic impact. This is
371 a well-studied river (Munné & Prat, 2004, 2011), which includes a pollution gradient
372 from pristine headwater reach, through site located downstream of a big reservoir or
373 salt mining, to urban and agricultural landscapes at lowlands. A sampling of
374 macroinvertebrates was done in 5 sites along the river (Múrria *et al.,* AIL2018).

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

382 *Lakes*

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

389 In France, diatom metabarcoding has been applied to assess the structure of diatom
390 community and the ecological status of the littoral zone of Lake Bourget (deepest

391 French lake). The structure of the assemblages based on the morphological (taxa lists)
392 and molecular (OTUs lists) identification of diatoms were well correlated. However,
393 the ecological status of the lake varied between these two methods since floristic
394 inventories differed significantly (Rivera *et al.*, 2018; Rivera *et al.,* AIL2018). The
395 main reason for this discrepancy was the incompleteness of the diatom reference
396 database (R-Syst::diatom) (Rimet *et al.,* 2016).

397 In Cantabria, five mountain lakes were sampled for molecular analysis of
398 environmental samples (water and sediment). The first is located at ca. 1870 m of
399 altitude in the Liordes Valley, a unique ecosystem in the *Picos de Europa* massif,
400 located in a glacial-karst depression surrounded by calcareous walls. The Lloroza
401 lakes (ca. 1800 m of altitude) are small lagoons of karstic nature located in *Picos de*
402 *Europa* National Park in the Cantabria province. Finally, the Enol and Ercina (at ca.
403 110m of altitude) are two glacial lakes forming the Covadonga lakes located within
404 the *Picos de Europa* National Park in the Asturias province. These samples are still
405 being processed (Sainz-Barain *et al.,* AIL2018).

406 In Siberia, the Oiskoe mountain lake is being studied with phytoplankton samples
407 through metabarcoding from a conservation perspective (Diaz-de-Quijano *et al*.,
408 AIL2018). Located in the Ergaki Natural Park, West Sayan Mountains, is a poorly
409 studied area due to its extreme climate with a wide range of annual temperatures (-
410 41°C to +32°C). The lake is surrounded by a mosaic landscape of bogs, sparse taiga
411 forest, scree and alpine tundra and biodiversity has particular adaptations to these
412 conditions (Anishchenko *et al*., 2015). However, human activities, namely tourism
413 and global warming in South Siberia and Central Asia, are the present threats to these
414 ecosystems.

415 *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 their being most of them 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.,

464 2003). The Cytochrome c (COI) is an amino acid sequence that is highly conserved in
465 eukaryotes, differing by only a few residues. There are robust universal primers for it
466 that recover most animal phyla, and thousands of reference sequences are available in
467 public databases such as BOLD and GenBank (Ratnasingham & Herbert, 2013).
468 (Herbert et al. 2013). However, the high variability in the third position of the COI
469 codons makes it difficult to design universal primers for metabarcoding DNA studies
470 (Ficetola et al., 2010). For fish identification, most used barcode markers in DNA
471 reference collections are the COI and cytochrome b (Cytb) genes, other mitochondrial
472 genes, which can confirm taxonomic identification at the species level. However,
473 some studies are showing that COI might not be the best option for assessing and
474 monitoring freshwater fish diversity using environmental DNA from water because
475 this marker might not contain suitably conserved regions (e.g., Deagle *et al*., 2014).
476 Instead, the potential of using the MiFish region from the ribosomal 12S is under
477 consideration (Miya et al., 2015; Filipe et al., AIL2018).

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

488 that includes hundreds of specimens of macroinvertebrate taxa from northeast
489 Portugal.

490 Macroinvertebrates and marine fish have been the target of comprehensive DNA
491 barcoding campaigns across multiple coastal ecosystems in continental Portugal. The
492 primary marker was the COI, occasionally supplemented by other markers (e.g.,
493 Borges *et al*., 2012). In the Lima estuary, DNA from meiofauna communities was
494 extracted from intertidal sediments. In this case, the target genes were the COI and
495 18S ribosomal RNA (18S rDNA) gene. MiSeq amplicon sequences were processed in
496 mothur (version 1.39.5, Schloss *et al*., 2009) by using appropriate bioinformatic
497 procedures; while the taxonomy of the processed sequences were assessed by blasting
498 against the full ntNCBI database (Fais *et al.,* AIL2018). This database was chosen due
499 to the lack of adequate reference sequences in better-known databases, such as BOLD
500 (Ratnasingham and Hebert, 2007) and Silva (Pruesse et al., 2007). In the French
501 coastal lagoons, the invertebrate communities were as well analysed from eDNA with
502 COI marker.

503 *18S, rbcL and 16S*

504 In the project BIOWAT-KIT a preliminary evaluation of different genomic regions
505 using publicly available sequence data was carried out in order to identify the best-
506 suited DNA barcode marker for the identification of 141 families of invertebrates
507 belonging to four different phyla (Platyhelminthes, Annelida, Mollusca, and
508 Arthropoda). Several primer pairs have been designed, including a degenerate primer
509 pair and a cocktail of group-specific primers, which will presumably amplify all the
510 target invertebrate taxa present in freshwater samples. Based on the results, the
511 mitochondrial 16S gene was selected for the DNA metabarcoding analysis of
512 freshwater invertebrate communities within this project, since it combines both

513 conserved regions suitable for primer design, and variable regions with good
514 taxonomic resolution at the family level (and potentially, also at the genus or species
515 level) (Pujante *et al.,* AIL2018).

516 In Thonon (France), the INRA team targeted several genes for diatoms (18S, *COI*,
517 *rbcL*) (Kermarrec *et al*., 2013). While COI is found in mitochondrial DNA of
518 eukaryotic organisms, the 18S is part of the ribosomal RNA of eukaryotes and the
519 ribulose-1,5-bisphosphate carboxylase/oxygenase (*rcbL*) is present in plants
520 chloroplasts. The *rcbL* showed to be the most suitable barcode for biomonitoring
521 purposes with diatoms (Kermarrec *et al.*, 2013; Kermarrec *et al.,* 2014; Pawlowski *et*
522 *al.,* 2016). Thus, DNA metabarcoding of periphytic diatom community samples from
523 Portuguese and French rivers included a step for DNA extraction using commercial
524 kit NucleoSpin® Soil and a second step for DNA sequencing with MiSeq system
525 (Illumina) using *rbcL* plastid gene (312 bp barcode) (Mortágua *et al.,* AIL2018,
526 Mortágua et al. 2019, Rivera *et al.,* 2018). Sample sequences obtained from
527 metabarcoding were then analysed using the software mothur (version 1.39.5, Schloss
528 *et al*., 2009). Taxonomic assignment of OTUs was based on the R-Syst::diatom
529 database (Rimet *et al.,* 2016, version 17-05-2017, http://www.rsyst.inra.fr/en). In
530 French lagoons, the process was similar, but the DNA extraction was done with the
531 kit Power Water DNA Isolation MOBIO® and sample sequences obtained from
532 metabarcoding were then analysed using the software QIIME (https://qiime2.org).

533 In Azorean lakes and thermal springs, DNA was extracted up to 24h after sample
534 collection, according to the gram-negative bacteria protocol of PureLink™ Genomic
535 DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), followed by amplification of genes
536 targeting 16S rDNA and cyanotoxins (Microcystin, Saxitoxin and Anatoxin-a) using
537 conventional PCR and electrophoresis protocols (Cordeiro *et al.*, AIL2018). All

538 protocols used were modified from existing ones available in the scientific literature
539 (Ouahid *et al*., 2005; Ballot *et al*., 2010; Ledreux *et al*., 2010; Rantala-Ylinen *et al*.,
540 2011; Casero *et al*., 2014).

541 Biofilms from central Portugal and their response to multiple stressors in mesocosms
542 were assessed through their OTUs composition in a study by Calapez *et al*.
543 (AIL2018). DNA was extracted from a portion of the biofilm using PowerSoil® DNA
544 Isolation Kit (Mobio Laboratories Inc., Carlsbad, CA, USA), followed by a PCR to
545 amplify rDNA genes for each studied biofilm community, using a Taq DNA
546 polymerase. The bacterial V3 region of 16S gene, fungi and eukarya of 18S gene
547 were amplified using universal primers pairs ITS1F-GC and ITS2, the V3 region of
548 bacterial 16S rDNA gene was amplified with the primer pair 338F-GC and 518R for
549 16S and Euk1A and Euk516r-GC for 18S. Then a Denaturing Gradient Gel
550 Electrophoresis (DGGE) was run for each community, conducted in a DCode system
551 (Bio-Rad, Hercules, CA, USA). DGGE images were converted, normalized, and
552 analysed with the software BioNumerics 7.6 (Applied Maths, Sint-Martens-Latem,
553 Belgium) to obtain the relative abundances according to gel band intensity (OTUs).

554 In the Russian lake Oiskoe, planktonic microeukaryotes were assessed before and
555 after ice-and-snow cover formation (Díaz-Quijano *et al*., AIL2018). The focus was set
556 on phytoplankton and general protists, but other eukaryotic actors of the microbial
557 loop, such as ciliates and fungi were assessed as well. General eukaryote primer pair
558 targeting the V4 region of the small subunit 18S rRNA gene was used (Balzano *et al.,*
559 2015). This is a modification of Stoeck's primer pairs (Stoeck *et al*., 2010), with an
560 extra degenerate nucleotide position, which allows haptophytes to be targeted.

561 Total biodiversity (from microorganisms to vertebrates) has also been addressed in
562 projects developed in Cantabria, with the addition of 16S and 18S primers for

563 prokaryotes and eukaryotes (Bact02 and Euka02 primers, respectively), besides COI
564 for macroinvertebrates (Sainz-Barain *et al.,* AIL2018).

565

566 **IV. New database entries**

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

570 *Fish and invertebrates*

571 For marine life, core COI reference databases for the most prominent groups of
572 Portuguese and Iberian fish and macroinvertebrates were made publicly available on
573 BOLD systems. Regarding fish, in addition to the Portuguese marine ichthyofauna
574 (Costa *et al.,* 2012), reference databases have been generated for the Mediterranean
575 (Landi *et al.,* 2014), the North Sea and British Isles species (Knebelsberger *et al.*
576 2014). A published compilation for all European marine fish species is available as
577 well (Oliveira *et al.,* 2016). For freshwater fish species, the reference database for
578 European species is almost complete concerning standard DNA barcodes (COI) and
579 public data can be found in GenBank and BOLD databases. However, there is only
580 very limited 12S sequence data available that can be used as a reference to
581 taxonomically annotate eDNA derived OTUs. Among the invertebrates there are
582 published databases and other scattered DNA barcode contributions available for
583 annelids, namely Polychaeta (Lobo *et al.*, 2016; Ravara *et al.,* 2017), for molluscs
584 (Gastropoda: Borges *et al.,* 2016; bivalve woodborers: Borges *et al.,* 2012), and for
585 crustaceans (e.g. Amphipoda; Lobo *et al.,* 2017b).

586 *Meiofauna*

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

592 *Diatoms*

593 For diatoms, R-Syst::diatom, a specific reference barcoding database has been
594 developed (http://www.rsyst.inra.fr/) (Rimet *et al.,* 2016) and was used in the studies
595 presented at AIL2018 conducted in Portugal by Mortágua et al. (AIL 2018; 2019) and
596 in France by Rivera *et al.* (AIL 2018). This database is open access and contains 18S
597 and *rbcL* barcodes. In addition, R-Syst::diatom provides information concerning
598 morphological diatom features (e.g., biovolumes, chloroplasts, etc.), ecological
599 features (taxa preference to pollution) and life-forms (mobility, colony-type). The
600 database is uploaded and curated every six months. The sequences obtained in the
601 Russian study are not attributed to any taxocenose-specific database but should
602 be made available to the builders of a cryophyllic diatom and green algae
603 ribosomic RNA database at the Helmholtz Centre for Polar and Marine Research
604 in Potsdam, Germany (shuang@awi.de).

605

606 **V. Multidisciplinary international networks**

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

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

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

631 Samples from the Cantabrian coast (Spain), Gulf of Lion (South France), Polynesian
632 ports and Spanish rivers are being processed in molecular facilities of the University
633 of Oviedo. DNA sequencing will be done at the Massive Sequencing Service Unit
634 from the IBBTEC (CSIC - *Universidad de Cantabria* – Sodercan). The University of
635 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.

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

665 **Ecology and conservation**

666 Molecular analyses in aquatic ecosystems brought not only new information but also
667 new questions. DNA barcoding studies on Portuguese marine life have been revealing
668 numerous cases of comparatively high intra-specific divergences, suggesting the
669 existence of considerable hidden diversity and putative cryptic species across diverse
670 marine taxa, including fish and major groups of invertebrates (Fais *et al.,* AIL2018).
671 These findings suggest that populations of marine organisms may be much more
672 structured than previously thought, calling for a continuous effort on the description
673 of the hidden diversity and further completion of the reference databases. In order to
674 improve the efficiency of amplification of COI barcodes from marine
675 macrozoobenthos, Lobo *et al.* (2013) developed a new pair of degenerate primers
676 with a broad scope of amplification success across a phylogenetically diverse range of
677 marine metazoan taxa.

678 In the very first study based on molecular data of freshwater diatom communities in
679 Portugal, the total number of diatom taxa identified was 125 from 88 river samples
680 which corresponded to about 41% of the number of taxa identified by using the light
681 microscope (Mortágua *et al.* AIL2018; Mortágua *et al*., 2019). These results,
682 somewhat unexpected, were in accordance with results registered in studies
683 performed in other countries (Vasselon *et al.*, 2017b; Rivera *et al*., 2018 and Keck *et*

684 *al*., 2018). A possible explanation might be the high number of unassigned reads,
685 which is a consequence of the incompleteness of the reference database.

686 The molecular approach was also found important in the detection of new
687 introductions of fishes and tracking introduction histories, which can be relevant for
688 designing proper management plans. It is the case of the species *P. phoxinus* that was
689 recorded for the first time in the Douro Basin. This species can be easily misidentified
690 as other species from the same genus when using only morphological identifications
691 in the field.,

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

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

703 In Russia, a unique dataset of early winter lake water microbial communities was
704 produced as winter dynamics are usually out of the scope of limnological studies in
705 Siberia, due to the harsh fieldwork conditions (Diaz-de-Quijano *et al.*, AIL2018). The
706 Cryptomycota clade LKM11, which was previously found in ice-covered lakes of
707 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.

A**mount 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. 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,

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

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

798

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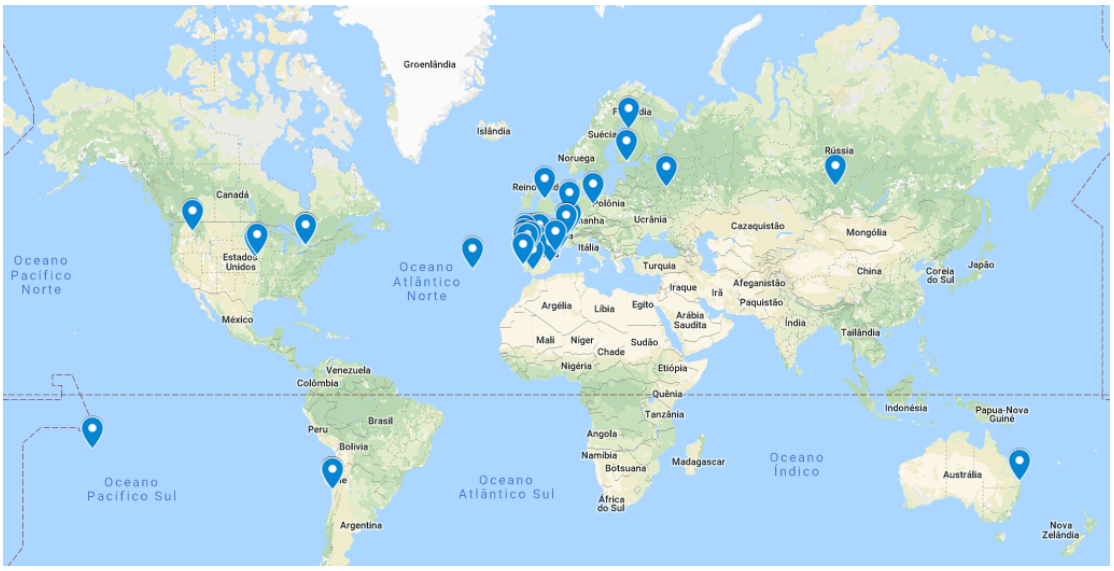
1202 **Table 1.** Biological groups, water bodies and barcode genes assessed in studies
1203 presented in the special session "The use of molecular tools in ecological and
1204 biodiversity assessment of aquatic ecosystems" of the XIX Congress of the Iberian
1205 Association of Limnology (AIL2018). *Grupos biológicos, massas de água e barcodes*
1206 *analisados nos estudos apresentados na sessão especial "O uso das ferramentas*
1207 *moleculares na avaliação ecológica e biodiversidade dos ecossistemas aquáticos",*
1208 *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-Barain *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	Cordeiro *et al*., AIL2018

		saxitoxinas, anaC, anaF for anatoxina, and mcyC, mcyD, mcyE, mcyG for microcistina	
Meiofauna (sediment)	Estuary – Lima river, Portugal	COI, 18S	Fais *et al.*, AIL2018



Legend to figure

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).*