ORIGINAL ARTICLE

Metabarcoding to monitor the crustacean zooplankton of a lake improves when using a reference DNA library from local samples

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ABSTRACT

Biodiversity surveys through morphology provide invaluable data to inform biological monitoring efforts, involving specialised taxonomic skills that are not always available. The revolution brought by the advent of metabarcoding associated to massive sequencing is currently seen as a potential advance, even if different approaches may often provide different results. Here we test if reliable results from metabarcoding can be obtained by i) basing the analyses on a detailed knowledge of the local diversity from morphology, ii) applying tools from DNA taxonomy to create a local reference library, ii) developing custom primers, taking as example the crustacean zooplankton of a subalpine lake in Northern Italy, Lake Maggiore. We support the idea that occurrences from metabarcoding can be reliable, especially with targeted primers, but we confirm that read numbers from massive sequencing could not be related to abundance of individuals in our analyses. Data from metabarcoding can thus be used to reliably monitor species occurrence in the lake, but not changes in abundance.

INTRODUCTION

Biological monitoring of aquatic habitats for assessment of environmental quality in Europe has been harmonised by the Water Framework Directive (WFD) in Europe to allow comparisons between the different national procedures, with the aim of achieving good ecological conditions for all the aquatic habitats, comparing the biota of each water body with a set of geographically delimited reference systems (Kallis and Butler, 2001). Such monitoring has been, and still is, performed using a morphological approach for the identification of the taxa of the so-called Biological Quality Elements (BQEs) (Simboura *et al*., 2005), taxonomically defined groups of organisms (e.g., families, orders, *etc*.) with known ecological requirements that can provide inference on ecological conditions based on their occurrence and abundance.

In addition to such European-scale national and international systems, other monitoring schemes have been developed for specific areas, for example for water bodies that are shared between countries (Leb, 2015), and for sites within the Long-Term Ecological Research (LTER) rationale (Lindenmayer and Likens, 2009; Magurran *et al*., 2010). These local monitoring schemes often consider additional BQEs, not included in the WFD, like for example zooplankton (Jeppesen *et al*., 2011).

A DNA-based approach on bulk samples, called metabarcoding, has been included in some specific projects, actions, and proposals for monitoring (Leese *et al*., 2016; Rey *et al*., 2020) to bypass several limitations existing in routine monitoring, where expert taxonomists cannot be available for all groups of organisms to be used according to national and international rules, and to include also other groups of organisms in the assessment of biological quality, like the zooplankton. Accurate taxonomic identification is notorious to be difficult for several taxa and thus to produce ambiguous and potentially subjective results, not allowing any convincing inference on quality assessment. For example, for some BQEs, the identification of taxonomic ranks higher than species is

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considered reliable (Birk *et al*., 2012). In other cases, different cryptic taxa within the same species groups may respond differently to the same environmental factors and stressors (Lucentini *et al*., 2011; Obertegger *et al*., 2014), diminishing the potential broad applicability of biological monitoring using such organisms. The use of DNA markers in biological monitoring with a rationale similar to that of the WFD could allow a more objective identification of taxa (a DNA sequence is unambiguous and not based on subjective approaches), provide reliable data to be subsequently used for other purposes, and potentially pose the basis for a deeper understanding of patterns and processes in biological diversity (Lim *et al*., 2016).

Notwithstanding such advantages, an abrupt switch from morphology-based to DNA-based biological monitoring is not considered a useful path to take (Collins and Cruickshank, 2013). To be able to take advantage of the historical morphological approaches, indexes, and records developed and gathered for the BQEs and to project them in the future use of DNA sequences on the same and other BQEs, a reference system should be put in place to match morphology and DNA, and to allow a comparison of metrics obtained with the two approaches to identify taxa (McManus and Katz, 2009).

The aim of the present study is to check whether DNA metabarcoding could be used to reliably identify occurrence and abundances of the crustaceans of the zooplankton of a subalpine lake, Lake Maggiore. The lake is shared between Italy and Switzerland, is subject to a multidecadal continuous monitoring program according to an international agreement between the two countries, the International Commission for the Protection of Italian and Swiss Waters (Commissione Internazionale per la Protezione delle Acque Italo-Svizzere, CIPAIS, https://www.cipais.org/) (Mosello *et al*., 2010), and is part of the European Long-Term Ecological Research (LTER DAIMS-SDR IT08-001-A). We focus on the crustaceans in the zooplankton, dealing with three steps: i) we sampled monthly for three years the zooplankton from the reference site used in monitoring program for CIPAIS and identified all species known from morphology from the site, ii) we developed a reference library through DNA barcoding and DNA taxonomy for all the crustaceans sampled and identified with a morphological approach in the zooplankton of the site in the lake; ii) we performed metabarcoding analyses in parallel from the same site with a set of common primers and with custom primers developed by checking *in silico* the DNA sequences obtained from the lake during the DNA taxonomy step; iv) we compared the different DNA metabarcoding data with the data obtained from the morphological survey on the same exact samples used for metabarcoding, to assess reliability of occurrence and abundances of the crustaceans in the zooplankton of the lake through DNA sequence data.

METHODS

Sampling

Sampling was performed in Lake Maggiore in a single site corresponding to the deepest zone of the lake (370m, in front of Ghiffa, VB, WGS84 coordinates: 45.9750, 8.6525), during survey campaigns performed by boat every month in 2019, 2020, and 2021, using the same approach developed for the continuous monitoring system in the lake (Arfè *et al*., 2019), carried on from that same site and with the same method since the 1940s (Manca *et al*., 2007b; Obertegger and Manca, 2011), officialised and standardised by the "Commissione Internazionale per la Protezione delle Acque Italo-Svizzere" (CIPAIS), the Italian-Swiss agreement that promotes, since 1978, monitoring activities aimed to survey and maintain the good quality of the water and biodiversity of Lakes Lugano and Maggiore (Mosello *et al*., 2010). Samples were collected vertically towing a plankton net of 80 µm mesh size, equipped with a flow meter, from a depth of 50 m to the surface, filtering about 1000 L of lake water, and stored in ethanol 96%. The occurrence of zooplankton in deeper layers is very rare and restricted to winter vertical mixing events, therefore the sampled water layer is representative of the net zooplankton population (Manca *et al*. 2000). A long historical series with detailed taxonomic knowledge on zooplankton exists from the same site, to be used to build a metabarcoding approach in biological monitoring, already knowing which species can be found in the lake in the different seasons.

Microscopic crustaceans (Cladocera and Copepoda) are among the most abundant, diverse, and well-known components of the zooplankton community in terms of biomass in lakes and ponds (Pace, 1986), and also in Lake Maggiore (Obertegger and Manca, 2011). They have important roles in the food web in lakes, as they link primary producers and macroscopic consumers (mainly fish), affecting both categories in terms of population and biomass (Borgmann *et al*., 1984; Carney and Elser, 1990; Vakkilainen *et al*., 2004). Zooplankton communities of Lake Maggiore have been studied since 1874 (Manca *et al*., 1992), with cladocerans and copepods always occurring in the water column of the lake (de Bernardi *et al*., 1988; Manca *et al*., 2008; Visconti *et al*., 2008).

Cladocerans in the reference site of the lake in Ghiffa are numerically ascribable mostly to *Daphnia*, namely to the species group including *Daphnia galeata* G. O. Sars, 1864 and *Daphnia longispina* O. F. Müller, 1776, whose populations dominated the cladoceran community in recent years (Visconti *et al*., 2008). The other species of cladocerans that are present in the lake are *Bythotrephes longimanus* (Leydig, 1860), *Diaphanosoma brachyurum* (Liévin, 1848), *Eubosmina longispina* Leydig, 1860 and

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Leptodora kindtii (Focke, 1844) (Manca *et al*., 2007b, 2007a). The copepod community of the site is composed by two species of Diaptomidae, *Mixodiaptomus laciniatus* (Lilljeborg in Guerne and Richard, 1889) and *Eudiaptomus padanus* (Burckhardt, 1900), and two of Cyclopidae, *Cyclops abyssorum* G. O. Sars, 1863 and *Mesocyclops leuckarti* (Claus, 1857) (Manca *et al*., 2008).

Sampling for morphological identification and estimation of taxa abundance was performed monthly during the whole period from January 2019 to December 2021. Morphological identification was performed only on adults (Kiefer, 1968; Kiefer, 1978; Margaritora, 1985; Einsle, 1993), given that naupliar and copepodite developmental stages of copepods cannot be reliably and easily identified in routine monitoring surveys, even if their species-specific taxonomic features have been already accurately described for the lake (Ravera, 1953).

Animals to be used to build the DNA barcoding reference library were extracted from different months across 2019, from the same samples used for morphological identification, in order to cover all species and their potential genetic variability through different seasons. Individuals that were morphologically identified to species level were sorted through light microscopy, fixed and stored in ethanol 96% at -20°C, ready for DNA extraction.

Sampling for DNA metabarcoding was performed monthly in 2019, 2020 and 2021, in parallel to the samples used for the morphological survey and the count of abundances. All metabarcoding samples have the corresponding sample with information from morphological identification. Bulk samples of animals from plankton tows, obtained with the same methods used for the morphological survey, were stored in buffer to be later processed for DNA extraction.

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Barcoding reference library

DNA was extracted from eight to 27 single animals for each identified morphological species. A fragment of the mitochondrial marker cytochrome c oxidase subunit I (COI) was amplified to obtain a reference library for all cladoceran and copepod species found in the zooplankton. DNA was extracted from single animals using a Chelex extraction protocol (Gómez *et al*., 2002). DNA from each individual was extracted in 35 µl of Chelex (InstaGene Matrix; Bio-Rad, CA, USA) with 1 µl protein kinase A, warming and shaking the solution for 30 minutes at 56°C with a final warming step of 10 minutes at 100°C. For each individual, a 658 base pairs fragment of the Folmer barcoding region of COI gene was amplified through PCR, using primers LCOI (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCOI (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*., 1994). Cycle conditions for PCR were the following: for COI fragment, initial denaturation at 95 °C for 5 min, followed by 42 cycles of 95 °C for 15 sec, 40 °C for 30 sec, 72 °C for 50 sec, and a final extension step of 72 °C for 5 min. All the chromatograms were checked for ambiguous nucleotide position and dubious indels using FINCHTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Forward and reverse sequences were merged in contigs using SEQUENCHER 4.1.4 (DNA sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA http://www.genecodes.com). All the sequences were aligned with MAFFT 7 (Kuraku *et al*., 2013), using the default automatic settings. Alignments were also checked using MESQUITE 3.04 (Maddison and Maddison, 2008) for absence of stop codons and correct amino acid translation, in order to minimise the potential for numts to be included.

From the alignments, we calculated the uncorrected genetic distances within and between species for the sequences from Lake Maggiore, and also in comparison to all the available sequences of the same species from GenBank. For a visual representation of distances within and between species and genera, we performed phylogenetic reconstructions using a Maximum Likelihood (ML) approach with PHYML software v3.0 (Guindon *et al*., 2010) with a GTR+invgamma evolutionary model.

DNA metabarcoding of bulk samples

Samples for DNA metabarcoding were preserved in a TRIS/EDTA/NaCl (each 0.1 M) buffer at -20°C until DNA extraction. For DNA extraction an aliquot of the bulk sample was added to the PowerBead tubes of the PowerSoil DNA extraction kit (Qiagen) and supplemented with 1% SDS and 250 µg ml^{-1} of Proteinase K and incubated at 56°C for 1 h. After this incubation the sample was homogenised in two cycles at 6000 rpm for 1 min (Precellys instrument). The next steps of the extraction were performed as suggested by the manufacturer of the kit (starting after the 10 min vortexing step) with the only modification that twice 50 µl of elution buffer were incubated for 15 min on the membrane before centrifugation. The first run of Illumina sequencing was performed on MiSeq platform using the 2 × 300 paired-end (PE) approach by IGA Technologies (REFERENCE) with general primers jgHCO2198, TAIACYTCIGGRTGICCRAARAAYCA, and mlCOIintF, GGWACWGGWTGAACWGTWTAYCCYCC (Leray *et al*., 2013) on the 12 monthly samples from 2019, obtained in parallel to the construction of the internal reference library from animals extracted from the same sampling survey. Two negative controls (Molecular Biology Grade Water, RNase/DNase-free water) and two positive controls (marine meiofauna) were included in the PCR run to check for problems of contamination, leaking, and tag jumping. Then, after *in silico* assessing the potential performance of the primers, we improved fit to the sequences we obtained thanks to the DNA barcoding, which created a local refer-

ence library, and thus developed the modified custom primer mlCOIintF_mod, GGNACBGGNTGRACNGTNTAYCCNCC. The new primer was obtained by adding additional uncertain positions to the previously published primer mlCOIintF. Metabarcoding using custom primer pair (mlCOIintF_mod and jgHCO2198) was performed on a selection of 21 samples collected from October 2019 to December 2021 with the same Illumina Miseq approach used for the general primer set. From the raw reads obtained from Illumina sequencing, adaptors and primers were clipped from the sequences using cutadapt v1.9.1 (Martin, 2011). The Usearch/Uparse pipeline was used for sequence assembly, quality filtration, chimera check and removal, and preparation of the data (Edgar, 2013), following the protocols of Martinez *et al*. (2020) for the construction of zero radius operational taxonomic units (zOTUs, Edgar, 2016). To quickly and easily distinguish between metabarcoding results when using common and custom primers, we named each unique sequence variant as 'Zotu' when obtained from the common primers and 'sq' when obtained from the custom primers. Taxonomic assignment was performed through BLAST in GenBank for each unique sequence to reach the phylum level. For the subset of Crustaceans analysed here in details, we assigned reads also to species level using two approaches. First, we compared them to GenBank using the best hit from a BLAST search, disregarding best hits with a similarity below 90%. Second, we used a phylogenetic approach, by aligning the unique sequences from Illumina (both 'Zotu' and 'sq') with the local reference library of all known species in the lake and then producing ML trees to look for the occurrence of monophyletic clades with sequences from both barcoding and metabarcoding. Currently, no software is known to outperform others and to produce excellently reliable taxonomic assignment at the species level, because of the poor eukaryotic reference datasets that limit software performance (Bik, 2021) and the existence of different group-specific thresholds between species (Bergsten *et al*., 2012; Magoga *et al*., 2021).

Test for abundances

Most of the current monitoring pipelines based on morphological data produce assessments of ecological quality of the aquatic biota by applying sophisticated methods accounting for abundances of species (Birk *et al*., 2012). In order to transition from biological monitoring through morphological approaches in species identification to metabarcoding with DNA-based approaches in what has been dubbed biological monitoring 2.0 (Baird and Hajibabaei, 2012), the ideal situation would be to have read abundances from Illumina sequencing being related to actual abundances of the different taxa. We used the results of the 21 samples of metabarcoding from Illumina sequencing using custom primers, the metabarcoding dataset with the most reliable occurrence data (see results), to test the possibility that read abundances in each of the 21 sample could be correlated with the abundance of individuals of the same species as counted in the corresponding 21 samples used for the morphological survey.

We used raw, untransformed read numbers in the final datasets as a metric of read abundance for each of the 21 samples, given that such numbers come from a series of standardisation and normalisation steps in the bioinformatics pipeline, allowing for meaningful comparisons. We compared such read numbers to the estimated densities of animals obtained from the traditional morphological surveys performed on the same 21 selected samples as a metric for species abundances. For Cladocera we analysed each species independently in the 21 samples, except for the case of two species of the *Daphnia galeata/longispina* kept as a single taxon because of the difficulty to separate them using morphology in routine monitoring. For Copepoda, due to the overwhelming number of unidentified juvenile stages (see section Results), we analysed abundance data at the family level, Cyclopidae and Diaptomidae. We assume that body size would not affect the amount of DNA from each individual, given that juveniles and adults of the different species in the dataset do not change in size by orders of magnitude as may be the case for example for some insect or fish species. We used Linear Models (LMs) with read numbers of each taxon (species or family) in each of the 21 samples as the response variable and density of animals of the same taxon as counted in the morphological survey of the same 21 samples as the explanatory variable in R 4.1.3 (R Core Team, 2022). Model fit was assessed with the R package 'performance' v 0.7.3 (Lüdecke *et al*., 2021).

RESULTS

Morphological survey

During the morphological survey performed from zooplankton samples collected every month from January 2019 to December 2021 we identified five taxa of Cladocera and four of Copepoda (Tab. 1). For the Cladocera we identified *Bythotrephes longimanus*, *Diaphanosoma brachyurum*, *Eubosmina longispina*, and *Leptodora kindtii*, in addition to the species complex *Daphnia galeata*/*longispina*. All animals could be assigned to one of these taxa, 72% of the individuals at species level, namely all animals except for those belonging to one of the two *Daphnia* species, *Daphnia galeata* and *Daphnia longispina*, not easily distinguishable during routine monitoring.

For the Copepoda, we identified *Cyclops abyssorum*, *Eudiaptomus padanus*, *Mesocyclops leuckarti*, and *Mixodiaptomus laciniatus*. Identification to species level for Copepoda could be performed only on 7% of the individ-

uals because of the large amount of animals at the juvenile stage, which are reliably identified only to family level in routine counts (Tab. 1).

Local reference library

COI sequences were obtained for all five taxa of Cladocera and four of Copepoda isolated in 2019 to build the local reference library. Overall, 74 sequences of Cladocera were obtained and uploaded in GenBank (MH321324-MH321397) and 65 for Copepoda (MN635799- MN635853 and MN635855-MN635864). A BLAST check confirmed species identifications for all the species with sequences that were already present in GenBank and the use of DNA taxonomy allowed the separation of the two species of *Daphnia* of the complex *D. galeata/longispina* for Cladocera (Supplementary Fig. S1). For three species, namely for the cladoceran *Eubosmina longispina* (Supplementary Fig. S1) and for the copepods *Eudiaptomus padanus* and *Mixodiaptomus laciniatus* (Supplementary Fig. S2), our sequences were the first ones to be uploaded to GenBank.

Metabarcoding using general primers

With the generic primer pair from the 12 samples collected in 2019, we obtained 101 unique sequence variants (325 base pairs (bp) long) that were unambiguously assigned to eukaryotes. They belonged mostly to Crustacea (n=64, Tab. 1), but also to Rotifera (n=25) and Protista (n=12).

For the Cladocera, of the species already known and sequenced in the lake, *Daphnia galeata*, *Daphnia longispina*, *Leptodora kindtii*, and *Bythotrephes longimanus* were successfully retrieved, could all be unambiguously identified with a BLAST search, and formed monophyletic clades with the sequences from the local reference library (Fig. 1). On the other hand, the metabarcoding approach failed in finding sequences for the other two species surely present in the lake in the same samples, *Eubosmina longispina* and *Diaphanosoma brachyurum*. Their absence from the metabarcoding results is not related to their potential absence from the samples, given that the subsamples used for the morphological analyses revealed that the two species were present and even abundant.

For the Copepoda, of the species of cyclopoids known and sequenced in the lake, *Cyclops abyssorum* and *Mesocyclops leuckarti* were found, unambiguously identified with a BLAST search, and formed monophyletic clades with the sequences from the local reference library (Fig. 2). Among the diaptomids, *Eudiaptomus padanus* could be identified through a comparison with our barcoding reference library. No sequence attributable to *Mixodiaptomus laciniatus* were found with metabarcoding, despite the presence of the species in the subsamples used for the morphological analyses. Other taxa of copepods were found, with few sequences (Fig. 2), but they could not be attributed to any known species through a BLAST search.

Overall, metabarcoding with common primers identified the occurrence of eight of the eleven known species. A BLAST search could attribute to species level only 20 out of the 64 unique sequence variants (31.2%); a com-

Tab. 1. Comparisons of results on species identification from morphology and DNA metabarcoding. Under 'morphology': 'yes' = taxon that can be unambiguously identified; 'unclear' = taxon that cannot be unambiguously identified; 'NA' = taxon that was not seen with the method. Under '# individuals: the sum of all estimated numbers of individuals in the morphological survey for each identifiable taxon is reported, but only for the 21 samples for which we also have results from Illumina sequencing with custom primers (to be used for the read/abundance comparison). Under 'metabarcoding': the number of unique sequences found for each taxon in the 21 analysed samples using custom primer pairs (mlCOIintF_mod and jgHCO2198); between parentheses the number of unique sequences found for each taxon in the 12 analysed samples using general primers (mlCOIintF and jgHCO2198).

Group	Species	Morphology	# individuals	# unique sequences (metabarcoding)
Cladocera	*Bythotrephes longimanus*	Yes	382	5 (2)
	Daphnia galeata	Unclear	13,141	3 (2)
	Daphnia longispina	Unclear		3 (2)
	Diaphanosoma brachyurum	Yes	19,544	1 (NA)
	Eubosmina longispina	Yes	12,942	3 (NA)
	Leptodora kindtii	Yes	254	5 (4)
Copepoda	*Cyclops abyssorum*	Yes	3,027	10 (3)
	Eucyclops macrurus	NA	NA	1 (NA)
	Eudiaptomus padanus	Unclear	24,785	96 (42)
	Mesocyclops leuckarti	Yes	1,279	14 (7)
	Mixodiaptomus laciniatus	Yes	103	4 (NA)
	Thermocyclops crassus	NA	NA	2 (NA)
	undetermined Cyclopidae	Yes	184,837	3 (1)
	undetermined Diaptomidae	Yes	218,044	4 (1)

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parison with the internal barcoding reference library improved taxonomic assignment to 62 out of the 64 sequences (96.9%).

Metabarcoding using custom primers

The custom primers were obtained by modifying the commonly used ones by adding more ambiguities by comparing their performance *in silico* on the alignment of sequences from the local reference library. These primers, applied to 21 zooplankton samples collected from October 2019 to December 2021, produced 302 unique sequence variants of 325 bp, unambiguously assigned to eukaryotes (Supplementary Data S1). They belonged mostly to crustaceans (n=154), but also to other Arthropoda (n=4), Rotifera (n=54), Cnidaria (n=1: the invasive species *Craspedacusta sowerbii*), and Protista (n=89).

All known Cladocera species in Lake Maggiore were found and identified, including those that were not detected with the generic primers: *Eubosmina longispina* and *Diaphanosoma brachyurum*. As for the Copepoda, all known species in Lake Maggiore were found, including *Mixodiaptomus laciniatus*, which was not identified with the generic primers. In addition, the use of optimized primers for metabarcoding of zooplankton samples led to further discoveries: in addition to seven unidentifiable sequences, few sequences of what could be *Eucyclops macrurus* (Sars G.O., 1863) in August 2021 (sequence sq1394 has 98.1% similarity with GenBank KC627334, KC627335, and KC627337) and *Thermocyclops crassus* (Fischer, 1853) in September and October 2020 (sequences sq351 and sq698 have from 99.2% to 100% similarity with GenBank MZ964921 and MZ964922) were found, but not seen through the morphological screening of the same samples.

Overall, metabarcoding with custom primers identified the occurrence of all the eleven known species from morphology, in addition to at least two other species not found during the morphological survey from the same samples. A BLAST search could attribute 44 out of the 154 unique sequence variants to species level (28.6%); a comparison with the internal barcoding reference library improved taxonomic assignment to 147 out of the 154 sequences (95.5%), leaving only seven sequences of copepods without a clear taxonomic identification.

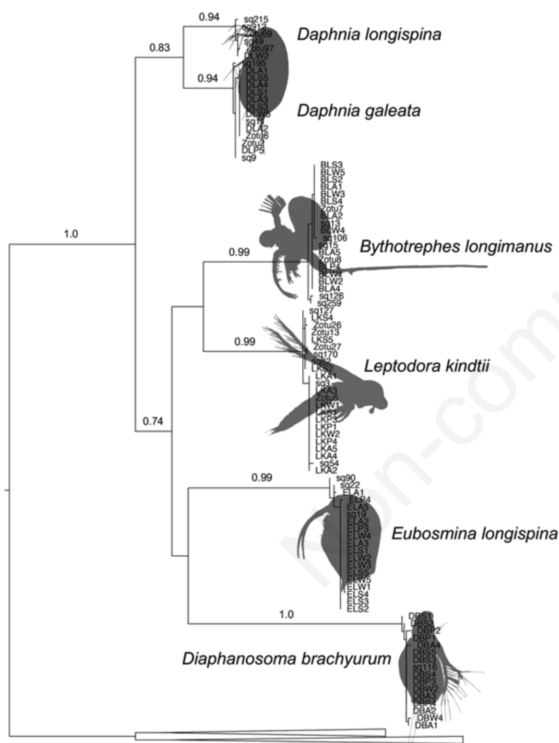

Fig. 1. Maximum Likelihood (ML) phylogenetic reconstruction for the COI sequences belonging to Cladocera merging the internal reference library (acronyms of species and sampling time, see Supplementary Fig. S1), metabarcoding from common primers (names starting with 'Zotu'), and metabarcoding from custom primers (names starting with 'sq'). Branch length is proportional to substitutions/sites in the scale bar, from a GTR + invgamma evolutionary model. Numbers on branches refer to aLRT support values, not reported for short terminal branches and for values below 0.7. The outgroups with the sequences from Copepoda are collapsed (see Fig. 2). Pictograms and species names are reported on the tree.

Abundance data

For Cladocera, the number of reads for each species (or species complex) could not be explained by the number of individuals in the same sample, if not for *Diaphanosoma brachyurum* (Tab. 2, Supplementary Fig. S3). For Copepoda, no explanation on read numbers at the family level was related to the abundance of individuals (Tab. 2, Supplementary Fig. S4), suggesting that read numbers cannot be easily equated to abundance data at any taxonomic level.

Tab. 2. Results of Linear Models (LM) with t and p-values for each model based on the effect of the abundance of individuals (from the morphological survey) on the number of reads (obtained with primer pair jgHCO2198-mlCOIintF_mod) for each taxon of Cladocera and Copepoda. Significant p-values are marked in bold.

Group	Species	t	p
Cladocera	*Bythotrephes longimanus*	-0.307	0.7620
	Daphnia galeata/longispina	0.505	0.6195
	Diaphanosoma brachyurum	4.109	**0.0006**
	Eubosmina longispina	1.069	0.2984
	Leptodora kindtii	1.402	0.1770
Copepoda	Cyclopidae	0.932	0.3628
	Diaptomidae	0.646	0.5263

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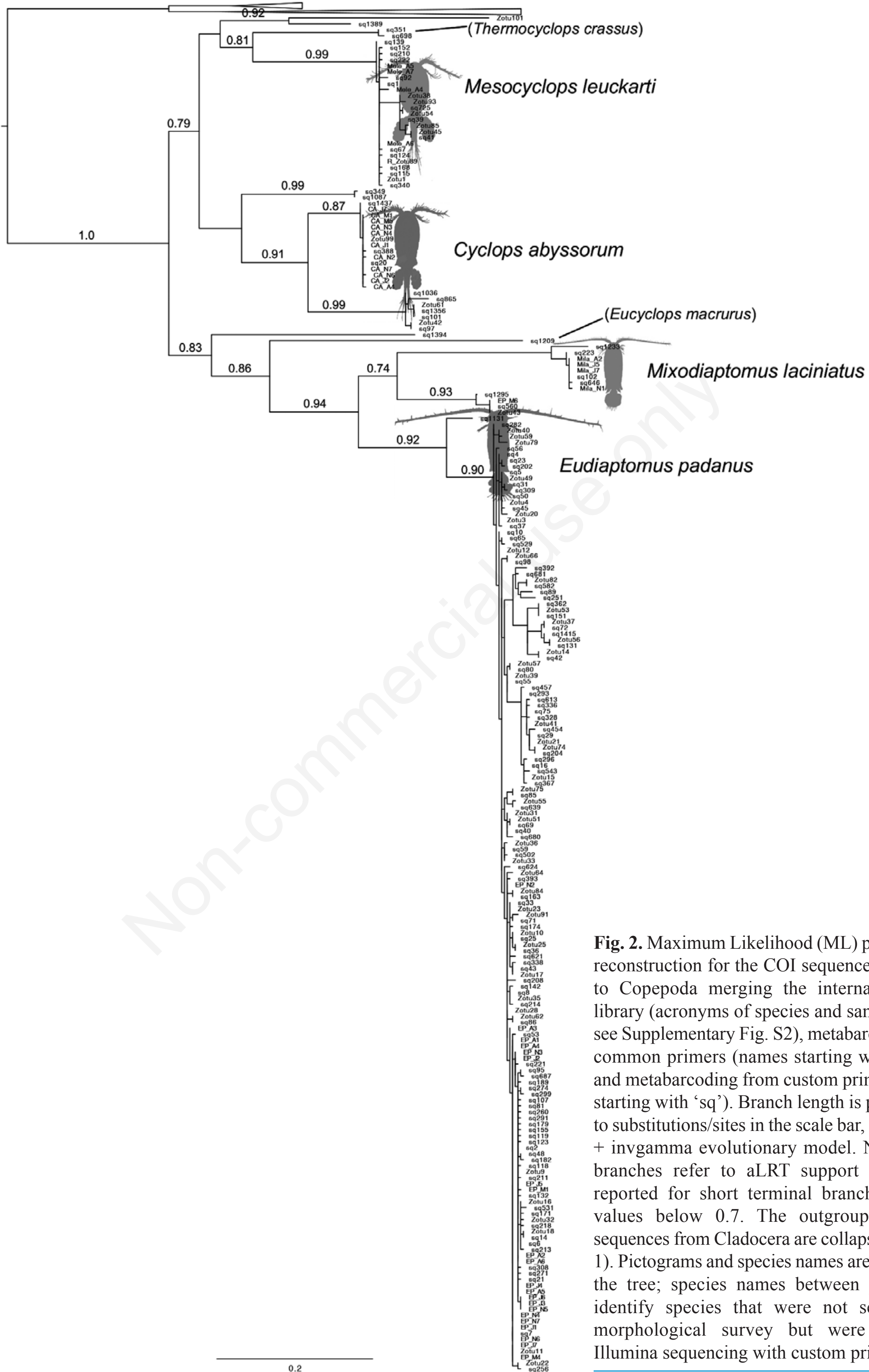

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Fig. 2. Maximum Likelihood (ML) phylogenetic reconstruction for the COI sequences belonging to Copepoda merging the internal reference library (acronyms of species and sampling time, see Supplementary Fig. S2), metabarcoding from common primers (names starting with 'Zotu'), and metabarcoding from custom primers (names starting with 'sq'). Branch length is proportional to substitutions/sites in the scale bar, from a GTR + invgamma evolutionary model. Numbers on branches refer to aLRT support values, not reported for short terminal branches and for values below 0.7. The outgroups with the sequences from Cladocera are collapsed (see Fig. 1). Pictograms and species names are reported on the tree; species names between parentheses identify species that were not seen by the morphological survey but were found by Illumina sequencing with custom primers.

DISCUSSION AND CONCLUSIONS

The most relevant result of our study is that the use of custom primers, developed specifically for the local genetic diversity of the target community, massively improves species detection from DNA metabarcoding. The use of common primers, already applied for studies of zooplankton crustaceans (Stefanni *et al*., 2018: Kiemel *et al*., 2022), did not reveal the whole expected species list known from the morphological survey. The use of custom primers allowed us to identify all the expected species. This may seem a tautological result, given that the custom primers matched the local species list and were exactly designed for that purpose. Yet, a series of potential PCR biases and additional problems may have prevented the amplification of DNA from all the species known to occur in the samples (Leray and Knowlton, 2017; Fonseca, 2018). The use of degenerate primers is known to reduce amplification bias (Krehenwinkel *et al*., 2017), and indeed, also in our case the degenerate custom primers had a better representation of the occurrence of local species diversity than commonly used general, less degenerate primers.

Identification to species level for the metabarcoding data from our custom primer was very good and even better than the morphological approach for Cladocera: 72% of the individuals could be assigned to species level from morphology (all except the two species of the *Daphnia galeata/longispina* complex), whereas 100% of the sequences could be unambiguously assigned to a species with distance-based and phylogeny-based approaches. The improvement in species identification for Copepoda from morphology to metabarcoding was even more extreme: only 7% of the individuals could be assigned to species level from morphology because of the large amount of juvenile stages that are identifiable only to family level in routine monitoring programs. Regarding metabarcoding using custom primers, 95.5% of the unique sequences (147 out of 154) were assigned to species level, including the identification of two species that were not found during the morphological survey. Random sampling during sequencing for metabarcoding is known to lead to low reproducibility of rare species, which may pass unnoticed (Leray and Knowlton, 2017): in our case, even species that were rare (or apparently absent) in the morphological survey were recovered with the custom primers.

It is known that under certain conditions of high primer match in rich communities with low evenness in the distribution of species abundance some primer pairs may also be able to provide reliable quantitative estimates (Piñol *et al*., 2019). Yet, in the case of the crustaceans of the zooplankton with the custom developed primers, even if occurrences were reliable, abundances were not. This is a common scenario and read abundance is not considered a suitable metric in eukaryotes, especially when using ribosomal or mitochondrial markers (Burki *et al*., 2021; Martin *et al*., 2022). For the use of common primers, not even occurrences were reliable, with several species, surely present in the samples, missing from the metabarcoding results. Our current understanding of the conditions affecting the quantitative performance of metabarcoding is still limited (Lamb *et al*., 2018): the conditions in Lake Maggiore and using our protocols did not allow for any possibility to estimate abundance of organisms from read numbers.

Overall, species composition from morphology is comparable to that obtained from DNA metabarcoding using custom primers, developed after screening the local genetic diversity. Given such potential reliability of DNA metabarcoding to describe community composition (Santoferrara, 2019), the approach has indeed been recently used to address ecological question related to drivers of ecological differences (Chain *et al*., 2016) and even impact of human activities (Martínez *et al*., 2020; Yang and Zhang, 2020). Our study confirms that such approaches could now be performed also using the crustaceans of the zooplankton of freshwater lakes, at least for the biogeographical area of the study site, for which the taxonomic coverage of the reference library is not too bad.

A further improvement on the method could be the creation of a better reference library to cover a broader taxonomic spectrum, including more families and genera, and more species (Bik, 2021; Buklin *et al*., 2021: Pappalardo *et al*., 2021): such reference dataset would allow the identification also of the few sequences that could only be attributed to undetermined copepods in our metabarcoding results. The barcoding initiatives of several countries already try to achieve such aim (Adamowicz, 2015; Geiger *et al*., 2016).

While building the reference library from DNA barcoding and DNA taxonomy for our study, we confirmed the presence of two mitochondrial lineages in the *Daphnia galeata/longispina* complex, most likely corresponding to the two nominal species *D. galeata* and *D. longispina*. It is thus possible that both species are present, even if hybridisation exists in the complex (Hobaek *et al*., 2004; Griebel *et al*., 2015) and the use of only one mitochondrial marker may provide misleading inference for the correct identification of the species.

Another result obtained by the metabarcoding analysis is the presence of unidentified copepod species, potentially present in the lake but not seen with the morphological survey. Another species of *Eudiaptomus* was previously found in the lake, *Eudiaptomus gracilis* (G.O. Sars 1863) (Visconti and Manca, 2010), but was not found during our morphological survey. Further studies in integrative taxonomy, collecting samples on purpose to perform detailed morphological analyses supported by the groups identified by DNA (Schlick-Steiner *et al*., 2010)

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would be able to give a name to the missing copepod taxa.

Other two copepod species, *Eucyclops macrurus* and *Thermocyclops crassus*, were found only by the metabarcoding performed with custom primers and not by the morphological survey. These species are historically very rarely present in the zooplankton of the deepest part of Lake Maggiore, and in low numbers (Manca *et al*., 2008). Yet, they are known from the lake and from nearby smaller water bodies (Giussani *et al*., 1990; Lepori, 2020) and their detection with the metabarcoding pipeline makes sense. Detection of littoral taxa in lake open water is not a rare phenomenon, because of horizontal migrations (Dussart, 1969; Hamza *et al*., 1993; Manca *et al*., 1998; Manca *et al*., 2007a; Visconti and Manca, 2010). Water warming and changes in thermal regime, with a more pronounced and durable thermal stratification, certainly enhance the possibility for these taxa to move to the lake open waters, even in the parts where the water column is deep (Wagner and Adrian, 2011). It would be difficult to find these species with morphological monitoring, especially if they are in low numbers. Yet, they were identifiable thanks to DNA metabarcoding.

An additional result of the DNA metabarcoding approach is the confirmation of the occurrence of an invasive alien species, the freshwater jellyfish *Craspedacusta sowerbii*, already known from the lake (Ramazzotti *et al*., 1962; Stefani *et al*., 2010; Morpurgo *et al*., 2021).

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DATA AVAILABILITY

GenBank Accession Numbers for barcoding sequences: MH321324-MH321397, MN635799- MN635853, and MN635855-MN635864.

Raw reads from Illumina sequencing: BioProject PRJNA909627.

REFERENCES

Adamowicz SJ, 2015. International Barcode of Life: Evolution of a global research community. Genome 58:151-162.

Arfè A, Quatto P, Zambon A, MacIsaac HJ, Manca M, 2019. Long-term changes in the zooplankton community of Lake Maggiore in response to multiple stressors: A functional principal components analysis. Water 11:962.

Baird DJ, Hajibabaei M, 2012. Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next generation DNA sequencing. Mol Ecol 21:2039-2044.

Bergsten J, Bilton DT, Fujisawa T, Elliott M, Monaghan MT, Balke M, et al., 2012. The effect of geographical scale of sampling on DNA barcoding. Syst Biol 61:851-869.

Bik HM, 2021. Just keep it simple? Benchmarking the accuracy of taxonomy assignment software in metabarcoding studies. Mol Eco Ress 21:2187-2189.

Birk S, Bonne W, Borja A, Brucet S, Courrat A, Poikane S, et al., 2012. Three hundred ways to assess Europe's surface waters: an almost complete overview of biological methods to implement the Water Framework Directive. Ecol Indic 18:31-41.

Borgmann U, Shear H, Moore J, 1984. Zooplankton and potential fish production in Lake Ontario. Can J Fish Aquat Sci 41:1303-1309.

Bucklin A, Peijnenburg KT, Kosobokova KN, O’Brien TD, Blanco-Bercial L, Cornils A, et al., 2021. Toward a global reference database of COI barcodes for marine zooplankton. Mar Biol 168:1-26.

Burki F, Sandin MM, Jamy M, 2021. Diversity and ecology of protists revealed by metabarcoding. Curr Biol 31:R1267-R1280.

Carney HJ, Elser JJ, 1990. Strength of zooplankton-phytoplankton coupling in relation to lake trophic state, p. 615-631. In: Tilzer MM, Serruya C (eds), Large Lakes. Brock/Springer Series in Contemporary Bioscience. Springer.

Chain FJJ, Brown EA, MacIsaac HJ, Cristescu ME, 2016. Metabarcoding reveals strong spatial structure and temporal turnover of zooplankton communities among marine and freshwater ports. Divers Distrib 22:493-504.

Collins RA, Cruickshank RH, 2013. The seven deadly sins of DNA barcoding. Mol Ecol Res 13:969-975.

de Bernardi R, Giussani G, Manca M, Ruggiu D, 1988. Long-term dynamics of plankton communities in Lago Maggiore (N. Italy). Int Ver Theor Angew Limnol Verhand 23:729-733.

Dussart B, 1969. Les Copépodes des Eaux continentales d'Europe occidentale. Tome II: Cyclopoides et Biologie. Paris: Editions N. Boublée and Cie; 292 pp.

Edgar RC, 2016. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. bioRxiv 081257.

Edgar RC, 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Meth 10:996.

Einsle U, 1993. Crustacea: Copepoda: Calanoida und Cyclopoida. Subwasserfauna bon Mitteleuropa. Bd. 8/Heft 4/Teil 1. Gustav Fischer Verlag, Stuttgart: 209 pp.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R, 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294-299.

Fonseca VG, 2018. Pitfalls in relative abundance estimation using eDNA metabarcoding. Mol Ecol Res 18:923-926.

Geiger MF, Astrin JJ, Borsch T, Burkhardt U, Grobe P, Hand R, et al., 2016. How to tackle the molecular species inventory for an industrialized nation—lessons from the first phase of the German Barcode of Life initiative GBOL (2012–2015). Genome 59:661-670.

Giussani G, de Bernardi R, Ruffoni T, 1990. Three years of expe-

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rience in biomanipulating a small eutrophic lake: Lago di Candia (Northern Italy). Hydrobiologia 200:357-366.

Gómez A, Serra M, Carvalho GR, Lunt DH, 2002. Speciation in ancient cryptic species complexes: Evidence from the molecular phylogeny of *Brachionus plicatilis* (Rotifera). Evolution 56:1431–1444.

Griebel J, Gießler S, Poxleitner M, Navas Faria A, Yin M, Wolinska J, 2015. Extreme environments facilitate hybrid superiority – The story of a successful *Daphnia galeata* × *longispina* hybrid clone. PloS One 10:e0140275.

Guindon S, Dufayard J, Lefort V, Anisimova M, Hordijk W, Gascuel O, 2010. New algorithms and methods to estimate Maximum-Likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307-321.

Hamza W, Ruggiu D, Manca M, 1993. Diel zooplankton migrations and their effect on the grazing impact in Lake Candia (Italy). In: Ringelberg J (ed), Proceedings Symposium "Diel vertical migration of zooplankton". Ergeb Limnol 39: 175-185.

Hobæk A, Skage M, Schwenk K, 2004. *Daphnia galeata* × *D. longispina* hybrids in western Norway. Hydrobiologia 526:55-62.

Jeppesen E, Noges P, Davidson TA, Haberman J, Noges T, Blank K, et al., 2011. Zooplankton as indicators in lakes: a scientific-based plea for including zooplankton in the ecological quality assessment of lakes according to the European Water Framework Directive (WFD). Hydrobiologia 676:279-297.

Kallis G, Butler D, 2001. The EU water framework directive: measures and implications. Water Policy 3:125-142.

Kiemel K, Weithoff G, Tiedemann R, 2022. DNA metabarcoding reveals impact of local recruitment, dispersal, and hydroperiod on assembly of a zooplankton metacommunity. Mol Ecol online ahead of print.

Kiefer F, 1968. Versuch einer Revision der Gattung Eudiaptomus Kiefer (Copepoda, Calanoida). Mem Ist Ital Idrobiol 24:9-160.

Kiefer F, 1978. Freilebend Copepoda – Die Binnengewässer. vol 26. Stuttgart: Schweizerbart'sche Verlagsbuchhandlung; 343 pp.

Krehenwinkel H, Wolf M, Lim JY, Rominger AJ, Simison WB, Gillespie RG, 2017. Estimating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. Sci Rep 7:1-12.

Kuraku S, Zmasek CM, Nishimura O, Katoh K, 2013. aLeaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. Nucleic Acids Re 41:22-28.

Lamb PD, Hunter E, Pinnegar JK, Creer S, Davies RG, Taylor MI, 2019. How quantitative is metabarcoding: A meta-analytical approach. Mol Ecol 28:420-430.

Leb C, 2015. One step at a time: international law and the duty to cooperate in the management of shared water resources. Water Int 40:21-32.

Leese F, Altermatt F, Bouchez A, Ekrem T, Hering D, Meissner K, et al., 2016. DNAqua-Net: Developing new genetic tools for bioassessment and monitoring of aquatic ecosystems in Europe. Res Ideas Outcomes 2:e11321.

Lepori F, 2020. Breve storia dello zooplancton da crostacei della zona pelagica del Lago di Lugano. Bollettino Società Ticinese Scienze Naturali 108:97-102.

Leray M, Knowlton N, 2017. Random sampling causes the low reproducibility of rare eukaryotic OTUs in Illumina COI metabarcoding. PeerJ 5:e3006.

Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, Boehm JT, Machida RJ, 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front Zool 10:34.

Lim NKM, Tay YC, Srivathsan A, Tan JWT, Kwik JTB, Baloğlu B, Meier R, Yeo DCJ, 2016. Next-generation freshwater bioassessment: eDNA metabarcoding with a conserved metazoan primer reveals species-rich and reservoir-specific communities. Roy Soc Open Sci 3:160635.

Lindenmayer DB, Likens GE, 2009. Adaptive monitoring: a new paradigm for long-term research and monitoring. Trends Ecol Evol 24:482-486.

Lucentini L, Rebora M, Puletti ME, Gigliarelli L, Fontaneto D, Gaino E, Panara F, 2011. Geographical and seasonal evidence of cryptic diversity in the *Baetis rhodani* complex (Ephemeroptera, Baetidae) revealed by means of DNA taxonomy. Hydrobiologia 673:215-228.

Lüdecke D, Ben-Shachar MS, Patil I, Waggoner P, Makowski D, 2021. performance: An R Package for Assessment, Comparison and Testing of Statistical Models. J Open Source Softw 6:3139.

Maddison W, Maddison D, 2008. Mesquite 2. A modular system for evolutionary analysis. Available from: http://www.mesquiteproject.org

Magoga G, Fontaneto D, Montagna M, 2021. Factors affecting the efficiency of molecular species delimitation in a species rich insect family. Mol Ecol Res 21:1475-1489.

Magurran AE, Baillie SR, Buckland ST, Dick JMP, Elston DA, Scott EM, Smith RI, Somerfield PJ, Watt AD, 2010. Long-term datasets in biodiversity research and monitoring: Assessing change in ecological communities through time. Trends Ecol Evol 25:574-582.

Manca M, Cavicchioni N, Morabito G, 2000. First observations on the effect of a complete, exceptional overturn of Lake Maggiore on plankton and primary productivity. Int Rev Hydrobiol 85:209-222.

Manca MM, Portogallo M, Brown ME, 2007a. Shifts in phenology of *Bythotrephes longimanus* and its modern success in Lake Maggiore as a result of changes in climate and trophy. J Plankton Res 29:515-525.

Manca M, Visconti A, de Bernardi R, 2008. Lo zooplancton del Lago Maggiore nel quinquennio 2003-2007: tendenze evolutive ed eccezioni alla luce dei cambiamenti globali. Biologia Ambientale 22:64-72.

Manca M, Torretta B, Comoli P, Amsinck SL, Jeppesen E, 2007. Major changes in trophic dynamics in large, deep sub-alpine Lake Maggiore from 1940s to 2002: A high resolution comparative palaeo-neolimnological study. Freshwater Biol 52:2256-2269.

Manca M, Beltrami M, Comoli P, Cavicchioni N, de Bernardi, 1998. Indagini sullo zooplancton. In: Istituto Italiano di Idrobiologia - CNR. Ricerche sull'evoluzione del Lago Maggiore. Indagini limnologiche. Campagna 1997. Relazione finale per il quinquennio. Commissione Internazionale per la Protezione delle Acque Italo-Svizzere; 52 pp.

Manca M, Calderoni A, Mosello R, 1992. Limnological research

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in Lago Maggiore: studies on hydrochemistry and plankton. Mem Ist ital Idrobiol 50: 171-200.

Margaritora FG, 1985. Fauna d'Italia. Cladocera. Calderini, Bologna; 399 pp.

Martin JL, Santi I, Pitta P, John U, Gypens N, 2022. Towards quantitative metabarcoding of eukaryotic plankton: an approach to improve 18S rRNA gene copy number bias. Metabarcoding Metagenomics 6:e85794.

Martin M, 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J 17:10-12.

Martínez A, Eckert EM, Artois T, Careddu G, Casu M, Curini-Galletti M, et al, 2020. Human access impacts biodiversity of microscopic animals in sandy beaches. Commun Biol 3:1-9.

McManus GB, Katz LA, 2009. Molecular and morphological methods for identifying plankton: what makes a successful marriage? J Plankton Res 31:1119-1129.

Morpurgo M, Schuchert P, Vorhauser S, Alber R, 2021. Occurrence of two distinct lineages of the freshwater jellyfish *Craspedacusta sowerbii* (Hydrozoa: Limnomedusae) in Italy. J Limnol 80:1974.

Mosello R, Bertoni R, Guilizzoni P, 2010. Limnological and palaeolimnological research on Lake Maggiore as a contribution to transboundary cooperation between Italy and Switzerland transboundary lakes and rivers, p. 160-167. In: Ganoulis J, Aureli A, Fried J (eds), Transboundary water resources management. A multidisciplinary approach. Weinheim: Wiley.

Obertegger U, Manca M, 2011. Response of rotifer functional groups to changing trophic state and crustacean community. J Limnol 70:231-238.

Obertegger U, Flaim G, Fontaneto D, 2014. Cryptic diversity within the rotifer *Polyarthra dolichoptera* along an altitudinal gradient. Freshwater Biol 59:2413-2427.

Pace ML, 1986. An empirical analysis of zooplankton community size structure across lake trophic gradients. Limnol Oceanogr 31:45-55.

Pappalardo P, Collins AG, Pagenkopp Lohan KM, Hanson KM, Truskey SB, Jaeckle W, et al., 2021. The role of taxonomic expertise in interpretation of metabarcoding studies. ICES J Mar Sci 78:3397-3410.

Piñol J, Senar MA, Symondson WOC, 2019. The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. Mol Ecol 28:407-419.

R Core Team, 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Available from: https://www.R-project.org/

Ramazzotti G, 1962. Ritrovamento della medusa dulciacquicola *Craspedacusta sowerbyi* nella regione del Lago Maggiore. Mem Ist ital Idrobiol 15:175-181.

Ravera O, 1953. Gli stadi di sviluppo dei Copepodi pelagici del Lago Maggiore. Mem Ist ital Idrobiol 7:129-150.

Rey A, Corell J, Rodriguez-Ezpeleta N, 2020. Metabarcoding to study zooplankton diversity, p. 252-263. In: Teodosio MA, Branco Barbosa AN (eds) Zooplankton Ecology. Boca Raton, CRC Press.

Santoferrara LF, 2019. Current practice in plankton metabarcoding: optimization and error management. J Plankton Res 41:571-582.

Schlick-Steiner BC, Steiner FM, Seifert B, Stauffer C, Christian E, Crozier RH, 2010. Integrative taxonomy: a multisource approach to exploring biodiversity. Ann Rev Entomol 55:421-438.

Simboura N, Panayotidis P, Papathanassiou E, 2005. A synthesis of the biological quality elements for the implementation of the European Water Framework Directive in the Mediterranean ecoregion: The case of Saronikos Gulf. Ecol Indic 5:253-266.

Stefani F, Leoni B, Marieni A, Garibaldi L, 2010. A new record of *Craspedacusta sowerbii*, Lankester 1880 (Cnidaria, Limnomedusae) in northern Italy. J Limnol 69:189.

Stefanni S, Stanković D, Borme D, de Olazabal A, Juretić T, Pallavicini A, Tirelli V, 2018. Multi-marker metabarcoding approach to study mesozooplankton at basin scale. Sci Rep 8:12085.

Vakkilainen K, Kairesalo T, Hietala J, Balayla DM, Bécares E, Van De Bund WJ, et al., 2004. Response of zooplankton to nutrient enrichment and fish in shallow lakes: A pan-European mesocosm experiment. Freshwater Biol 49:1619-1632.

Visconti A, Manca M, 2010. The invasive appearance of *Eudiaptomus gracilis* (G.O. Sars 1863) in Lago Maggiore. J Limnol 69:353-357.

Visconti A, Manca M, de Bernardi R, 2008. Eutrophication-like response to climate warming: An analysis of Lago Maggiore (N. Italy) zooplankton in contrasting years. J Limnol 67: 87-92.

Wagner C, Adrian R, 2011. Consequences of changes in thermal regime for plankton diversity and trait composition in a polymictic lake: a matter of temporal scale. Freshwater Biol 56:1949-1961.

Yang J, Zhang X, 2020. eDNA metabarcoding in zooplankton improves the ecological status assessment of aquatic ecosystems. Environ Int 134:105230.

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