




# Stable isotope analysis reveals trophic segregation between the invasive zebra mussel *Dreissena polymorpha* and the native duck mussel *Anodonta anatina* in Lake Trasimeno (Italy)

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**Abstract** Non-indigenous freshwater bivalves negatively affect invaded ecosystems through different mechanisms, including inter-specific competition for trophic resources. Here, we investigated in Lake Trasimeno (Central Italy) the diet of the invasive *Dreissena polymorpha* and the native *Anodonta anatina*.  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  stable isotopes were measured in winter and summer in bivalves, phytoplankton, and sedimentary organic matter (SOM); the relative dietary contributions of the two resources were determined using Bayesian mixing models. To elucidate the different carbon and nitrogen pools characterizing the study site, isotopic analyses were extended to zooplankton and to representatives of the benthic flora

and macroinvertebrate fauna. Independently from the season, the two bivalves showed a limited trophic overlap, as mixing models indicated for *D. polymorpha* a diet based primarily on phytoplankton, while *A. anatina* relied mainly on SOM. Dietary differences were less marked in summer, when comparable isotopic values characterized phytoplankton and SOM. In winter, conversely, the trophic differentiation between the two species was more evident, and corresponded with a significant enrichment in SOM  $\delta^{13}\text{C}$  values, likely due to a substantial contribution of carbon deriving from decaying macrophytes. Whether differences in ecological and behavioral traits alone can explain the observed trophic segregation between the two species, or if they have actively shifted their diet to reduce competition for food is discussed. We conclude emphasizing the need of an advanced resolution of the influence of non-indigenous species on

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the flux of energy and matter in invaded lentic systems, including Lake Trasimeno.

**Keywords** Biological invasions · Trophic competition ·  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  · Mixing models · Carbon dynamics · Lentic systems

## Introduction

With the exception of Antarctica, freshwater bivalves (including the orders Unionida, Venerida, Mytilida, and Myida) are distributed throughout all continents where they are acknowledged to exert a critical influence on the structure and function of lotic and lentic ecosystems (Lopes-Lima et al., 2018). Indeed, besides providing a dominant contribution to the benthic biomass, bivalves can affect the abundance of bacteria, particulates, and primary producers in the water column through top-down effects, ultimately influencing nutrient cycling (Taylo et al., 2015; Vaughn, 2018; McDowell & Sousa, 2019 and literature cited). In addition, they can act as ecosystem engineers, altering the physical structure of the benthic environment via bioturbation or creating colonizable biogenic substrates (Vaughn & Hakenkamp, 2001; Boeker et al., 2016; Ilarri et al., 2019).

Among freshwater bivalves, Unionida have declined dramatically in recent decades and are currently considered one of the most threatened taxonomic groups worldwide (Dudgeon et al., 2006; Lopes-Lima et al., 2014b; Ferreira-Rodríguez et al., 2019). The remarkable declines in spatial distribution, abundance, and species diversity have been related to a suite of anthropogenic disturbances, including habitat loss and fragmentation, water quality degradation, overexploitation, and climate change; the introduction of invasive species has been emphasized as a further, important threat to the conservation of native Unionida (Lopes-Lima et al., 2017, 2018; Böhm et al., 2021). Invasion impacts on native bivalves have been generally related with a number of factors, including differential ability to respond to abiotic (e.g., environmental stress) and biotic pressures (e.g., predators including parasites), modification of the physical or biogeochemical conditions of the substrate and water column, as well as competition for space (Sousa et al., 2014; Bielen et al., 2016; Özgo et al., 2020; Taskinen et al., 2021). In addition, a number of reviews

and long term studies have suggested that invasive bivalves—dreissenid mussels in particular—may be superior competitors in food acquisition due to, e.g., more flexible diets, or higher ability and efficiency to exploit trophic resources (Higgins & Vander Zanden, 2010; Sousa et al., 2014; Strayer and Malcom, 2018). Evidence supporting this scenario is still relatively limited (Makhutova et al., 2013; Novais et al., 2016; Douda & Čadková, 2018; Modesto et al., 2021), and further experimental investigations are needed to verify under field conditions the potential occurrence of exploitative food competition for invasive and native bivalves.

Here, we compared the diets of the invasive zebra mussel *Dreissena polymorpha* (Pallas, 1771) (Myida) and of the native duck mussel *Anodonta anatina* (Linnaeus, 1758) (Unionida) occurring in sympatry in Lake Trasimeno (Central Italy). Complete information on the biology, ecology, and invasion history of *D. polymorpha* is provided in Karatayev et al. (2007) and in Nalepa and Schloesser (2019). In brief, the species is characterized by a high fecundity ( $2.7\text{--}10 \times 10^5$  eggs produced per female per reproductive season), with planktonic free-swimming larvae and benthic sessile adult stages, reaching a typical shell length of 20–30 mm in 3–4 years. In the last two centuries, *D. polymorpha* has spread from the native Ponto-Caspian Basin toward North American and European freshwaters at alarming rates (Karatayev et al., 2015). The invasion has caused considerable concern worldwide due to the impacts observed in both lotic and lentic environments, related to, e.g., negative effects on the structure of native communities as well as on energy and element fluxes across trophic levels (Ricciardi et al., 1996; Burlakova et al., 2000; Caraco et al., 2006; Sousa et al., 2011). In Italy, the dreissenid was first reported from Lake Garda in the early '70s (Giusti & Oppi, 1972); it was subsequently observed in Lake Trasimeno in 1999 (Spilinga et al., 2000), probably introduced by transfer of recreational boats, fish restocking, and aquaculture practices (Charavgis and Cingolani, 2004). The species is currently found almost ubiquitously in the basin on rocks and artificial substrates as well as on soft bottoms at high densities (up to 200,000 ind.  $\text{m}^{-2}$ : Lancioni and Gaino, 2006; Goretti et al., 2020b). *Anodonta anatina*, as other unionids, has a complex life cycle involving internal fertilization, larval stages (glochidia) brooded by females (or hermaphrodites)

in gill chambers, and subsequent release ( $3\text{--}4 \times 10^5$  glochidia per individual) as fish ectoparasites completing their metamorphosis to young mussels in the host (Kat, 1984; Niemeyer, 1993). Adults are characterized by an indeterminate growth and can live up to 13 years reaching a shell length of more than 100 mm (Zieritz and Aldridge, 2011; Müller et al., 2021). The species has a pan-European distribution (Graf, 2007) and occurs in lakes and slow-flowing lotic environments throughout northern and central Italy, including Lake Trasimeno (Froufe et al., 2017).

*Dreissena polymorpha* and *Anodonta* spp. are suspension feeders with diets primarily based on phytoplankton (Bastviken et al., 1998; Bontés et al., 2007; Naddafi et al., 2007; Lopes-Lima et al., 2014a) that can be supplemented with a diverse spectrum of alternative resources, including organic particulate resuspended from bottom sediments (Raikow & Hamilton, 2001; Strayer et al., 2004; Vaughn et al., 2008; Cole and Solomon, 2012). Exploitative competition for food has been repeatedly suggested to explain the negative impacts exerted by the zebra mussel on native unionid bivalves including species of the genus *Anodonta* (Strayer and Smith, 1996; Baker and Levinton, 2003; Strayer & Malcom, 2018). In the past decade, the analysis of bulk carbon and nitrogen stable isotopes (SIA hereafter) has provided valuable insights into the origin and nature of bivalve trophic ecology (among others, Atkinson et al., 2010; Brauns et al., 2021). Accordingly, in the present study, we measured  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  stable isotope values of soft tissues of *D. polymorpha* and *A. anatina* from a littoral site in Lake Trasimeno and of their potential pelagic and benthic trophic resources, i.e., phytoplankton and organic matter of sedimentary origin (SOM hereafter). Bayesian mixing models were further used to quantify the relative contribution of the resources to the diets the two bivalve species and, ultimately, verify their degree of dietary overlap and potential trophic competition.

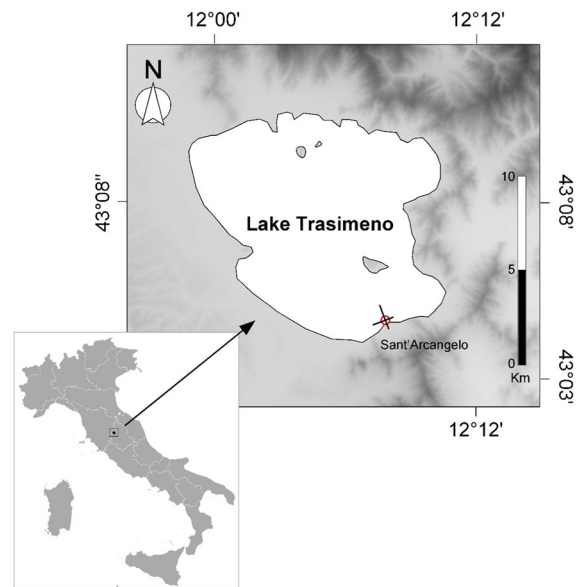
Given the shallowness of Lake Trasimeno and its susceptibility to sediment resuspension events (see further in the next section), we posited that SOM may contribute to the diet of the bivalves in particular in winter months, when phytoplankton abundance is low and wind-induced resuspension of bottom sediments increases remarkably (Ludovisi & Gaino, 2010; Bresciani et al., 2020). To this end, isotopic analyses were repeated in August and February to verify

the effect of seasonal variations in the availability of pelagic and benthic organic matter sources on the dietary habits of the two species. In addition, to contextualize the results and provide a more comprehensive understanding of the contribution of different sources to carbon and nitrogen dynamics in the lake littoral zones, stable isotope analysis was extended to zooplankton together with other representative plant and invertebrate taxa occurring at the study location.

## Material and methods

### Site description

The study was performed in Lake Trasimeno ( $43.133283^\circ\text{N}$ ,  $12.100064^\circ\text{E}$ , Central Italy; Fig. 1). Details on the lake's morphometric and hydrological characteristics can be found in Ludovisi & Gaino (2010) and in Bresciani et al. (2020). In brief, the basin is located 257 m above sea level and is the largest laminar lake in Italy ( $124 \text{ Km}^2$ ). It is shallow (average depth: 4.7 m, maximum depth: 6.3 m), has a single artificial outlet, and is fed by several ephemeral creeks. Given the relatively small extent of the watershed ( $396 \text{ Km}^2$ ), its hydrological regime is driven by



**Fig. 1** Lake Trasimeno. The figure includes a digital elevation map of the area surrounding the basin; the location where the study was performed is also indicated

precipitation, and strong seasonal and inter-annual oscillations in water level and quality are observed (Ludovisi & Poletti, 2003; Ludovisi & Gaino, 2010). The lake is mesoeutrophic and phosphorous-limited, and late-summer phytoplankton blooms are typically observed. In addition, it is polymictic with no significant thermal stratification during the year and frequent wind-driven sediment resuspension events in winter months (Ludovisi & Gaino, 2010; Gaino et al., 2012; Bresciani et al., 2020).

The littoral zones are generally muddy, with dense beds of aquatic macrophytes belonging to the genera *Stuckenia*, *Myriophyllum*, and *Vallisneria* extending seasonally in particular along the southern coasts of the lake (Marchegiano et al., 2017). The macroinvertebrate community is composed of a diverse assemblage of annelid, mollusc, insect, and crustacean taxa including the invasive decapod *Procambarus clarkii* (Girard, 1852) and amphipod *Dikerogammarus villosus* (Sowinsky, 1894) (Goretti et al., 2014; VV.AA., 2015; Catasti et al., 2017; Goretti et al., 2020b; Mancini et al., 2021). Native bivalves comprise the sphaeriid *Sphaerium corneum* (Linnaeus, 1758) and the unionids *Unio elongatulus* C.Pfeiffer, 1825, *Anodonta anatina*, *A. cygnea* (Linnaeus, 1758), and *A. exulcerata* Porro, 1838 (VV.AA., 2015; Froufe et al., 2017).

### Sample collection

Sampling operations were carried out on August 20th, 2015, and on February 4th, 2016, in the southern sector of the basin in the locality of Sant'Arcangelo (43.089788° N, 12.156246° E; Fig. 1). To collect phytoplankton and zooplankton under conditions of high-water clarity and minimum sediment resuspension, sampling dates corresponded with days always preceded by at least 72 h of good weather and low/negligible wind speed. The sampled area, representative of the general benthic conditions characterizing the littoral environments of the lake (Marchegiano et al., 2017; Goretti et al., 2020a), was located in a shallow embayment (approximate mean depth = 1 m) with muddy bottoms and artificial rocky shores. The riparian vegetation was mainly represented by stands of the common reed *Phragmites australis* (Cav.) Trin. ex Steud. 1841 while *Myriophyllum spicatum* L., *Stuckenia pectinata* (L.) Böerner, and *Vallisneria spiralis* L. dominate among submerged macrophytes. In winter, large natural accumulations of leaf litter and

organic detritus originating from the aforementioned floral species are generally found along the shores and bottoms (Mancinelli et al., 2018).

At each sampling occasion, plankton samples were taken at approximately 50 m from the coast from a pier extending in the embayment. Five 6-L samples of surface water (0–50 cm) were collected by a hand-held Ruttner bottle and transported to laboratory for phytoplankton analyses in refrigerated containers. In addition, five 200-L samples of surface water were collected using a diaphragm suction pump and filtered in situ on a 50 µm mesh size screen for zooplankton analyses.

Thirty individuals of *D. polymorpha* were randomly scraped from submerged rocks and artificial structures, while *A. anatina* specimens were hand-collected by wading. Since sampling operations imposed a significant disturbance of the embayment soft bottoms, ultimately making increasingly difficult the detection of specimens, five *Anodonta* individuals were collected per sampling occasion. A pond net (mesh size = 1 mm) was swept five times through submerged macrophytes and leaf litter accumulations to collect samples of the dominant plant and macroinvertebrate species. After collection, all floral and faunal samples were placed, depending on individual size, in Falcon tubes or in other plastic buckets in filtered lake water and sealed. In addition, samples of the superficial sediment layer (6 replicates per season) were collected using a methacrylate core (400 mm length, 114 mm φ) driven into the sediment to a depth of approximately 10 cm. The core was extracted after sealing its upper end and the overlying water was removed by aspiration to avoid resuspension. Subsequently, the superficial layer (0–1 cm) of each core was gently scooped with a plastic spoon and collected in Falcon tubes, while the remaining sediment was transferred in plastic bags. All collected samples were stored in refrigerated containers (4 °C) until transfer to the laboratory.

### Laboratory procedures

Water samples collected for phytoplankton analysis were filtered through GF/C fiber glass filters (1.2 µm mesh size). The samples collected for zooplankton analysis were centrifuged in order to remove floating particles and filamentous algae, prior to be filtered through GF/C fiber glass filters (1.2 µm mesh size).

For both phytoplankton and zooplankton, the material collected on the GF/C filters was examined using an inverted microscope ( $\times 200$  magnification for phytoplankton and  $\times 40$  for zooplankton) to qualitatively confirm the dominance of phyto- or zooplanktonic organisms over other sestonic material. Once examined, the material retained by the filters was gently scraped with a scalpel and dried in Eppendorf tubes at  $60\text{ }^{\circ}\text{C}$  for at least one week. Identical drying conditions were applied to all the remaining plant and animal samples. Plants were identified to species level and rinsed in distilled water to remove extraneous materials. All sediment samples were sieved on a 1-mm screen to collect invertebrates for later identification and analysis. Superficial sediment layer samples were sieved and homogenized, while leaf litter and other macroscopic detrital particles (coarse particulate organic matter, CPOM hereafter) retained in the sieve were collected. All plant and sediment samples were eventually dried ( $60\text{ }^{\circ}\text{C}$ ,  $> 1$  week).

In general, invertebrates were identified to species level using conventional taxonomic keys and enumerated; they were subsequently kept in distilled water for 12 h to clear gut contents and euthanized by thermal shock ( $- 80\text{ }^{\circ}\text{C}$  for 10 min). Subsequently amphipods, decapods, chironomids, and oligochaetes (see Results) were individually dried ( $60\text{ }^{\circ}\text{C}$ ,  $> 1$  week). A calliper was used to measure to the nearest mm the shell length of euthanized *D. polymorpha* specimens as the distance between posterior to anterior tips along the median axis; bivalves were then dissected and had their foot excised and dried ( $60\text{ }^{\circ}\text{C}$ ,  $> 1$  week). Noticeably, for *A. anatina*, classical taxonomic keys were inadequate in providing an unquestionable identification of the sampled individuals, given the high intraspecific variability in morphometric traits generally observed in species belonging to the genera *Anodonta* (Guarneri et al., 2014; Riccardi et al., 2019). Accordingly, after shell length measurement, the excised foot of each individual was divided into two subsamples. The first subsample was dried as previously described; the second was preserved in ethanol 70% at  $- 20\text{ }^{\circ}\text{C}$  and subsequently subjected to molecular species attribution performed through PCR–RFLP (PCR-restriction fragment length polymorphism) on the nuclear locus ITS1 (internal transcribed spacer) specifically chosen to avoid DUI (doubly uniparental inheritance) problems. Total DNA was isolated individually in duplicate from the

foot subsample using the Wizard® Genomic DNA Purification Kit (Promega) by means of a modified protocol (Lucentini et al., 2010) and the quantity and quality of DNA were assessed on 1% agarose gel and by spectrophotometric analysis, as already used for other bivalve species (Lucentini et al., 2010). Amplification and digestion of ITS1 region were carried out following Zieritz et al. (2012) to unequivocally assign each individual to a species by means of a validated PCR–RFLP protocol. Restriction products were checked by electrophoresis in 2.2% TBE buffer-agarose gel containing SafeView Nucleic Acid Stain (NBS Biologicals) and visualized under UV light. The totality of the examined *Anodonta* specimens was identified as *A. anatina*.

#### Stable isotope analysis

All oven-dried samples were ground to a fine powder with a mortar and pestle. Each sediment sample was preventively split into two aliquots, one of which was acidified (HCl, 2 N) using the drop-by-drop procedure to remove carbonates (Jacob et al., 2005). Consequently, subsamples of different sizes were taken from each taxon/matrix [i.e., macrophytes:  $3.1 \pm 0.1$  mg; sediment:  $29.1 \pm 0.1$  mg (non-acidified) and  $9.9 \pm 0.1$  mg (acidified); phytoplankton and zooplankton:  $2.8 \pm 0.4$  mg; bivalves, amphipods, decapods, oligochaetes, and chironomids:  $2.1 \pm 0.1$  mg] and pressed into ultra-pure tin capsules (Costech Analytical Technologies). Multiple specimens were pooled when single individuals did not provide a sufficient mass for the subsample (see Table 1 in Results). Carbon and nitrogen stable isotope values were determined using an Elemental Analyser (Thermo Scientific Flash EA 1112) connected with an Isotope Ratio Mass Spectrometer (Thermo Scientific Delta Plus XP). Concentrations of total carbon (C) and nitrogen (N) were expressed as  $\text{g Kg}^{-1}$  tissue dry weight; isotopic values were expressed in conventional per mil  $\delta$  notation in relation to international standards (PeeDee Belemnite for carbon and atmospheric  $\text{N}_2$  for nitrogen). Analytical precision based on the standard deviation of replicates of internal standards (International Atomic Energy Agency IAEA-NO-3 for  $\delta^{15}\text{N}$  and IAEA-CH-6 for  $\delta^{13}\text{C}$ ) was  $0.2\text{‰}$  for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . Acidified samples were used to measure sediment  $\delta^{13}\text{C}$  values, while untreated samples were used for  $\delta^{15}\text{N}$  values.



**Table 1** Trophic sources (including primary producers) and consumers collected in Lake Trasimeno in summer and winter

Trophic sources	Phylum	Acronym	Summer	Winter
<i>Chara globularis</i> Thuill	Charophyta	cha	5	—
<i>Cladophora glomerata</i> (L.) Kütz	Chlorophyta	cl	5	—
<i>Myriophyllum spicatum</i> L	Tracheophyta	my	5	—
<i>Stuckenia pectinata</i> (L.) Börner	Tracheophyta	st	3	—
<i>Vallisneria spiralis</i> L	Tracheophyta	va	4	—
Leaf litter / plant detritus		CPOM	—	3
Phytoplankton		ph	5	5
Sedimentary organic matter		SOM	6	6
Consumers				
<i>Anodonta anatina</i> (Linnaeus 1758)	Mollusca	an	5	5
<i>Branchiura sowerbyi</i> Beddard, 1892	Annelida	br	3 (8)	3 (7)
<i>Chironomus plumosus</i> Linnaeus, 1758	Arthropoda	chi	3 (22)	3 (17)
<i>Dreissena polymorpha</i> (Pallas, 1771)	Mollusca	dr	5 (30)	8 (30)
<i>Echinogammarus veneris</i> (Heller, 1865)	Arthropoda	ec	7 (16)	4 (9)
<i>Procambarus clarkii</i> (Girard, 1852)	Arthropoda	pr	6	—
Zooplankton		zo	5	5

The acronyms used in Fig. 2 to indicate the different taxa are included. Sample sizes for stable isotope analysis are reported; for consumers, the number of specimens originally collected is indicated in brackets

Some animal taxa showed a C:N ratio higher than 3.5–4 (Table S1, online information), thus indicating a considerable contribution of lipids to tissues carbon pool (Post et al., 2007). Lipids are depleted in  $^{13}\text{C}$  compared to proteins and carbohydrates and may significantly bias  $\delta^{13}\text{C}$  estimations (Logan et al., 2008). Accordingly, in samples with C:N > 3.5,  $\delta^{13}\text{C}$  values were corrected for lipid content using the correction algorithms based on tissue C:N ratios proposed by Syväranta & Rautio (2010) for zooplankton and by Post et al. (2007) for the remaining samples.

#### Data analysis

In general, values in the text are expressed as means  $\pm$  1SD if not otherwise specified. All statistical procedures were implemented in the R statistical environment development (ver. 4.1.1; R Development Core Team, 2021). Two-tailed Student's *t*-tests ( $\alpha=0.05$ ) were used to check for seasonal or inter-specific differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in phytoplankton, SOM, and in the two bivalves; data were tested for conformity to assumptions of variance homogeneity (Cochran's *C* test) and normality (Shapiro–Wilks test) and transformed when required. Given the relatively low sample sizes (Table 1), the R software *pwr* (Champely et al., 2020) was used to perform a power analysis for each test showing a significant outcome (see Results). Without exceptions,

the analyses indicated that the power to detect the observed effects at the 0.05 level was always > 93%.

A two-member Bayesian mixing model was implemented to determine the proportional contribution of phytoplankton and SOM to the diet of each bivalve species using the R package SIMMR (Parnell, 2020). SIMMR includes an updated Bayesian mixing algorithm based on the SIAR package (Parnell et al., 2010) to produce a probability distribution that represents the likelihood a given food source contributes to the consumer's biomass. In addition, similar to SIAR, it produces a range of feasible solutions to the mixing problem to which are assigned credibility intervals (CIs) (in this study, 95% CI; Parnell et al., 2010). Within the SIMMR framework,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were adjusted for one trophic level. The identification of appropriate trophic enrichment factors (hereafter TEF) is a key issue in isotopic ecology, as they significantly affect the output of mixing model procedures (Phillips et al., 2014). Taxonomy and the nature of the diet are among the sources of variability that may influence TEFs (Vanderklift and Ponsard, 2003; Caut et al., 2009; Brauns et al., 2018), imposing, when possible, the use of specifically determined values (see, e.g., Annabi et al., 2018; Twining et al., 2020). The carbon and nitrogen TEF values for one trophic level used here (i.e.,  $\Delta\delta^{13}\text{C}=0.8\pm 1\%$ ,  $\Delta\delta^{15}\text{N}=3.6\pm 0.9\%$ ,  $n=8$ ) were calculated as the average of the values collated by Brauns et al. (2018)

from laboratory studies on marine bivalves feeding on phytoplankton, implemented with data from Kasai et al. (2016) on the freshwater venerid *Corbicula sandai* Morelet, 1886 (Table S2, online information). 10,000 simulations per model were run; Gelman diagnostics were carried out on models output to determine whether confidence intervals were comprised between 1 and 1.1, and a higher number of simulations were performed if necessary.

To express the results of the simulations only in terms of mean contributions of each source with standard deviations may hide multimodality or the extent of variations in dietary preference within consumer populations (Semmens et al., 2013). Accordingly, for each potential resource, the results were presented as mean contributions as well as probability densities of proportional dietary contributions using the density plot function in SIMMR. Posterior probabilities of similarity (POS), defined as the Bayesian probability that two proportional dietary contributions are identical (Blasco & Blasco, 2017), were estimated to verify for the two bivalve species whether in each sampling occasion phytoplankton and SOM provided significantly different contributions to the respective diets. In addition, since the sample size of bivalve consumers employed here for isotopic analysis (5–8; Table 1) represents the lower boundary considered appropriate for the implementation of a two-member Bayesian mixing model procedure (Ward et al., 2011; Phillips et al., 2014; see also Kadoya et al., 2012), the R package *samplesim* (Casajus et al., 2021) was used to investigate the effect of sample size on estimates and precision of stable isotope mixing solutions. In brief, the package allows to modify samples sizes assuming a normal distribution of a given mean and standard deviation. Samples of different sizes are created from the distribution, and mixing proportions are estimated using the SIAR algorithm for multiple replicates of each sample size. Here, we used 999 replicates with sample sizes = 5, 10, 20, 40, and 80.

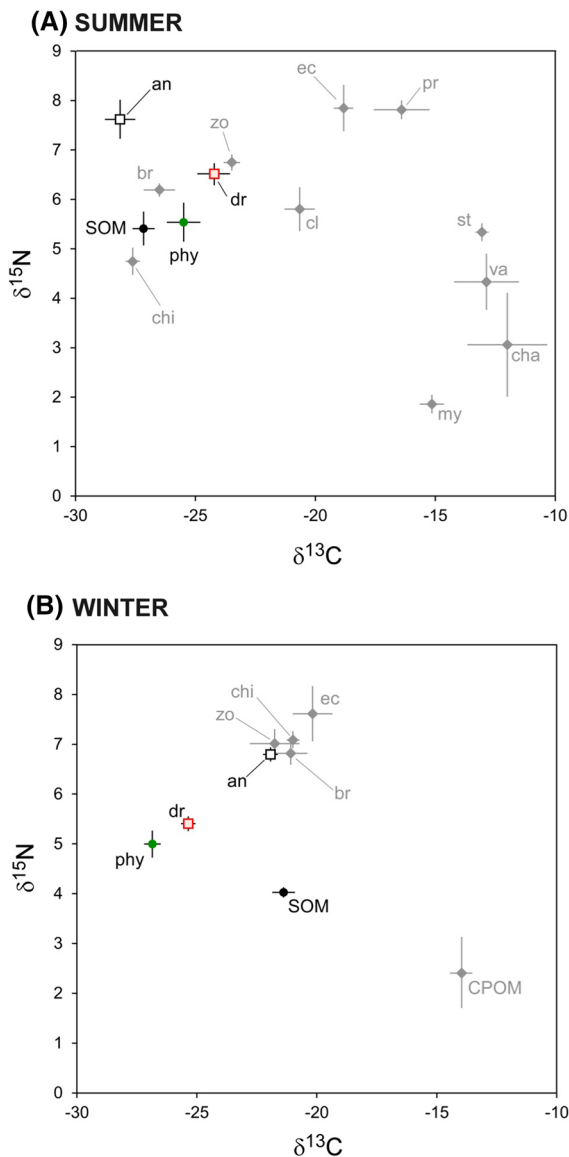
Concentration dependencies were not incorporated in the models because elemental concentration values in SOM were expected to be extremely diluted given the low percentage of organic matter (max 7% in dry weight; estimated as weight loss on ignition at 450 °C for 6 h; Mancinelli unpublished data); therefore, using concentration dependencies would have unrealistically and abnormally increased the contribution of this source (Raoult et al., 2018).

## Results

*Dreissena polymorpha*, *Anodonta anatina*, phytoplankton, zooplankton, and SOM samples were collected on both August and February, together with the dipteran *Chironomus plumosus* (Linnaeus, 1758), the amphipod *Echinogammarus veneris* (Heller, 1865), and the non-indigenous oligochaete *Branchiura sowerbyi* (Beddard, 1892) (Table 1). Five species of aquatic macrophytes and six juvenile specimens of the non-indigenous Louisiana crayfish *Procambarus clarkii* (ranging between 24 and 32 mm in total length measured from the tip of the rostrum to the rear edge of telson; Mancinelli, personal observation) were collected only in August, while CPOM was collected in significant amounts only in February (Table 1). On average, *D. polymorpha* individuals collected in February were significantly larger than in August ( $17.6 \pm 5.9$  vs.  $13.2 \pm 3.6$  mm shell length; *t*-test,  $t=4.3$ ,  $P<0.0001$ , 58 d.f.), while for *A. anatina* specimens, differences in size between the two sampling occasions were negligible ( $94.7 \pm 13.9$  vs.  $88.4 \pm 11.8$  mm in February and August, respectively;  $t=1.1$ ,  $P=0.34$ , 8 d.f.).

In August, phytoplankton and SOM showed comparable  $\delta^{15}\text{N}$  values ( $5.5 \pm 0.4\text{‰}$  vs.  $5.4 \pm 0.3\text{‰}$ ; *t*-test,  $t=0.5$ ,  $P=0.62$ , 9 d.f.), but differed in  $\delta^{13}\text{C}$  ( $t=4.5$ ,  $P=0.003$ , 9 d.f.), with more depleted values observed for SOM than for phytoplankton ( $-27.2 \pm 0.5\text{‰}$  vs.  $-25.7 \pm 0.4\text{‰}$ ; Fig. 2A). Macrophytes, in contrast, showed highly enriched  $\delta^{13}\text{C}$  values (Fig. 2A), ranging between  $-20.7 \pm 0.6\text{‰}$  [*Cladophora glomerata* (L.) Kütz.] and  $-11.9 \pm 1.7\text{‰}$  (*Chara globularis* Thuill.), and variable  $\delta^{15}\text{N}$  values ranging between  $1.9\text{‰}$  (*Myriophyllum spicatum*) and  $5.8\text{‰}$  (*Cladophora glomerata*). Depleted  $\delta^{13}\text{C}$  values consistent with those of SOM and phytoplankton were observed for zooplankton, as well as for the deposit-feeders *Chironomus plumosus* and *Branchiura sowerbyi*; in contrast, crustaceans (i.e., *Echinogammarus veneris* and *Procambarus clarkii*) showed enriched  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values consistent with those generally determined for macrophytes (Fig. 2A).

In February, phytoplankton showed negligible variations in  $\delta^{15}\text{N}$  compared to August ( $t=1.27$ ,  $P=0.12$ , 8 d.f.), while a limited ( $1.2\text{‰}$  on average), yet significant depletion was observed for  $\delta^{13}\text{C}$  ( $t=2.39$ ,  $P=0.02$ , 8 d.f., Fig. 2B). Conversely, SOM



**Fig. 2**  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  isotopic bi-plot illustrating the carbon and nitrogen values (means  $\pm$  1SD) of *Dreissena polymorpha* and *Anodonta anatina* as compared with those of phytoplankton, zooplankton, and SOM in summer (A) and in winter (B). For the sake of comparison,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of macrophytes (summer), CPOM (winter), the amphipod *Echinogammarus veneris* (both seasons), juveniles of the decapod *Procambarus clarkii* (summer), the chironomid *Chironomus plumosus* (both seasons), and the oligochaete *Branchiura sowerbyi* (both seasons) are included. Taxa are indicated using the acronyms listed in Table 1; for those taxa characterized by C/N values  $> 3.5$ ,  $\delta^{13}\text{C}$  values have been lipid-corrected (see text for details and Table S1 in online information)

**Table 2** Summary of the results of Bayesian stable isotope mixing models: posterior mean and median (50%) estimates together with 2.5, 25, 75, and 97.5% credibility intervals (CI) of proportions of phytoplankton in the diet of *Dreissena polymorpha* and *Anodonta anatina* in summer and in winter

	<i>Dreissena polymorpha</i>		<i>Anodonta anatina</i>	
	Summer	Winter	Summer	Winter
Mean	0.7	0.83	0.3	0.22
2.50%	0.14	0.72	0.03	0.07
25%	0.56	0.8	0.12	0.17
50%	0.77	0.83	0.23	0.22
75%	0.89	0.87	0.42	0.28
97.50%	0.97	0.94	0.86	0.38

Summary data for SOM can be expressed as one minus phytoplankton data and are not reported

isotopic values varied significantly between seasons, showing more depleted  $\delta^{15}\text{N}$  values ( $t = -9.53$ ,  $P < 0.0001$ , 10 d.f.) and enriched  $\delta^{13}\text{C}$  values ( $t = 21.4$ ,  $P < 0.0001$ , 10 d.f.) in February than in August. Carbon isotopic values, in particular, were enriched by approximately 5.8‰, and were consistent with those observed for living macrophytes in August (Fig. 2A) and for CPOM collected in February (Fig. 2B).

$\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in *Dreissena polymorpha* showed a temporal pattern of variation generally mirroring that of phytoplankton (Fig. 2A and 2B), even though the species showed more depleted  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in February than in August (t-tests,  $P$  always  $< 0.05$ ). Similarly,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in *Anodonta anatina* varied remarkably between the two sampling occasions (Fig. 2A and 2B; t-tests,  $P$  always  $< 0.05$ ). In particular, a significant enrichment in  $^{13}\text{C}$  was observed in February, consistent with that observed for SOM (Fig. 2B); noticeably, similar patterns of variation characterized the zooplankton as well as *C. plumosus* and *B. sowerbyi* (Fig. 2B). The results of SIMMR indicated that in August phytoplankton was included in the diet of both *D. polymorpha* and *A. anatina* (Table 2; Fig. 3A); however, its contribution varied considerably, being dominant for *D. polymorpha* (70% and 77%, mean and median proportional contribution), while being approximately 30% (23% median contribution) for *A. anatina*, for which SOM appeared to prevail in the diet (Table 2). Noticeably, for *D. polymorpha* the probability densities of the proportional dietary contributions of phytoplankton and SOM overlapped considerably (Fig. 3A);



furthermore, the posterior probabilities of similarity test indicated a non-significant difference ( $P=0.21$ ). In contrast, *A. anatina* showed more segregated, even though marginally non-significant ( $P=0.09$ ) proportional dietary contributions (Fig. 3A). Noticeably, a substantial effect of sample size was observed on mixing solutions, as simulations indicated that depending on the number of analyzed individuals phytoplankton might have reached a median proportional contribution of up to 88% and less than 13% in the diet of *D. polymorpha* and *A. anatina*, respectively (Fig. S1, online information).

Compared with August, in February the trophic segregation between the two species was more evident, with *D. polymorpha* relying mainly on phytoplankton, and *A. anatina* primarily exploiting SOM (Table 2). Additionally, the probability densities of phytoplankton and SOM proportional dietary contributions showed no overlap for both bivalves, the similarity probability was negligible (*D. polymorpha*:  $P=0.002$ ; *A. anatina*:  $P<0.0001$ ), and no considerable sample size-related effects were observed, as the estimated proportional contributions of the resources remained virtually unchanged with an increase in the sample size of the consumers (Fig. S1).

## Discussion

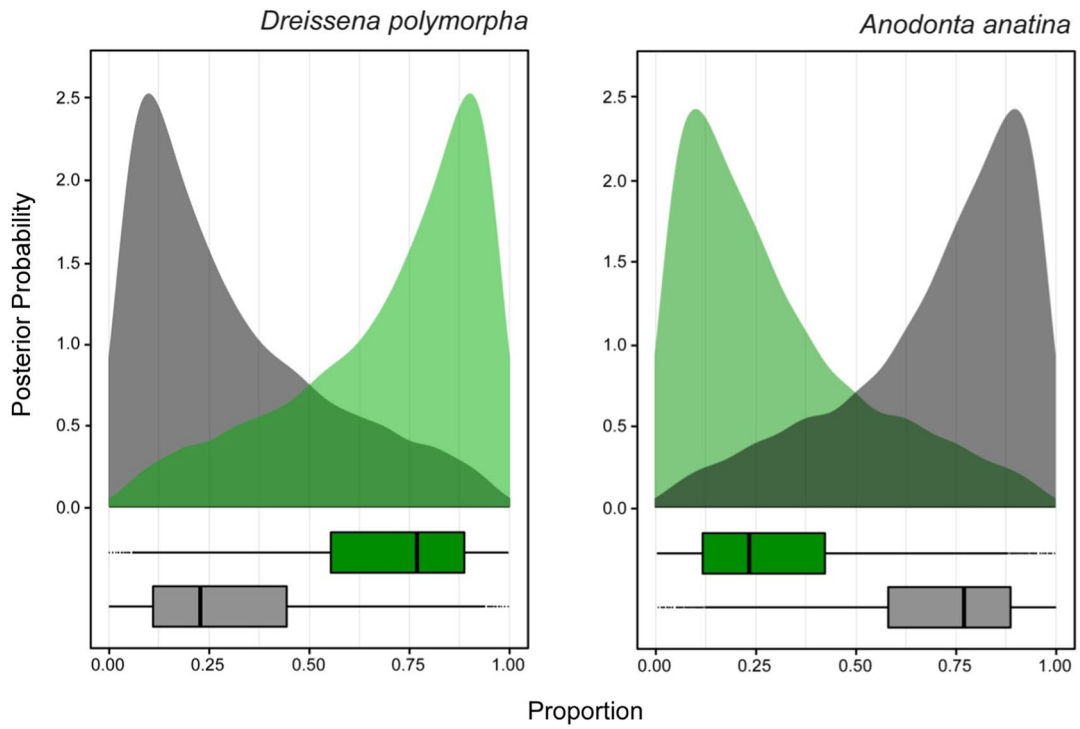
Our isotopic investigation indicated that in Lake Trasimeno, independently from the season, the invasive *Dreissena polymorpha* and the native *Anodonta anatina* showed different trophic habits, with phytoplankton and SOM prevailing in the diet of the dreissenid and the anodontid, respectively. Dietary differences were particularly apparent in February (Table 2; Fig. 3B; see also Fig. 2B). In August, the respective proportional contributions of the two resources were remarkably dissimilar in terms of mean and median values (Table 2), yet both the mixing models procedure and the similarity tests on posterior probabilities highlighted a notable uncertainty in the results, in particular for *Dreissena* (Fig. 3A).

A comprehensive understanding of these findings requires to cast them within the background provided by the isotopic data on additional animal and plant taxa collected during the study. In summer, it is apparent that two main carbon pools characterized the benthic system of the study location. The first

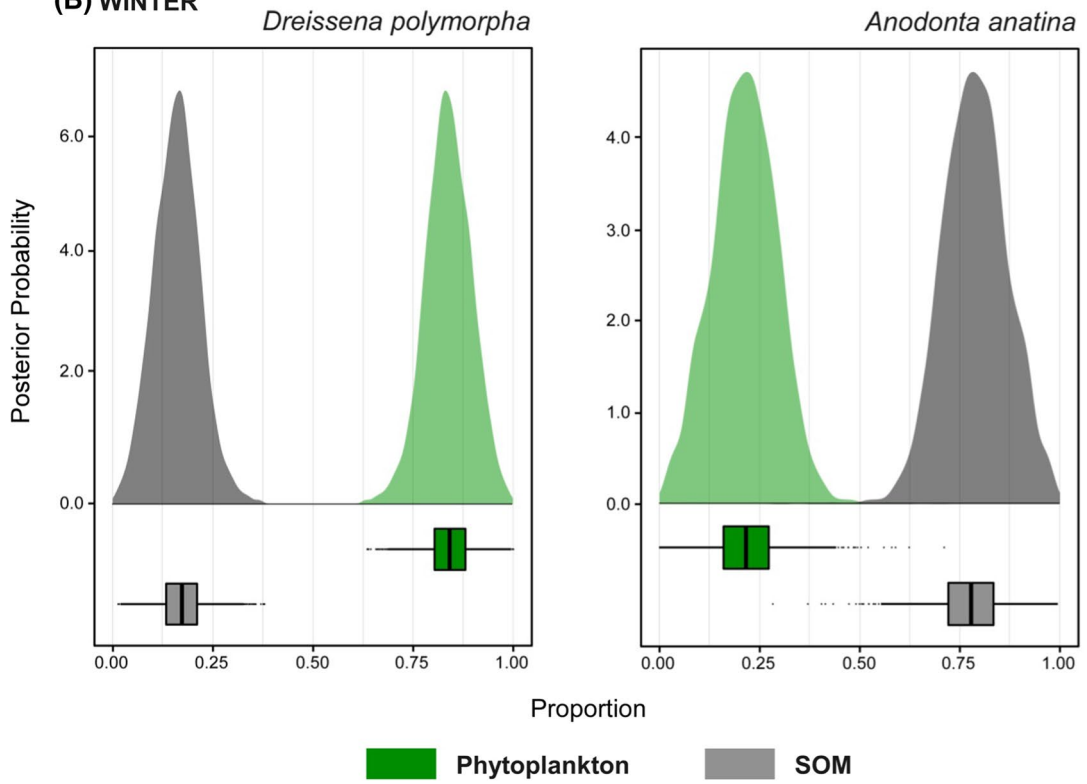
was represented by  $^{13}\text{C}$ -enriched living macrophytes (Fig. 2A; see also Fig. 4 for a graphical synthesis), likely to support second-level crustacean consumers, such as the herbivorous/detritivorous amphipod *Echinogammarus veneris* and juveniles of the omnivore *Procambarus clarkii* (Mancinelli et al., 2002; 2007; Alcorlo & Baltanás, 2013). The second pool included  $^{13}\text{C}$ -depleted phytoplankton and SOM, with overlapping  $\delta^{15}\text{N}$  values and differing significantly in  $\delta^{13}\text{C}$  by approximately 1.4‰ (Fig. 2A). Indeed, the similar isotopic values of the two sources can explain the uncertain results of the mixing model output (Fig. 3A). The use of mixing models to quantify animal diets requires the sources to have isotopically distinct values; small differences (e.g., 1‰ or lower), even when statistically significant, may have no practical ecological implications, ultimately hampering the robustness of the results (Phillips et al., 2014). In these respects, the dual isotopic approach adopted here should include in future investigations additional biochemical methodologies based on, e.g., fatty acids (Makhutova et al., 2013; Fujibayashi et al., 2016) or a wider spectrum of isotopic markers (Weber et al., 2017) to allow for a more robust resolution of the summer diet of *D. polymorpha* as well as of *A. anatina*. In addition, future studies should also analyze a larger number of individuals. Together with resources showing limited isotopic differences, relatively low sample sizes in consumers can determine outputs showing highly diffuse source contributions (Brett, 2014; Phillips et al., 2014), as observed here in August. The simulation test on the effect of the sample size of the two bivalves actually indicated that a larger number of analyzed individuals might have increased the robustness of the mixing model results obtained in August for both *D. polymorpha* and *A. anatina* (Fig. S1). In contrast, in February, the simulation test confirmed the robustness of the results independently from the sample size (Fig. S1). It is apparent, however, that future analyses carried out on larger sample sizes will allow researchers to investigate, e.g., size-related dietary changes or seasonal variations in the isotopic niche of the two bivalves, not addressed here and generally overlooked in freshwater bivalves [but see Suh & Shin, 2013 for the estuarine *Ruditapes philippinarum* (Adams & Reeve, 1850)].

Noticeably, the observed isotopic similarity between phytoplankton and SOM may suggest a

**(A) SUMMER**



**(B) WINTER**



**Fig. 3** Posterior probability distributions of the contributions of phytoplankton and sedimentary organic matter (SOM) to the diet of *Dreissena polymorpha* and *Anodonta anatina* in summer (A) and in winter (B). Bar plots represent posterior model estimates of contributions in terms of median, interquartile range, and max/min values; for further details on the results of Bayesian stable isotope mixing models, see Table 2

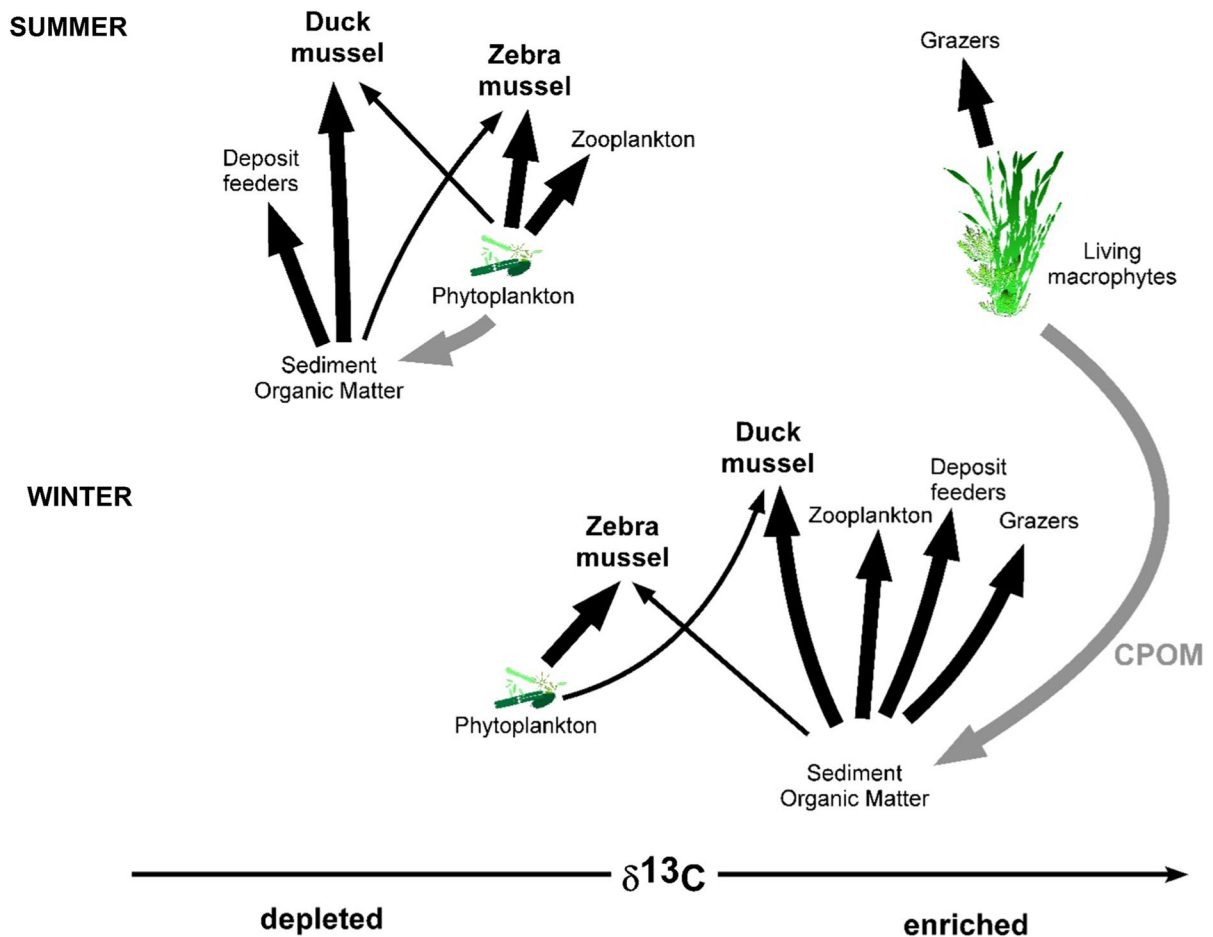
summer pelagic–benthic coupling (sensu Soetaert et al., 2000), which *D. polymorpha* and other suspension feeders possibly contribute to through bio-deposition of feces and pseudo-feces (Gergs et al., 2009; Ozersky et al. 2012; but see further in the text). The accumulation of fresh organic matter in superficial sediments after phytoplanktonic blooms (common in August in Lake Trasimeno: Ludovisi and Gaino, 2010; Bresciani et al., 2020; see also Xu et al., 2019 and literature cited for examples from other shallow lentic systems) can be considered as the main causative mechanism of the coupling. This hypothesis is partially supported by the non-significant differences in C:N ratios (SOM =  $13 \pm 4.6$ ; phytoplankton =  $8.8 \pm 1.2$ ; *t*-test with separate variance estimates: *t* = - 2.1, *P* = 0.09, 5.6 d.f.) and by the limited depletion in  $\delta^{13}\text{C}$  values between SOM and phytoplankton, consistent with what is generally observed during early diagenetic processes of pelagic organic matter in freshwater systems (e.g., Lehmann et al., 2002).

The general picture that emerges in summer is that phytoplankton supports directly *D. polymorpha* and indirectly, through pelagic–benthic coupling, *A. anatina*, together with other depositivores such as *Branchiura sowerbyi* and *Chironomus plumosus* (Fig. 4). It is worth mentioning that the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of *C. plumosus* were significantly more depleted than those of SOM (Fig. 2A; *t*-tests, *P* < 0.05 for both elements), suggesting that the trophic link may be mediated by  $^{13}\text{C}$  and  $^{15}\text{N}$ -depleted methanotrophic bacteria (Jones et al., 2008; Tsuchiya et al., 2020).

In February, conversely, while phytoplankton showed depleted  $\delta^{13}\text{C}$  values comparable with those observed in August,  $\delta^{13}\text{C}$  in SOM was significantly higher, an enrichment likely to be determined by macrophyte-derived decaying CPOM (Fig. 2B; Fig. 4). Under these conditions, the trophic segregation between the two bivalves was more apparent (Table 3; Fig. 3B) and further corroborated by post hoc similarity tests.

The habitat preferences of the two species may explain their dietary differences. *Dreissena polymorpha* can form clumps (“druses”) and colonize unconsolidated sediments, but, as observed here and in previous studies performed in Lake Trasimeno (Lancioni & Gaino, 2006), they are generally attached to natural and artificial hard substrata, thus filter-feeding in the water column (Mellina & Rasmussen, 1994). In contrast, *A. anatina* lives buried in sediment superficial layers or at the water–sediment interface, where it can show a relatively high mobility (Schwalb & Pusch, 2007). Additionally, anodontids can forage by pedal feeding, i.e., by using the foot to sweep detrital and colloidal particles, microphytobenthos, and bacteria from the sediment surface directly into the shell (Nichols et al., 2005; Brendelberger & Klauke, 2009).

Nevertheless, SOM provided an average contribution between 20 and 30% to the diet of *D. polymorpha* depending on the season (Fig. 3; Table 3). A relatively high trophic plasticity has already been indicated in *D. polymorpha*, and a dual reliance on both phytoplankton and SOM has been shown in both lacustrine and riverine populations (Garton et al., 2005; Cole & Solomon, 2012). Wind-induced resuspension events in Lake Trasimeno occur year-round yet are more frequent and intense in winter (Havens et al., 2009; Ludovisi and Gaino, 2010; Gaino et al., 2012); hence, *D. polymorpha* may adopt in this season a strategy of selective feeding, as already highlighted for the species on phytoplankton (e.g., Baker et al., 1998; Vanderploeg et al., 2001; Naddafi et al., 2007). A relatively high trophic plasticity has been also indicated for *A. anatina*, and laboratory investigations have shown that phytoplankton, if available, can be efficiently consumed by the duck mussel (Bontés et al., 2007; Dionisio Pires et al., 2007). In spite of this, the species showed in this study independently from the season a net prevalence of SOM in its diet. Even though the already mentioned behavioral and ecological dissimilarities can explain the dietary differences observed between the two species, these may also be interpreted as the result of a trophic segregation adopted to minimize competition for food. In particular, it can be hypothesized that the high efficiency of *D. polymorpha* in feeding on phytoplankton (Roditi et al., 1996; Dionisio Pires et al., 2004) gives rise to a competitive advantage to *D. polymorpha* with respect to *A. anatina* for this food source, imposing to the latter a SOM-based diet. Further controlled



**Fig. 4** Graphical summary of seasonal variations in trophic relationships (black arrows) and transfers of material (gray arrow) linking basal resources (i.e., phytoplankton, sediment-

tary organic matter, and macrophytes) with *Dreissena polymorpha*, *Anodonta anatina*, and other invertebrates in the benthic environment of Lake Trasimeno littoral zones

feeding experiments may help in clarifying the actual nature of the interaction between the two bivalves, as well as between *D. polymorpha* and zooplankton: the bivalve's clearance on phytoplankton may negatively affect pelagic consumers (Jack & Thorp, 2000; Kissman et al., 2010), a potential interaction suggested by the consistency in their  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values observed here in summer (Fig. 2A).

A further aspect to be considered is that the  $^{13}\text{C}$ -enrichment in SOM reflected in those of *B. sowerbyi* and *C. plumosus*, as well as in zooplankton (Fig. 3B), a result that can be explained considering the feeding habits of dominant zooplanktonic species in Lake Trasimeno. Spring–summer zooplankton is typically characterized by strictly herbivorous species [mainly *Bosmina longirostris* (O.F.Müller,

1776), *Daphnia galeata* (Sars, 1864) and *Diaphanosoma brachyurum* (Liévin, 1848)] and to a minor extent by copepods of the genus *Cyclops*, having a herbivorous–detritivorous generalist diet; conversely, *Cyclops* spp. generally prevail in winter zooplankton (Hamza et al., 1995; Ludovisi et al., 2005). Such a seasonal taxonomic differentiation in zooplankton was confirmed by the qualitative observations made to verify the nature of the seston (see Material and Methods), with summer samples showing a greater abundance of cladocerans than winter samples, dominated by *Cyclops*.

It is worth noting that the general scenario discussed here is based on the assumption that the isotopic composition of an organism is linked only to that of its diet. In principle, non-trophic factors may

have contributed to the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of *D. polymorpha* and *A. anatina* in summer and winter. For example,  $\delta^{13}\text{C}$  in both freshwater and marine bivalves has been indicated to be influenced by seasonal shifts in energy allocation strategies (Geist et al., 2005; Paulet et al., 2006), and reproduction-related  $\delta^{13}\text{C}$  enrichments have been often observed in summer (Malet et al., 2007). In addition, both *D. polymorpha* and *A. anatina* reduce remarkably their metabolic activity and filtration rates under low temperature conditions (Schneider, 1992; Lurman et al., 2014). This implies that in winter both species may experience periods of starvation, and fasting in bivalves may cause a considerable  $\delta^{15}\text{N}$  enrichment in soft tissues (Yokoyama et al., 2005). In our study, the occurrence of both the aforementioned phenomena cannot be excluded; however, it is likely that they did exert a secondary influence on the isotopic values of *D. polymorpha* and *A. anatina*, as they both showed depleted  $\delta^{13}\text{C}$  and enriched  $\delta^{15}\text{N}$  values in summer as compared to winter (Fig. 2).

In conclusion, the present study provided novel information on the trophic habits of the invasive *D. polymorpha* and of the native *A. anatina* in Lake Trasimeno, and a number of issues were advanced on carbon dynamics between the benthic and pelagic compartments in the lacustrine littoral zone (Fig. 4). The results of our investigation may provide a useful framework to investigate in future the impact of *D. polymorpha* as well as of other invasive species on the flux of energy and elements in the basin. However, our results considered as preliminary, and additional, year-round investigations are required to verify their generality. Specifically, the dietary patterns observed in the present study in winter and summer need to be verified on a seasonal or even shorter temporal scale, given the irregularity of wind-induced resuspension events and the high monthly variability in abundance and composition of the phytoplanktonic assemblage characterizing Lake Trasimeno (Taticchi, 1992; Havens et al., 2009; Bresciani et al., 2020).

Noticeably, the limited sampling effort applied in the present context allowed the collection, together with *D. polymorpha*, of two other non-indigenous invertebrates, i.e., *B. sowerbyi* and *P. clarkii*. *Branchiura sowerbyi* impact on invaded systems has received to date scant attention, while considerable information is available on the ecological impacts of *P. clarkii* on native benthic invertebrates, including

anodontid bivalves (Meira et al., 2019 and literature cited). However, the actual effects of *P. clarkii* on *A. anatina* in Lake Trasimeno are to date unexplored. In addition, the Chinese pond mussel *Sinanodonta woodiana* (I.Lea, 1834), invasive in Italy (Cilenti et al., 2019), has been repeatedly recorded in Lake Trasimeno after 2017 (Froufe et al., 2017; Goretti et al., 2020b). Also for this introduced anodontid, no information is available on its impacts on native bivalve species occurring in the lake. Thus, beside providing reference information on trophically mediated interactions between *D. polymorpha* and *A. anatina*, this study may also provide an incentive toward a more advanced resolution of the role played by native bivalve species in the flux of energy and matter in Lake Trasimeno, and how it is directly or indirectly influenced by introduced invertebrate and fish species.

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**Data Availability** Enquiries about data availability should be directed to the authors.

#### Declarations

**Conflict of interest** The authors have not disclosed any competing interests.

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