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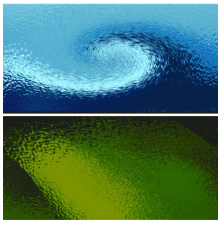

RESEARCH ARTICLE

Estimation of benthic macroinvertebrates taxonomic diversity: testing the role of sampling effort in a Mediterranean transitional water ecosystem.

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Abstract

1 - The accurate evaluation of benthic macroinvertebrate taxonomic diversity in transitional water ecosystems is strictly related to sampling effort and, usually, biomonitoring protocols define the sampling effort needed to the elaboration of a specific ecological indicator. The time-lag between the sampling event and the final assessment of ecological status, and to overall costs for sampling, personnel and sample treatment suggest a reduction of sampling effort.

2 - How to simplify methods and to reduce efforts without compromising the ecological validity of taxonomic diversity indicators is a topic recurrently debated in the procedures for sampling protocol implementation. Regarding this topic, the identification of optimal sample unit size (SUS) and sieve mesh size (SMS) is still lacking, mainly for benthic macroinvertebrates of Mediterranean transitional water ecosystems.

3 - The present study analyzes the effect of the increasing the sampling effort in terms of sample unit size (SUS; 0.0225 m^2, 0.0450 m^2, 0.0675 m^2, 0.0900 m^2) and sieve mesh size (SMS; 0.5 mm, 1 mm, 2 mm) on the estimation of taxonomic diversity in a Mediterranean lagoon. Benthic macroinvertebrates were collected in September 2009 at two locations, considering a perturbed and a relatively unperturbed study site of Lesina lagoon (South-East Italy). Samples were sieved on a column of three decreasing mesh sizes of sieves. Taxonomic richness (S), Shannon–Weaver index (H'), Simpson index (λ) and Taxonomic distinctness (TD) were calculated for each study site, SUS and SMS combination, and replicate. The difference between perturbed and relatively unperturbed site was tested according to the variation of sampling effort using three-way ANOVA tests.

4 - As expected, the accuracy of the results increased with increasing of SUS and SMS, the difference between perturbed and relatively unperturbed study site were always highlighted by each taxonomic diversity index, independently by used SUS and SMS. The variation of taxonomic diversity indicators seems to depend mainly by used sieve mesh size suggesting the reduction of sampling effort through the reduction of sample unit size.

5 - Finally, this contribution could be useful in harmonizing sampling methodologies for the cost-effectiveness taxonomic diversity estimation and biomonitoring programs.

Keywords: benthic macroinvertebrates, taxonomic diversity estimation, sample unit size, sieve mesh size, sampling effort, Mediterranean transitional waters, Lesina lagoon.

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Introduction

The estimation of taxonomic diversity is a major step in the implementation of ecological indicators, in biological monitoring, and for the assessment of the ecological status of aquatic ecosystems and, ultimately, in the detection of several kinds of natural and anthropogenic impacts (Pearson and Rosenberg, 1978; Rosenberg and Resh, 1993; Karr and Chu, 1999; Heino *et al.*, 2007; Orfanidis *et al.*, 2007, 2008; Evagelopoulos *et al.*, 2008; Ponti *et al.*, 2009). The accuracy of taxonomic diversity estimation and the effectiveness of biomonitoring programs, based on the analysis of benthic macroinvertebrate assemblages, are strictly related to the sampling design and effort, whereas their feasibility depends on the time spent for sample collection and processing and sustainable costs (Basset *et al.*, 2004; Basset *et al.*, 2008a; Aarnio *et al.*, 2010; Oliveira *et al.*, 2011; Pinna *et al.*, 2013). Moreover, the Water Framework Directive (WFD; EC, 2000), which represents the legislative basis for the management and protection of European water bodies, suggests to the Member States rapid and cost-effectiveness sampling and fast procedures, in the evaluation of the biodiversity of the aquatic ecosystems.

Therefore, the simplification of methodologies and effort allocation are topics recurrently debated among researchers, aiming at sampling the broadest range of macroinvertebrates using the fastest and inexpensive technique, while guarantying the accuracy and reproducibility of the obtained results (Metzeling and Miller, 2001; Vlek *et al.*, 2006). So far, the reduction of the sampling effort has been achieved by limiting the number of samples or restricting the number of organism collected and measured (Metzling and Miller, 2001; Vlek *et al.*, 2006), using a higher taxonomic resolution (e.g., identification of genus or family; Dauvin *et al.*, 2003) or analyzing only the most significant fraction of benthic macroinvertebrates selected by body size (Pinna *et al.*, 2013).

Nevertheless, in literature, studies on optimal sample unit size (SUS) and/or sieve mesh size (SMS) needed to collect representative and accurate benthic macroinvertebrate samples are mostly lacking in transitional water ecosystems. Indeed, an ideal mesh size and sample unit size are the ones with favourable cost/effectiveness ratio, in which samples demand little time for processing and are fully representative of the benthic macroinvertebrates community of the sampling area (Buss and Borges, 2008).

The aim of this study was to investigate how a reduction in the SUS and an increase in the SMS affect the macroinvertebrates community, in two different study sites (SS) of a Mediterranean lagoon. The effects on macroinvertebrates community were tested using four different SUS (0.0225 m^2, 0.0450 m^2, 0.0675 m^2, 0.0900 m^2) and three alternative SMS (0.5 mm, 1 mm, 2 mm), and measured on common taxonomic diversity indices in both study site.

Material and methods

Sampling area

Lesina lagoon (41.88°N; 15.43°E) is a non-tidal and mesohaline transitional water ecosystem, located on the SE Italian coastline (Fig. 1a). The lagoon has an extended and narrow shape, elongated in the east-west direction (24.4 km long), and is connected with the Adriatic Sea by means of natural and artificial channels interspersed with sand dunes. The two major channels are named Acquarotta and Schiapparo. It covers an area of 51.4 km^2 and an average depth of 0.8 m; the catchment area is about 600 km^2 (Vignes *et al.*, 2009 and references therein). More information about Lesina lagoon description and ecological characteristics are available

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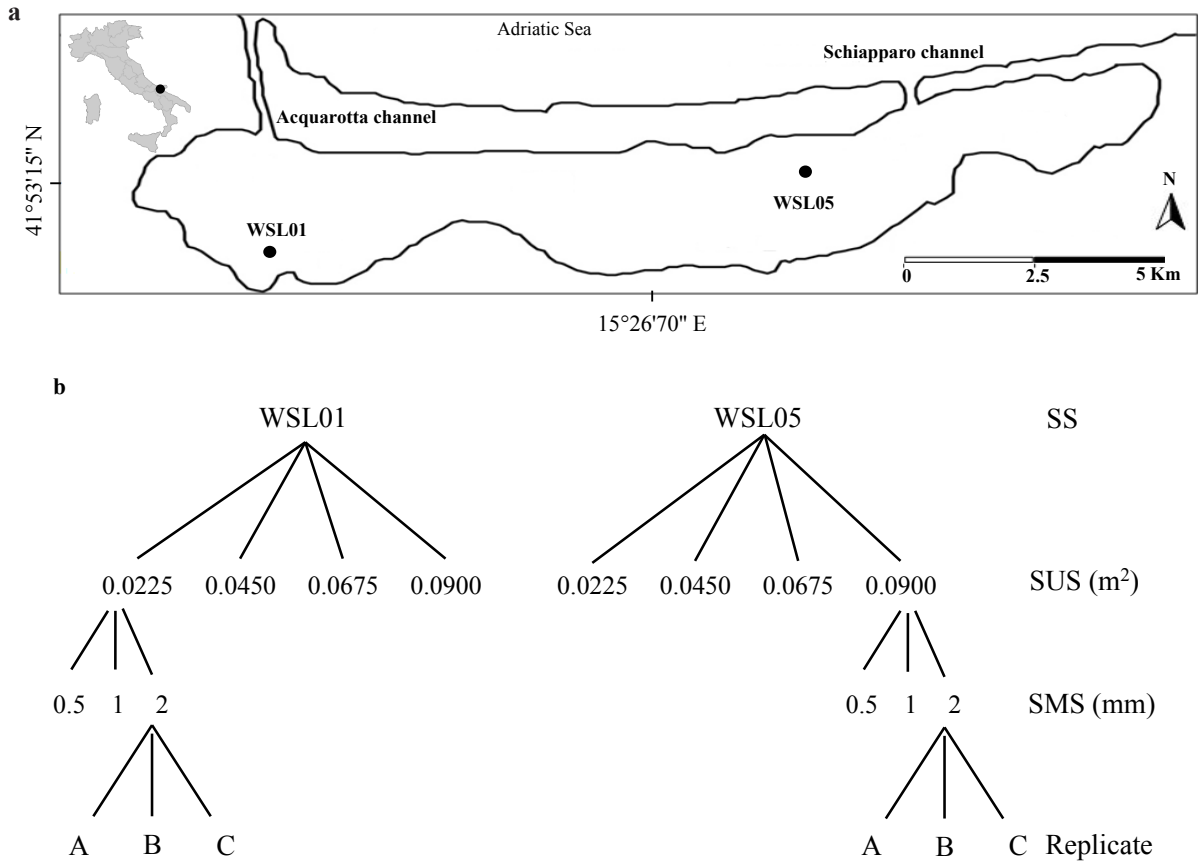

Figure 1. Map of the Lesina lagoon (SE Italy) with study sites (a), and sampling design used in this study (b). WSL01 is a perturbed study site and WSL05 is a relatively unperturbed site (from Borja *et al.*, 2011). In the sampling design two study sites (SS), four sample unit sizes (SUS, m^2), and three sieve mesh sizes (SMS, mm) were accounted. Three replicate were randomly sampled for each SUS and SMS. In total, in the two study sites were established twelve sampling effort conditions (4 SUS x 3 SMS).

in the literature (e.g., Mancinelli and Rossi 2001; Nonnis Marzano *et al.*, 2003; Manini *et al.*, 2005; Specchiulli *et al.*, 2009; Pinna *et al.*, 2013); in general, the benthic macrofauna and macroflora of the lagoon are similar to other Adriatic transitional water ecosystems (e.g., Mancinelli and Rossi, 2001; Menéndez *et al.*, 2003; Mancinelli *et al.*, 2005, 2007; Orfanidis *et al.*, 2008; Ponti *et al.*, 2008; Mancinelli *et al.*, 2009; Mancinelli, 2010; Potenza and Mancinelli, 2010; Mancinelli, 2012; Mancinelli *et al.*, 2013; Pinna *et al.*, 2013). Potentially, the lagoon is characterized by a low vulnerability to human activities; however, urban and agricultural wastewater discharges enter the lagoon particularly in the western area of the lagoon, leading to well know pulse eutrophication events (Vignes *et al.*, 2009; Borja *et al.*, 2011). During summer 2008, a strong dystrophic crisis occurred in the western area of the lagoon, resulting in hypoxic conditions for a few weeks over an area up to 2 km^2, significantly affecting all ecosystem compartments (Specchiulli *et al.*, 2009). Nutrient load from wastewaters, reduced hydro-dynamism and extreme climate events have been advocated as major causes of the dystrophic events

(Vignes *et al.*, 2009; Basset *et al.*, 2013).

Sampling design and laboratory procedures

The field sampling campaign was carried out in September 2009 within the activities of the FP7 WISER project, at a perturbed site (WSL01) and a relative unperturbed site (WSL05) of the Lesina lagoon (Fig. 1a). The perturbation level of the study sites was derived by Borja *et al.* (2011). Four sample unit sizes (0.0225 m^2, 0.0450 m^2, 0.0675 m^2, 0.0900 m^2), three sieve mesh sizes (0.5 mm, 1 mm, 2 mm), and three replicates for each SUS and SMS combination were established in the two study sites (Fig. 1b). The replicates for each SUS was randomly sampled from the two study sites using an Ekman-Birge grab sized 0.0225 m^2 (Fig. 1b).

Each sample was sieved through a column of 0.5 mm, 1 mm and 2 mm sieve mesh sizes (®Retsch GmbH, Germany) and the organisms retained were fixed in 4% buffered formalin. Physical and chemical parameters were also recorded, using a hand-held multiprobe (YSI 556) and a Secchi disk (Table 1). Total pressure was obtained as the sum of pressure level (1: low, 2: medium, 3: high) for each pressure type observed in the two study site (from Borja *et al.*, 2011; Table 1).

Table 1 - Geographical position and physical-chemical parameters of Lesina lagoon study sites. (*)Total Pressure derived by Borja *et al.* (2011).

	Study sites	
	WSL01	**WSL05**
Latitude (N)	41°51'56.6"	41°53'32.5"
Longitude (E)	15°20'35.3"	15°28'56.5"
Depth (cm)	-100	-120
Temperature (°C)	24.0	24.4
Conductivity (µS/cm)	22231	20450
Salinity (psu)	18.06	17.28
Oxygen (mg/l)	7.59	6.24
Transparency (cm)	-70	-120
Organic content (%)	4.7	14.0
***Total Pressure**	10	4

Benthic macroinvertebrates were washed, manually sorted and identified at the lowest possible taxonomic level using a stereo microscope (Leica MZ6). All individuals were subsequently counted, measured individually (total length) and weighted to the nearest 1µg to get the dry weight, after desiccation in a stove at 60 °C for 72 h (Pinna and Basset, 2004; Basset *et al.*, 2012). The individual body size of each specimen was expressed as individual biomass after calculation of the individual ash free dry weight (AFDW, mg), excluding the ash content percentage from each specimen's dry weight; ash content was obtained using dry specimens pooled according the taxonomic category and burned by a muffle furnace at 450 °C for 12 h (Borja *et al.*, 2011). All data were used to build up twelve datasets (four SUS x three SMS). In each dataset, specimens were identified as "2mm" if they were retained by 2 mm sieve mesh size; as "1mm" if they were retained on 2 mm and 1 mm sieve mesh sizes; as "0.5mm" if they were retained on 2 mm, 1 mm and 0.5 mm sieve mesh sizes.

Statistical analysis

To visualize the effects of increasing of the sampling effort in terms of SUS and SMS on the macroinvertebrates community, four diversity indices commonly used in benthic ecology were performed, based on equitability, dominance and species richness. To this aim taxa richness (S), Shannon–Weaver index (H') (Shannon and Weaver, 1963), Simpson (λ) (Simpson, 1949) and Taxonomic distinctness (TD) (Warwick and Clarke 1995, 1998) were calculated from density matrix data using PRIMER 6 software from Plymouth Marine Laboratory (Clarke and Gorley, 2006). All indices were measured at replicate level and averaged for SUS and SMS. H' was

calculated using the logarithm for a base 2. Three-way analysis of variance (Three-way ANOVA) was used to test the null hypothesis of no differences between study site, among sample unit sizes and among sieve meshes, according to a full factorial design. Sampling design considered three fixed factors, namely: study site (SS, with two levels), sample unit size (SUS, with four levels) and sieve mesh size (SMS, with three levels) (Fig.1b). This analysis was performed with STATISTICA software, version 8.0. Prior to the analyses, data were $x^{1/3}$ transformed to meet the assumptions of normality and homogeneity of variances.

Results

In the more detailed combination of SUS and SMS (0.0900 m^2 x 0.5 mm) a total of 17 taxa were identified being 7 Anellida (Polychaeta), 4 Arthropoda, (3 Crustacea and 1 Insecta), and 6 Mollusca (4 Bivalvia, 2 Gastropoda) (Table 2). The total number of taxa was lower in the WSL01 (12 taxa) than WSL05 (14 taxa), and the same difference was observed in the other combinations. *Ecrobia ventrosa* was the dominant specimen in more detailed combination of SUS and SMS, with a frequency slightly less than 92% (18,377 individuals), whereas the other taxa collectively accounted for 8%. Of these taxa twelve not exceed 1% (Table 2).

The specimens collected belong to four functional feeding groups (FFG): suspension/filter feeders dominated by taxa richness, accounting for 41% with an abundance of 5%, scrapers/shedders accounted for 18% in taxa richness whereas dominated by abundance with 92%, deposit/detritus feeders accounted for 29% in taxa richness and 2% in abundance, predators 12% in taxa richness and 1% in abundance (Fig. 2).

In general, the scraper *E. ventrosa* was the most abundant species, the suspension/filter feeder was the group with higher number of taxa (7) with 1056 individuals (Fig. 2).

The taxonomic richness exhibited an increase with increasing in SUS values in all the sieve meshes considered, with 2 mm values higher than 1 mm and 0.5 mm values, both in WSL01 and WSL05 (Fig. 3). A significant difference was observed between study site, among sample unit size within each study site and among sieve mesh size, as expected, since taxonomic richness is highly sensible to sampling effort. There was

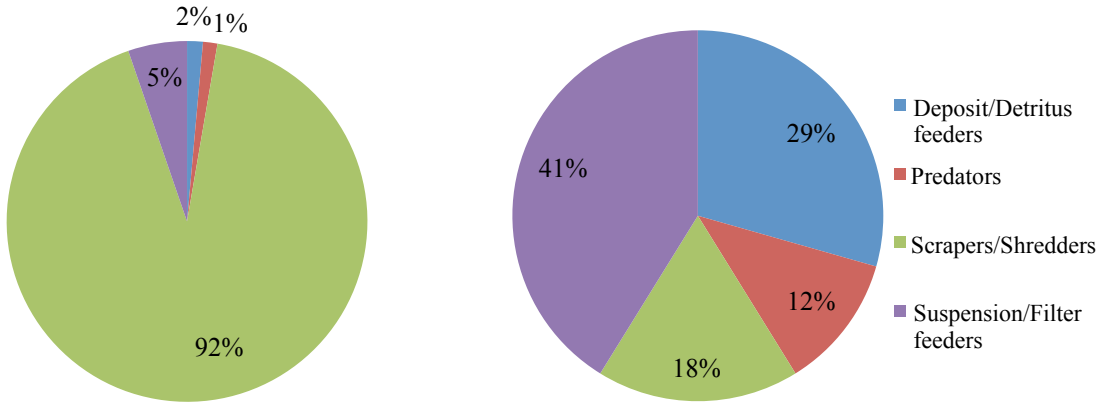

Figure 2. Pie charts of the proportion of the number of individuals (A) and taxa (B) in the following functional feeding groups. D: deposit/detritus feeders, P: predators, Scr/Shr: scrapers/shredders, S: suspension/filter feeders. Percentage values being for more detailed combination of SUS and SMS (0.0900 m^2 x 0.5 mm).

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Table 2 - Species list of the macroinvertebrate community collected in the more detailed combination of SUS and SMS (0.0900 m^2 x 0.5 mm), and relative frequency of each species, in the two study sites are reported. D: deposit/detritus feeders, P: predators, Scr/Shr: scrapers/shredders, S: suspension/filter feeders.

Phylum	Class	Subclass/Order	Family	Genus	Taxa	FFG	Frequency (%)
Anellida							
	Polychaeta						
		Clitellata			Oligochaeta	D	0.34
		Phyllodocida					
			Nereididae				
				Alitta	*Alitta succinea*	P	1.00
				Neanthes	*Neanthes* sp.	P	0.30
		Sabellida					
			Serpulidae	Ficopomatus	*Ficopomatus enigmaticus*	S	1.43
		Scolecida					
			Capitellidae				
				Capitella	*Capitella capitata*	D	0.01
				Heteromastus	*Heteromastus filiformis*	D	0.20
		Spionida					
			Spionidae				
				Polydora	*Polydora ciliate*	D	0.01
Arthropoda							
	Insecta						
		Diptera	Chironomidae	Chironomus	*Chironomus salinarius*	D	0.87
	Malacostraca						
		Amphipoda	Corophiidae	Corophium	*Corophium* sp.	S	0.01
			Gammaridae	Gammarus	*Gammarus aequicauda*	Scr/Shr	0.02
		Isopoda					
			Sphaeromatidae	Lekanesphaera	*Lekanesphaera hookeri*	Scr/Shr	0.08
Mollusca							
	Bivalvia						
		Euheterodonta incertae sedis					
			Cardiidae	Cerastoderma	*Cerastoderma glaucum*	S	0.01
			Semelidae	Abra	*Abra segmentum*	S	2.72
		Mytiloida					
			Mytilidae				
				Musculista	*Musculista senhousia*	S	0.01
				Mytilaster	*Mytilaster minimus*	S	1.12
	Gastropoda						
		Basommatophora					
			Planorbidae	Planorbis	*Planorbis* sp.	S	0.01
		Littorinomorpha					
			Hydrobiidae	Ecrobia	*Ecrobia ventrosa*	Scr/Shr	91.92

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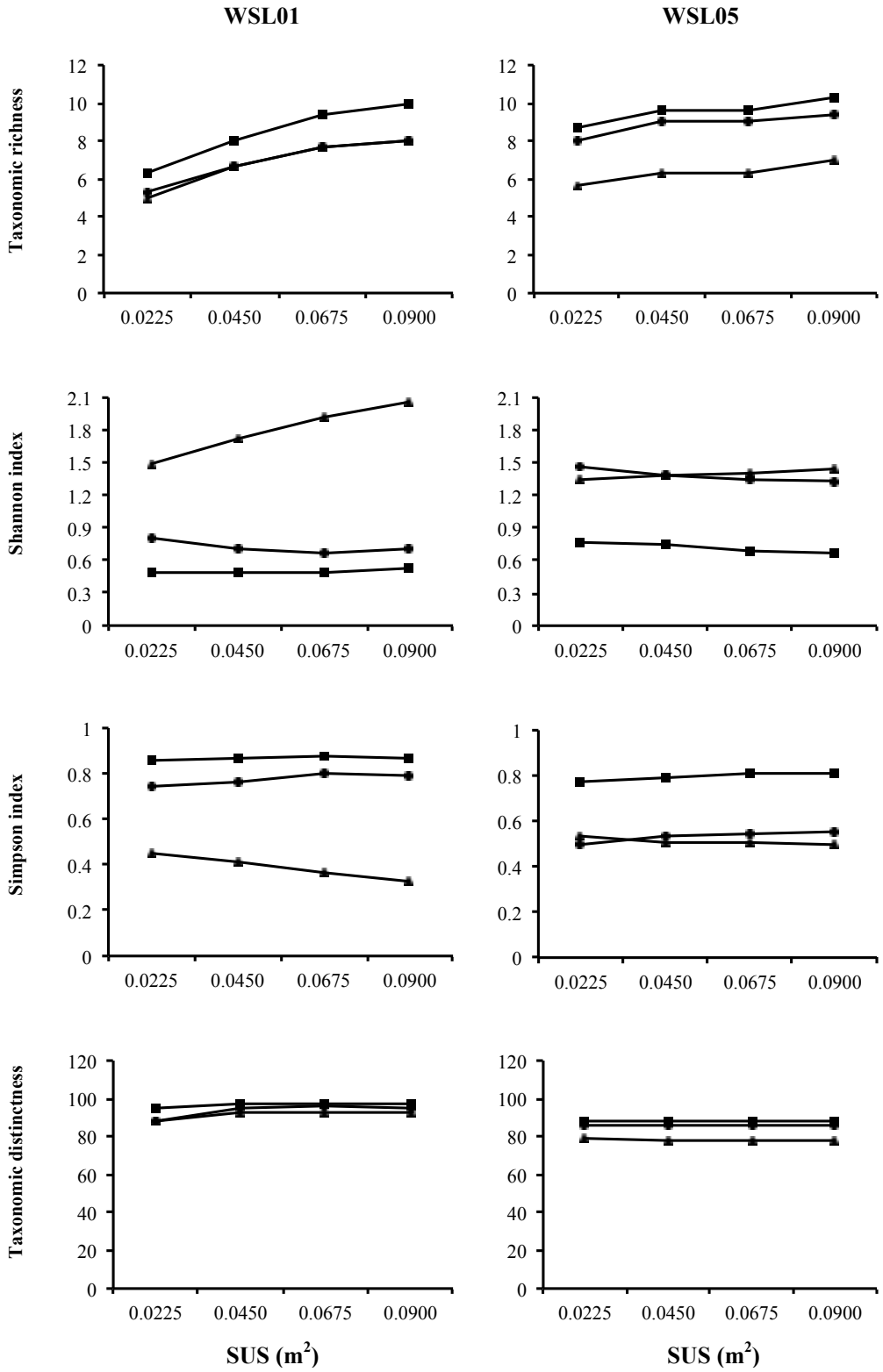

Figure 3. Line charts of the taxonomic diversity indices mean values computed for the twelve combinations (SUS x SMS), in WSL01 and WSL05 study sites (■ refers to 0.5 mm sieve mesh; ● refers to 1 mm sieve mesh; ▲ refers to 2 mm sieve mesh).

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also a significant interaction between study site and sieve mesh size (Table 3; Fig. 3). The Shannon index in this study showed higher values in the 2 mm sieve mesh than 1 mm and 0.5 mm in all SUS considered (Fig. 3). Similarly to the taxonomic richness, there was a high significant difference between study sites, likewise among sieve mesh sizes within each study site. No variability instead among sample unit size, whereas a significant interaction there was between study site and sieve mesh size (Table 3; Fig. 3).

The Simpson index, here used as a dominance index, showed higher values in

Table 3 - Results of three-way ANOVA of study site, sample unit size and sieve mesh size effects on taxonomic diversity indices in WSL01and WSL05. Bold values indicate significant tests for $p < 0.05$.

Source of variation	SS	df	MS	F	p
Taxonomic richness					
Study site (SS)	13.347	1	13.347	8.214	**0.006**
Sample unit size (SUS)	51.819	3	17.273	10.630	**<0.001**
Sieve mesh size (SMS)	70.194	2	35.097	21.598	**<0.001**
SS*SUS	9.708	3	3.236	1.991	0.128
SS*SMS	18.361	2	9.181	5.650	**0.006**
SUS*SMS	0.806	6	0.134	0.083	0.998
SS*SUS*SMS	0.417	6	0.069	0.043	1.000
Residual	78.000	48	1.625		
Shannon index					
Study site (SS)	0.446	1	0.446	5.717	**0.021**
Sample unit size (SUS)	0.039	3	0.013	0.168	0.918
Sieve mesh size (SMS)	11.687	2	5.844	74.880	**<0.001**
SS*SUS	0.113	3	0.038	0.483	0.695
SS*SMS	3.488	2	1.744	22.348	**<0.001**
SUS*SMS	0.380	6	0.063	0.811	0.567
SS*SUS*SMS	0.098	6	0.016	0.210	0.972
Residual	3.746	48	0.078		
Simpson index					
Study site (SS)	0.075	1	0.075	7.396	**0.009**
Sample unit size (SUS)	0.001	3	0.000	0.022	0.996
Sieve mesh size (SMS)	1.763	2	0.882	86.704	**<0.001**
SS*SUS	0.004	3	0.001	0.130	0.942
SS*SMS	0.402	2	0.201	19.782	**<0.001**
SUS*SMS	0.037	6	0.006	0.612	0.720
SS*SUS*SMS	0.004	6	0.001	0.071	0.998
Residual	0.488	48	0.010		
Taxonomic distinctness					
Study site (SS)	1702.838	1	1702.838	187.726	**<0.001**
Sample unit size (SUS)	67.951	3	22.650	2.497	0.071
Sieve mesh size (SMS)	640.792	2	320.396	35.321	**<0.001**
SS*SUS	110.310	3	36.770	4.054	**0.012**
SS*SMS	101.730	2	50.865	5.607	**0.006**
SUS*SMS	14.178	6	2.363	0.261	0.952
SS*SUS*SMS	18.697	6	3.116	0.344	0.910
Residual	435.402	48	9.071		

the 0.5 mm sieve mesh than 1 mm and 2 mm sieve meshes, both in WSL01 and WSL05 (Fig. 3). Similarly to Shannon index, this index exhibited a high variability between study site and among sieve mesh size within each study site. In contrast, no differences were observed among sample unit size levels, while a significant interaction there was between study site and sieve mesh size (Table 3; Fig. 3).

The taxonomic distinctness, estimating the average path length between individuals including those from the same species, showed similar values among sample unit sizes with WSL01 higher than WSL05 (Fig. 3). This index varied significantly among study sites and sieve mesh sizes. There was also a significant interaction between study site and sample unit size and between study site and sieve mesh size (Table 3; Fig. 3).

Discussion

The choice of the SUS and SMS in the assessment of the taxonomic diversity is of crucial importance for the effectiveness of the sampling method, and subsequent management considerations (Carter and Resh, 2001; Buss and Borges, 2008).

The results of this study showed that sieve mesh size affected all the diversity indices considered, and the taxonomic richness, in both study sites (Aarnio *et al.*, 2010; Barba *et al.*, 2010; Couto *et al.*, 2010; Pinna *et al.*, 2013). On the contrary, no large differences were detected with increasing the surface of the sample unit size in both study sites, except for the taxonomic richness, which is highly influenced by different sample sizes and sampling effort, according to Warwick and Clarke (1998) and Gray (2000). These results suggest that it is not always necessary to sample such a large sample unit size or maximize the sampling effort to achieve an accurate representation of the macroinvertebrates community using the taxonomic diversity indices. Moreover, most of the studies analyzing the reduction of the sampling effort are based on the comparison of results obtained by varying either the sieve mesh or the sample size, whereas no attempts have been made to test the combination of both sieve mesh and sample size. Thus, the present results are novel and have no parallel in literature.

In WSL01, and to a lesser extent in WSL05, increasing the sampling area, the taxa richness increased proportionally, as theoretically expected, plotting a cumulative curve, in accordance with Lyons *et al.* (1992). Similar results were obtained by Oliveira *et al.* (2011), who studied the influence of the increase of the subsample size (e.g., number of quadrates sampled) in stream ecosystems. The Shannon index reported a high diversity with larger sieve mesh size (2 mm), whereas a low diversity occurring with smaller sieve mesh sizes (0.5 mm and 1 mm), mainly in WSL01, due to the dominance of certain species (e.g., *E. ventrosa*). The difference among sieve meshes are not in agreement with observations provided by Barba *et al.* (2010), who compared macroinvertebrates captured in the 1 mm sieve mesh and one filtered through it in stream ecosystems. Differently from Barba *et al.* (2010), however, our assessments are in agreement with Couto *et al.* (2010) and with Pinna *et al.* (2013), the latter comparing 0.5 mm, 1 mm and 2 mm sieve meshes in a lagoon ecosystem, whereas the former compared 0.5 mm and 1 mm sieve meshes in an estuarine ecosystem.

The Simpson index based on the dominance exhibited a pattern complementary to Shannon index, showing higher diversity for 2 mm sieve mesh than 1 mm and 2 mm sieve meshes, in both study sites.

Taxonomic distinctness measures differently from other indices are independent from sample unit size and sieve mesh size and add information about taxonomic distance among present species (Warwick and Clarke, 2001). However, Taxonomic distinctness seemed to

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show a similar sensitiveness compared to the other diversity indices tested, in according to other studies (Salas *et al.*, 2006; Verissimo *et al.*, 2012).

Moreover, all the diversity indices considered were able to discriminate between the perturbed and relatively unperturbed study site (Basset *et al.*, 2008b; Ponti *et al.*, 2008; Borja *et al.*, 2011); despite some authors point out that most of these measures, except taxonomic distinctness can be highly influenced by different sample sizes, sampling effort, habitat type or complexity, and do not show monotonic behaviour in response to environmental pressures (Warwick and Clarke, 1998, Wilkinson, 1999, Rogers *et al.*, 1999; Gray, 2000; Salas *et al.*, 2006).

Conclusions

In conclusion, variations in taxonomic diversity estimations seem to depend mainly on sieve mesh size, suggesting that a reduction in sampling effort through the reduction of sample unit size may provide reliable and accurate results while minimizing sampling costs. This type of simplification is absolutely necessary for cost-effectiveness and practical biomonitoring.

Anyway, the final choice of sieve mesh size and sample unit size should be based on the specific goals and hypotheses of a given taxonomic diversity biomonitoring program, and should account for precision, and cost-effectiveness, as well as type of habitats sampled and target taxa etc., as suggested by many authors (Meyer *et al.*, 2011).Finer meshes and larger sample size combination may result more accurate; however, the drawback of using finer meshes is that early instars, better retained by fine mesh nets, are not the best indicators of environmental conditions, because these organisms have not been in place long enough to respond to chronic condition. Even if in Mediterranean coastal areas many non-native species are already recorded (Mancinelli *et al.*, 2013), the effect of simplifying the procedures in the sampling protocols on non native species, which are affecting taxonomic diversity of transitional waters, is to date unexplored.

Moreover, the present contribution could be useful in harmonizing sampling methodologies for the cost-effectiveness taxonomic diversity estimation, and the development of rapid biomonitoring protocols using benthic macroinvertebrates in transitional water ecosystems.

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