Exploratory study for the optimization of sampling effort in a non-vegetated lagoon within a Mediterranean wetland (Albufera Natural Park, Valencia, Spain)

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Abstract

The analysis of macroinvertebrate communities is essential in aquatic ecology studies. Nonetheless, sample collection, processing and species determination of macroinvertebrates are extremely time-consuming and require huge efforts. Moreover, despite the crucial need of refined sampling protocols, investigations on viable benchmark sampling efforts are still scarce. This study provides a preliminary analysis on the optimisation of the sampling effort required to study macroinvertebrate communities from rice fields. Twenty core sediment samples were collected from a non-vegetated Mediterranean lagoon (rice field) in Valencia (Spain), and their macroinvertebrate community assemblages were obtained. Characterisations of the minimum number of samples needed for both faunistic inventories and environmental quality assessments were carried out using diversity indexes (number of taxa, Shannon, Simpson, and Margalef indexes), ecological indicators (Nutritional Mode Index, IMN) and several species richness estimators (Chao 1, Chao 2, Jacknife 1, Jacknife 2, ACE, ICE, Bootstrap and EstimateS 9.1.0). Our results indicated that in surveys in which the taxa richness of the communities is the objective, 20 samples or even more could be needed. However, when the objective of the study is to assess the environmental quality by means of ecological indexes such as the IMN, three samples could be an acceptable benchmark. Our findings, despite being limited by our experiment conditions, can provide methodological guidelines for ecological assessments in Mediterranean rice fields and shallow non vegetated lagoons. Further research involving multiple study areas and seasonal patterns will help meliorate the accuracy of this protocol and refine sampling efforts in wetlands.

Keywords: Macroinvertebrates; sampling effort; IMN; wetland; rice fields; Mediterranean.

1. Introduction

Invertebrates play a principal role in the ecological functioning of inland aquatic ecosystems, notably wetlands and transitional habitats (Griffiths, 1991; Anderson et al., 2013). They are essential components involved in the health of these environments (Zilli and Marchese, 2011), and many authors have pointed out their importance as the basis of food chains that sustain vertebrates (McQueen et al., 1986; Cyr and Downing, 1988; Batzer et al., 1993). They also participate in the litter decomposition and cycling of organic matter and nutrients (Batzer and Wissinger, 1996; Wallace and Webster, 1996), and contribute to the maintenance of the biochemical balance within freshwater ecosystems. Given their importance, macroinvertebrates are used as indicators of the health of aquatic environments (Hellawell, 1978; Alba Tercedor and Sánchez-Ortega, 1988; Wallace & Webster, 1996; Pinna et al., 2007), and several countries have established them as a compulsory component to be considered in the ecological assessment of freshwater ecosystems (Smith et al., 1999; WFD, 2000; Gresens et al., 2009).

During the last five decades, issues such as accuracy, variability and representativeness of the data obtained in biological surveys have been a cornerstone in ecological investigations (Magurran, 1988; Gotelli and Colwell, 2001; Oertli et al., 2002; Ramos-Merchante and Prenda, 2017). Special attention has been paid to the sampling design, the methodology employed, and if quantitative or qualitative data are needed depending on the purpose of the study (Anderson et al., 2013). In freshwater ecology studies, habitat diversity, spatial variation in the distribution of organisms (patchiness), substrate types, size and motility of subjects are some of the most relevant factors (Meyer et al., 2011). Overall, three basic aspects play a vital role in shaping the different methodological approaches found in the literature: sampling devices, spatial sampling design, and sampling effort (number of replicates and sample size) (Lorenz et al., 2004).

With regard to the sampling device, Surber sampling nets have been widely used in rivers (Gillies et al., 2009), while pond netting, horizontal activity traps, grabs, dredges, coring devices and artificial substrates have been used for lentic systems (Biggs et al., 1998; Batzer et al., 2001; García Criado and Trigal, 2005; Whiles and Goldowitz, 2005; Becerra et al., 2008; Anderson et al., 2013). Interestingly, Meyer et al. (2011), highlighted that wetlands pose sampling challenges because of the predominance of soft substrata and vegetation, and several studies focused on the comparison of different sampling methods for invertebrate communities (Turner and Trexler, 1997; García Criado and Trigal, 2005; Becerra et al., 2008; Pinna et al., 2017). These studies showed that some devices such as sweep nets

and stovepipes can offer reliable results (representative number of taxa captured), while others, such as corers (tubes of 5-10 cm diameter) could lead to more biased results. However, agreement on the need to use several complementary procedures exist, emphasizing the relevance of using more than one sampling technique to obtain a better description of biodiversity and environmental quality status (Anderson et al., 2013). Despite these aspects, corers still provide reliable sampling tools in non-vegetated lagoons, given these habitats host the vast majority of macroinvertebrates within the bottom substrate (Valdecasas et al., 2010). For instance, corer devices have been used in the ECOFRAME scheme for the assessment of the ecological status of shallow lakes (Moss et al., 2003), and in other studies on macroinvertebrate communities (Miracle et al., 2006; Sahuquillo et al., 2007**).**

With regard to spatial distribution, Morrisey et al. (1992), reported that abundances of infauna in soft sediments are patchy at a range of spatial scales, from a meter up to several kilometres. In addition, these authors reported more significant differences between the number of taxa at the smaller than at the larger spatial scales, concluding that distributional patterns at different scales are not the same for all the invertebrate groups. Anderson et al. (2013) suggested that since working with a high number of samples may be laborious, the sampling design must consider a balance between adequate sample sizes and the resources needed to process and handle the samples.

Another crucial point is the sampling effort necessary to obtain representative samples according to the objectives of different types of studies. In inventory studies, it is assumed that the maximum effort must be applied to ensure a vast collection of taxa within a specific habitat. On the other hand, in environmental quality monitoring surveys, sampling protocol optimization is essential to ensure standardized procedures for ecological assessments and reduce direct monitoring costs (Vlek et al., 2006). These authors also state that in these kind of investigations the number of replicate samples is not relevant, since usually only one multihabitat sample used to be taken.

Ecological monitoring often involves multihabitat sampling procedures that incorporate the composition of multiple subsamples into a single sample (Barbour et al., 2006; Becerra et al., 2008). Nonetheless, the sampling effort is rarely justified in the vast majority of works in lotic and lentic systems involving biodiversity, environmental studies or both. Within the ECOFRAME project, Moss et al. (2003) established a standardized methodology of 10 random core samples for soft-bottomed communities, but the authors did not justify this number. In their adaptation of the Helawell Index to Iberian Peninsula (IBMWP) to assess the water quality of rivers, Alba-Tercedor and Sánchez Ortega

(1988) proposed collecting samples until no further specimens of new families were captured (sample observation was performed in the field). While widely employed in ecological assessments of Mediterranean rivers, the *in situ* IBMWP protocol involves a very high sampling effort which has been found to produce a slight underestimation when compared to the diversity assessment carried out in the laboratory (Bonada et al., 2002). In addition, it has been noted that the number of replicates or the sample size may have a different influence on each of the ecological metrics applied (Lorenz *et al.*, 2004; Vlek *et al*., 2006), affecting representativeness and rigour.

Lorenz et al. (2004) pointed out that macroinvertebrate-based bioassessment surveys involve several key factors such as the method applied for sample collections, the procedure of sorting the sample and counting the specimens, and the taxonomic precision during the identification. Regarding the latter aspect, Gotelli and Colwell (2001) reported that species accumulation and rarefaction curves become asymptotic sooner when the taxonomic level is higher (i.e. family vs. genus or species), stressing the need to reach a pragmatic balance between effort and accuracy.

Overall, standardised and sufficiently detailed approaches are scarce, and very little knowledge still exists around minimum benchmark sampling efforts in freshwater ecology studies (Cobelas et al., 2005). Here, we expand this knowledge by testing the sampling effort of core-based benthic invertebrate surveys from a non-vegetated shallow lagoon (rice field), an ecosystem that plays a key ecological role in the Mediterranean region (Fasola and Ruiz, 1996; Rossi et al., 2003). The aim of this work is to provide a preliminary investigation on the sampling effort required for studies on faunistic diversity, and for biomonitoring surveys for ecological characterization, in a Mediterranean plant-free shallow lagoon.

2. Materials and methods

2.1. Study area

The field work was carried out at a rice field (study area = 5307 m^2) in the El Saler district within the Albufera Natural Park (Valencia, Spain) (Fig. 1), on the 18th of January, 2018. This area is intensely dedicated to rice growing, and rice fields are interconnected through channels, forming a continuous wetland of 30,000 hectares, with periodical flooding and drying. The paddy orchards are flooded from September to February, being this aspect of special interest for biodiversity and trophic source conservation (Lupi et al., 2013). The collection of samples was carried out at the end of wintry

144 inundation, which is the period of the annual cycle when the macroinvertebrate community is the most
145 stable. The values of the nutrient concentrations were: ammonium: 0.3 mg/L; nitrite: <0.01 mg/L; nitrate:
146 0.5 mg/L; phosphates: 1 mg/L. The system revealed alkaline (pH: 8.3) and aerobic (dissolved oxygen:
147 10.7 mg/L) conditions with high conductivity values (2352 µS/cm), typical of environments subjected to
148 periodic evaporation as is the case of rice fields.
149
150 *2.2. Macroinvertebrates sampling*
151 Macroinvertebrate sampling was carried out collecting a sample every 10 m along two parallel transects
152 following a zigzag pattern designed prior to fieldwork (Fig. 1). The study area was fairly uniform and
153 homogeneous. Sampling points were considered as independent (following Simple Random Sampling
154 (SRS) (de Vries, 1986)).
155 Twenty sediment samples were taken with a methacrylate cylinder (5.2 cm inner diameter),
156 which was pushed into the sediment and the upper 10 cm of the column was finally analysed. This
157 technique had the objective of collecting those organisms that were strictly associated with substratum,
158 and the total number of samples (20) was decided on the basis of the information gathered from other
159 freshwater studies working with replicates (Barbour et al., 1999; Vlek et al., 2006; Gillies et al., 2009).
160 Samples were fixed in 5% formaldehyde in the field and washed and sieved with a 250 µm net in
161 the laboratory. Retained specimens were preserved in 70% ethanol and were later sorted from the
162 sediment. Specimens were counted and identified under a stereomicroscope (Leica MZ16) to the lowest
163 taxonomic level possible with the help of specific taxonomic keys (Brinkhurst and Jamieson, 1971;
164 Tachet et al., 1987; Tachet et al., 2000; Kotov and Ferrari, 2010; Oscoz et al., 2011). The faunal checklist
165 was drawn up according to the scientific and taxonomic information from the Catalogue of Life (Roskov
166 et al., 2013), and Fauna Europaea (de Jong, 2013). Family Daphniidae (Daphnia *sp*.) and Order
167 Cyclopoida were excluded from the study due to their nektonic tendency (Galassi et al., 2009; Tiberti,
168 2011). The results were treated according to two approaches: 1) analysis of the taxonomy richness (for
169 faunal inventories) and 2) calculation of an ecological index for the environmental quality assessment.
170
171 *2.3. Analysis of sampling effort for environmental quality estimation*
172 The ecological status of our system was assessed through the Nutritional Mode Index (hereafter
173 referred to as IMN, for consistency with Rueda et al. (2005) and Rueda and Hernández (2008)). The IMN

focuses on the characterisation of macroinvertebrate trophic assemblages, given alterations in the aquatic ecosystems can condition the distribution and relative abundance of nutritional groups and provide crucial information about the functional composition of biotic communities (Statzner et al., 2001).

The calculation of the IMN involves a series of steps. First, macroinvertebrate taxa are grouped according to their way of collecting food (as well as the size and nature of the ingested resources) and eleven nutrition groups, following the classification proposed by Tachet et al. (1987), are created: H = Herbivorous; O = Omnivorous; D = Detritivores; P = Predators; Rm = Grazers; Rs = Scrapers; F = Filterers; L = Limnivorous; ChH = Herbivorous-sucking; ChP = Predators-sucking; S = Scrapers-sucking (see Appendix 1 for further details). Next, the abundances of the nutrition groups per each sample are converted to their relative abundance (in %) and two new variables are calculated from this dataset. For the first variable, we count the number of nutrition groups which are more abundant than 0 % and we continue to count these at progressive increments of 1% until 15%. The sum of these counts is the variable 'total positives'. For the second variable, we count the number of nutrition groups with an abundance equal to 0% and more than 40%, 45%, 50%, 60%, 70%, 80%. The sum of these counts is the variable 'total negatives'. The final IMN value is the difference between the 'total positives' and the 'total negatives'.

Similar to other biological quality indexes like IBMWP (Alba-Tercedor and Sánchez-Ortega,1988; Alba-Tercedor et al., 2004), IBGN (AFNOR, 1992), TV (Verneaux and Tuffery, 1967), QBR (Munné et al., 1998) and IHF (Pardo et al., 2002), the IMN follows the rules and recommendations set out in the Third Technical Seminar organized by the European Community on "Biological methods for assessing water quality" (Ghetti and Bonazzi, 1980), and the Framework Directive on the water policy area (EC, 2000), establishing environmental quality classes (Appendix 2). In order to obtain a better graphic vision, environmental quality classes are assigned to different colours.

As an ecological status index, the IMN should correctly reflect the diversity dynamics of the community under study (Bongers, 1990; Allegro and Sciaky, 2003). In order to test the variability and representativeness of the IMN, correlations with diversity indexes (D_{si}, H' and D_{mg}), were calculated using the non-parametric Spearman's rho test (Kolmogorov-Smirnov test revealed a non-normal distribution for the IMN values ($Dn = 0.308$; $P < 0.01$).

204 *2.4. Diversity metrics and statistical analysis*

205 Three of the widest employed diversity indexes in ecology, Simpson (Dsi) (Simpson, 1949),
206 Shannon (H') (Shannon, 1948; Shannon and Weaver, 1949) and Margalef (Dmg) (Margalef, 1983), were
207 calculated per each sample through the software Past, version 2.17 (Hammer et al., 2001). The
208 effectiveness of the sampling procedure (Colwell and Coddington, 1994) was tested through seven non-
209 parametric species richness estimators (Chao 1 (Chao 1984, 1987), Chao 2 (Chao 1984, 1987), first-order
210 Jackknife (Jackknife 1) (Burnham and Overton 1978, 1979; Heltshe & Forrester, 1983; Smith and van
211 Belle, 1984), second-order Jackknife (Jackknife 2) (Burnham & Overton, 1978, 1979; Smith and van
212 Belle, 1984; Palmer, 1991), ACE (Chazdon et al., 1998; Chao et al., 2000), ICE (Chazdon et al., 1998;
213 Chao et al., 2000) and Bootstrap (Smith and van Belle,1984)) calculated by means of the open access
214 EstimateS 9 software application (Colwell, 2019). These parameters helped estimation of 'true' number of
215 taxa (Colwell & Coddington, 1994), and the quotient between the number of taxa obtained through the
216 sampling and the estimator values (transformed into relative percentages) allowed assessment of the
217 representativeness.

218 Matlab software version 8.3 (R2014a) (MATLAB, 2014) was employed to work at a matrix level
219 and calculate the mean (median and standard deviation) of the taxa among all the possible sample
220 combinations. First, taxa were ascribed to trophic groups and IMNs per each sample were calculated,
221 allowing a preliminary investigation on the range of values obtained. Second, mean and standard
222 deviation values of the IMN for each sample combination (first, samples were combined and second, the
223 IMN was calculated) among the entire pool of samples were obtained through the *combnk* function with
224 the Matlab software version 8.3 (R2014b). Third, an empirical sample-based rarefaction curve was
225 obtained to test the variability of the IMN values. Finally, in order to extrapolate rarefaction curves
226 beyond the empirical sample dataset (Colwell, 2004; Colwell et al., 2012), quadruple rising (by 100
227 randomizations) was applied to sample size, and an 80-sample pool was obtained. This rarefaction curve,
228 created through the free EstimateS 9 software (Colwell, 2019), used the multinomial model detailed in
229 Shen et al. (2003).

230 Distributions of IMN values and trophic assemblages were investigated by means of principal
231 component analysis (PCA), through the software Past version 2.17 (Hammer et al., 2001). Kolmogorov-
232 Smirnov normality tests (D_n), homogeneity tests between samples (Pearson's Chi-squared test (χ^2)) and

correlations among diversity indices (no. of taxa, Dsi, H' and Dmg) and the values of the IMN (Spearman's rho correlation (R_s)) were all obtained through the software SPSS version 20 (IBM, 2011).

3. Results

3.1. Taxa richness and sampling effort

Among the 20 samples considered (named B1 to B21, B10 does not exist), 13 taxa spread over two phyla, five classes, one subclass, four orders, eight families, three subfamilies, one genus, 1 species complex and 1 species (Table 1). The most abundant taxa were class Ostracoda (49.36%) and family Tubificidae (44.82%), accounting for 94.18% of all the specimens found within the 20 samples. The least abundant taxa were subfamily Tanypodinae and family Ilyocryptidae (0.08%). The taxa with the highest average density within samples (mean ± SD ind./m^2) were class Ostracoda (13997 ± 9396) and family Tubificidae (12711 ± 6856 ind./m^2), while family Ilyocryptidae and subfamily Tanypodinae displayed the lowest average density (24 ± 111 ind./m^2) (Table 1). The highest density (45847 ind./m^2) was found in sample B1, whereas the lowest was observed in B13 (9169 ind./m^2). Samples B1 and B12 had the largest number of taxa (7), contrary to the least diverse sample B13 which hosted only two groups (Table 1).

Results from the homogeneity test showed that the samples were not part of the same pool (χ^2 = 622.346, $P < 0.01$), suggesting that diversity richness would get bigger as the sampling effort increases. The percentage of sampling effectiveness (the probable number of existing taxa) varied between 74.07% (Jackknife 2) and 93.62% (ACE) (Table 2). The minimum average number of taxa was 3.2 ± 1.1, which corresponded to a sampling effort of only one sample. As the sampling effort increased, higher values of the taxa richness were obtained, with the maximum value (11 taxa) being reached with an effort of 20 samples (Fig. 2). Accordingly, an extrapolation of taxa richness beyond the empirical data set was carried out, showing a curve with an asymptotic trend, more marked as from an effort of 20 samples (Fig. 6).

3.2. IMN dynamics and sampling effort

The IMN values varied between 5 (sample B11) and 43 (sample B12), and their average value was 26 (Fig. 3). Sample B12 was the most diverse according to the Shannon (H' = 1.212) and Margalef (D_{mg} = 1.306) indexes, and B5 was the most diverse on the Simpson Index (D_{si} = 0.6371) (Fig. 3). Sample

B11 showed the lowest values of Shannon (H' = 0.1584) and Simpson indexes (D_{si} = 0.07133), and B7 was the least diverse on the Margalef Index (D_{mg} = 0.2233) (Fig. 3).

The number of individuals (N) did not correlate with the values of the IMN per each sample (R_s = 0.344; P > 0.05), indicating that IMN values are independent of abundance. Contrarily, positive linear correlations between the values of the IMN and the number of taxa (R_s = 0.752; P < 0.01), Shannon (R_s = 0.624; P < 0.01), Margalef (R_s = 0.660; P < 0.01) and Simpson (R_s = 0.468; P < 0.05) indexes were found.

The PCA ordination indicated that fourteen out of the twenty samples laid within the positive quadrant of the two axes (Fig. 4), confirming a functional homogeneity dominated by omnivorous and fewer predators and limnivores. B9 and B12 (the samples with the highest IMN values: 30 and 43 respectively) were the samples furthest apart in the Axis 2, showing increased affinities towards filterer and scraper feeding habits. In contrast, samples with the lowest IMN values B11 (IMN:5) and B13 (IMN:7) displayed nutritional affinities closer to limnivory and predation.

The IMN-based rarefaction curve (Fig. 5) revealed a more marked asymptotic tendency than the taxa accumulation curve (Fig. 2). The average IMN value in correspondence of the minimum sampling effort (one sample) was 19.01 ± 0.76, followed by a gradual increase that reached its maximum (23.13 ± 4.89) in correspondence to the effort of 18 samples. As expected, standard deviation and outliers for the calculated average IMN values gradually lowered as the number of samples increased, finally disappearing in correspondence with the maximum sampling effort.

The analysis of the average values of the IMN revealed two different ecological characterizations (Fig. 6): highly stressed environment (class V) with a very simplified trophic web, when only one sample was considered; very stressed environment (class IV) with a simplified trophic web, obtained with a sampling effort of two to twenty samples (Appendix 2) (Rueda et al., 2005; Rueda & Hernández, 2008).

4. Discussion

4.1. Faunistic inventories in Mediterranean rice fields

This study was planned as an exploratory research on the sampling effort required in invertebrate aquatic community studies from a Mediterranean rice field. Data processing was carried out using a pool of 20 benthic samples (considered as replicates) based on the sampling efforts reported in the freshwater ecology literature (García-Criado and Trigal, 2005; Miracle et al., 2006). In fact, while some studies

293 indicated a sampling effort ranging from three to five samples (Barbone et al., 2012; Orwa et al., 2013),
294 several other investigations employed pools of up to twenty samples. For instance, Becerra et al. (2008)
295 employed twenty traps to maximize the total taxa richness and minimize sampling effort in heavily
296 vegetated wetlands. Oliveira et al. (2011) utilized a 20-unit sample to determine the optimum
297 subsampling effort in streams of South-east Brazil, while Gillies et al. (2009) collected sixteen and
298 seventeen samples from two Australian rivers with the same purpose.

299 Our results highlighted that if the objective of a survey is to state the taxa richness, the maximum
300 sampling effort (20 replicates) is recommended. Mathematical modelling was necessary to validate the
301 pool of samples and test our findings further. The percentage of representativeness obtained through the
302 non-parametric taxa estimators revealed that the maximum diversity was not reached. Interestingly, as
303 shown in the extrapolation (Figure 6), by doubling the sampling effort (40 samples), only 12.59 taxa on
304 average were detected, supposing a minor increase (14.5 %) compared to the significant effort required.
305 This trend was confirmed as the sampling effort increased, with the highest richness (13 taxa, accounting
306 for just a 3.3 % increase compared to the previous case) being detected with a 60-sample design.
307 Confidence intervals widened as the sample size increased (Fig. 6), but this was due to the multinomial
308 design (see Shen et al., 2003 for further details) employed for the extrapolation (Colwell et al., 2012).

309 Overall, our results indicated that our pool provided a robust and representative set of samples to
310 investigate taxonomical diversity in rice fields. Our findings also suggest that faunistic studies in
311 environments such as non-vegetated lagoons would require an extremely high sampling effort, for
312 example 20 samples or even more. This is because reports on species diversity require cataloguing of all
313 the potential taxa inhabiting a specific area. Gillies et al. (2009) argued that the likelihood of collecting a
314 taxon is related to its abundance within the pool. Similarly, taxa that have a widespread distribution are
315 also more likely to be collected than those with a narrow distribution. As new taxa are found, even if rare
316 or with low detectability, an increasingly bigger effort is required to collect and consider them within the
317 entire pool of replicates. This becomes more evident in communities with homogeneously distributed
318 species (three taxa amount to 95.82% of all the organisms found), which is the case of the present study.
319 Indeed, additional studies from more diverse Mediterranean rice fields will help test the applicability of
320 our results and improve the accuracy of faunistic inventories in these vital semi-natural environments.
321

322 *4.2. Sampling effort and ecological assessment*

Whilst the Water Framework Directive (EC, 2000; WFD, 2000;; EC, 2008) suggests rapid and cost-effective sampling procedures, the identification of minimum sampling efforts is a basic step to improve bio-assessment protocols. We tested the ratio between sampling effort and efficiency through the use of the IMN, an index based on the ecological assumption that nutritional modes are strictly related to trophic web complexity, as well as to environmental stability or its perturbation (Alba-Tercedor, 1988; Statzner et al., 2001). One of its advantages, unlike the majority of indices applied to lentic systems, is that the methodological protocol of the IMN does not require a species-specific characterisation (Rueda et al., 2005; Rueda & Hernández, 2008). Among the nutritional groups analysed for this study, omnivores - a trophic category that has been widely reported in rice fields (Olsson et al., 2008; Winckler et al., 2017) - dominated the macroinvertebrate community. Correlation analyses between the IMN and major diversity indexes indicated that the IMN acts as a rigorous and reliable tool for the ecological assessment of shallow non-vegetated lagoons, as already found for springs (Rueda et al., 2013) and reservoirs (Rueda et al., 2008).

Our findings on the sampling optimisation showed that efforts of up to 11 samples could potentially expose the risk of leading to wrong ecological assessments (lower whiskers of the boxplot falling into the wrong class, Fig. 5), while from 12 samples onwards all the values (excluding the outliers) fell in the right categorisation (class IV, Fig. 5). However, this 12-sample benchmark would imply extremely time-consuming procedures and very expensive budgets for just one system, especially when we consider that ecological indexes are designed to assess and compare the quality of multiple aquatic environments (Chessman et al., 2007). On the other hand, a sampling effort of just one sample led to a wrong ecological assessment (average IMN values falling into the class V), stressing the need to reach an optimum sampling effort/efficiency ratio for robust sampling protocols.

Interestingly, a two-sample sampling effort illustrated a representative average ecological characterisation of the system (class IV, same as per the sampling effort from three to 20 samples). However, the median corresponded to class V instead of class IV (Fig. 5), and this could lead to misleading interpretations, given the most frequent value did not correspond to the correct class characterisation (class IV (Fig. 5)). However, a sampling effort of three samples showed that the mean and median fitted into the class IV (and none of the outliers fell in the wrong categorisation (Fig. 5)), suggesting that three replicates can be considered as an appropriate sampling effort. Notably, this protocol

based on three replicates has been frequently used in freshwater ecology studies, but it is rarely well-founded (Gillies et al., 2009).

Our findings provide evidence that when biological metrics are used in Mediterranean non-vegetated shallow lagoons, three core sediment samples allow an adequate assessment of the ecological status. Despite the fact that our results cannot be extrapolated to other habitats, this study raises a reflection on sampling designs, and can provide useful guidelines to elaborate accurate collecting protocols in similar types of systems. Indeed, we consider that the present contribution is useful for harmonizing sampling efforts and optimizing cost-effectiveness in monitoring programs on non-vegetated aquatic environments such as rice fields. Further research involving multiple study areas and seasonal patterns will help meliorate the accuracy of the methodological protocols and refine sampling efforts in wetlands.

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