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Assessing the ecological status of a Mediterranean river: benthic invertebrates and diatoms as complementary bioindicators

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ABSTRACT

Assessing the ecological status of a Mediterranean river: benthic invertebrates and diatoms as complementary bioindicators

Freshwater is a fundamental component in the health and well-being of humans and ecosystems, and for socio-economic development. Therefore, it has become essential to manage it in a balanced way due to its importance. To provide some insights into this topic, we evaluated the ecological status and water quality of a Mediterranean river through the analysis of both benthic diatoms and invertebrates' communities, as well as physicochemical and hydromorphological parameters. The assessment of the physicochemical water quality indicated the presence of organic pollution, especially during spring and summer. The evaluation of the physical habitat revealed slight transformations in the river's channel and an impacted riparian zone unable to function as a barrier to the anthropogenic impacts. Moreover, both diatoms and benthic invertebrates' communities showed that the river still has some sampling sites classified below the "Good" ecological status as proposed by the Water Framework Directive, with populations dominated mostly by ubiquitous and tolerant *taxa* to organic pollution. The results obtained here could contribute to create future research projects in order to provide integrated management solutions to improve the river's ecological status.

Key words: ecological status, Water Framework Directive, organic pollution, riparian zone, partial least squares regression

RESUMO

Avaliação do estado ecológico de um rio mediterrânico: invertebrados bentônicos e diatomáceas como bioindicadores complementares

A água doce é uma componente fundamental para a saúde e bem-estar dos seres humanos e dos ecossistemas bem como para o desenvolvimento socioeconómico. Devido à sua importância é essencial geri-lo de uma maneira equilibrada. De forma a abordar este tópico, avaliamos o estado ecológico e a qualidade de um rio mediterrânico através da análise das comunidades de diatomáceas e de invertebrados bentónicos, bem como de parâmetros físico-químicos e hidromorfológicos. A avaliação da qualidade físico-química da água indicou a presença de poluição orgânica, principalmente durante a primavera e o verão. A avaliação do habitat revelou pequenas transformações no canal do rio e uma zona ripária impactada, incapaz de funcionar como uma barreira aos impactos antropogénicos. Além disso, tanto a comunidade de diatomáceas como de invertebrados bentónicos mostraram que o rio apresenta alguns locais de amostragem com classificação inferior ao 'Bom' estado ecológico, tal como proposto pela Diretiva-Quadro da Água, com populações dominadas principalmente por taxa ubíquos e tolerantes à poluição orgânica. Os resultados obtidos são importantes para criar futuros projetos de investigação, de forma a obter soluções de gestão integrada para melhorar o estado ecológico do rio.

Palavras chave: *estado ecológico, Diretiva-Quadro da Água, poluição orgânica, zona ripária, regressão parcial de mínimos quadrados*

INTRODUCTION

Rivers are a fundamental component for the development of human populations and are, consequently, subjected to several anthropogenic impacts (Lainé *et al.*, 2014). The quality of freshwater is considered a global concern of increasing proportions, since the risks of its degradation are directly translated into socioeconomic impacts. In fact, the disruption of freshwater ecosystems is undermining the capacity of environment to provide basic water-related services (e.g., purification, storage, provisions) (UN, 2014). Since degraded ecosystems can no longer regulate and restore themselves due to the loss of resilience, studies predict that half of river basins in the European Union will be affected by an increase spread of water scarcity and stress by 2030 (EC, 2012; UN, 2014).

In 2000, the implementation of the Water Framework Directive (WFD; EC, 2000) radically shifted the way that surface water quality was measured, with biological communities (fish, benthic invertebrates, phytobenthos, and macrophytes) being favored over the more limited aspects of the physicochemical parameters (Hering *et al.*, 2010). For this reason, the anthropogenic concept of water as a resource was abandoned, in favor of a broader ecological view, where water is reflected as the ecosystems' support.

In the European Union, the WFD requires that all water bodies reach a "Good" ecological status by 2027, considering the four biological quality elements (BQEs) (benthic invertebrates, fish, macrophytes, and phytobenthos) as the central assessment component, supported by both physicochemical and hydromorphological parameters. Even though there have been some regional successes in improving water quality, there is no data suggesting an overall improvement on a global scale (UN, 2012).

Benthic diatoms (Bacillariophyceae Dangeard, 1933) are considered valuable indicators of environmental conditions in river and stream ecosystems, given the fact that they respond directly and sensitively to many physical, chemical, and biological changes. Moreover, these eukaryotic algae constitute a fundamental link between primary (autotrophic) and secondary (heterotrophic) production, and form a vital component of aquatic ecosystems (Tan *et al.*, 2017). Nowadays, diatoms are extensively used to assess ecological conditions in streams and rivers around the world (e.g., Juggins *et al.*, 2016; Larras *et al.*, 2017; Tan *et al.*, 2017), due to their high abundance and taxonomic diversity, ubiquitous distribution, simple and inexpensive sampling, and fast and predictable responses to environmental disturbances through changes in their composition and abundance (Tan *et al.*, 2017). However, benthic diatoms are less sensitive to hydromorphological pressures, with researchers resorting to other biological elements, such as benthic invertebrates (Juggin *et al.*, 2016). Indeed, invertebrates interact locally with their surrounding environment since their distribution, occurrence, and abundance are highly dependent on the predominant environmental characteristics, thus reflecting disturbances at the ecosystem level. As in most natural aquatic ecosystems, there is a rich diversity of benthic invertebrates that provide a range of structural and functional measures or metrics (e.g., tolerance to organic pollution, habitat preferences, and composition/richness/equitability measures) used to evaluate the rivers' ecological status (Lainé *et al.*, 2014).

Therefore, the authors in this work investigated which main environmental pressures influenced both benthic invertebrate and diatom communities of Sousa River (Porto, Portugal), while still evaluating its ecological status. The most predominantly pressures found in Sousa River are point and non-point sources of pollution from surrounding agricultural areas and small livestock units, domestic and industrial untreated effluents from wastewater treatment plants and channelization.

MATERIALS AND METHODS

Study area and sampling sites

The Sousa River (Porto, Portugal) originates in a small town of the Felgueiras municipality (Porto District, Portugal), at an elevation above 400 m and belongs to the Douro River's watershed. The river, which flows through open valleys associated with various igneous rocks (e.g., granites, granodiorites and schists) and intrenched meander valleys related to the presence of quartzitic rocks (e.g., quartzites and conglomerates), has an overall length of 95 km and a watershed area of 557 km^2.

Concerning land use, in the region of the Sousa River's watershed, monocultures of eucalyptus (15.85 %), temporary irrigated crops (12.31 %) and discontinuous urban tissue (10.10 %) predominate while native forests, constituted by *Quercus* sp., only correspond to 0.044 %, almost the same area covered by invasive forests (0.043 %).

The watershed of the Sousa River is classified in the river type "N1 > 100 km^2", within the Northern Portugal Rivers of Medium-Large Dimension (watershed area: > 100 km^2). This type of rivers reflects the climate of the Northern part of the country with high annual mean precipitation (1196.35 ± 347.30 mm) and low annual mean temperatures (12.62 ± 1.23 °C).

The four sampling sites were chosen in an up-downstream gradient near anthropic pressures, such as, industrial and agricultural areas and urban centers (Fig. 1). The first site (SR1) was located close to the river's spring, in Felgueiras (41° 20' 10.1" N, 8° 12' 15.7" W); the second site (SR2) was set further downstream (41° 11' 29.5" N, 8° 20' 06.0" W); the third sampling point (SR3) was located under the NATURA 2000 Network at the "Valongo" site (41° 07' 43.1" N, 8° 26' 01.7" W) and the last one was located very close to the mouth of Sousa River (41° 05' 54.7" N, 8° 29' 38.5" W).

Physicochemical and hydromorphological parameters

Water physicochemical parameters were monitored in situ during biological samplings. Conductivity (Cond; µS/cm), dissolved oxygen (DO; mg/L), pH, total dissolved solids, (TDS; mg/L) and water temperature (Temp; °C) were measured

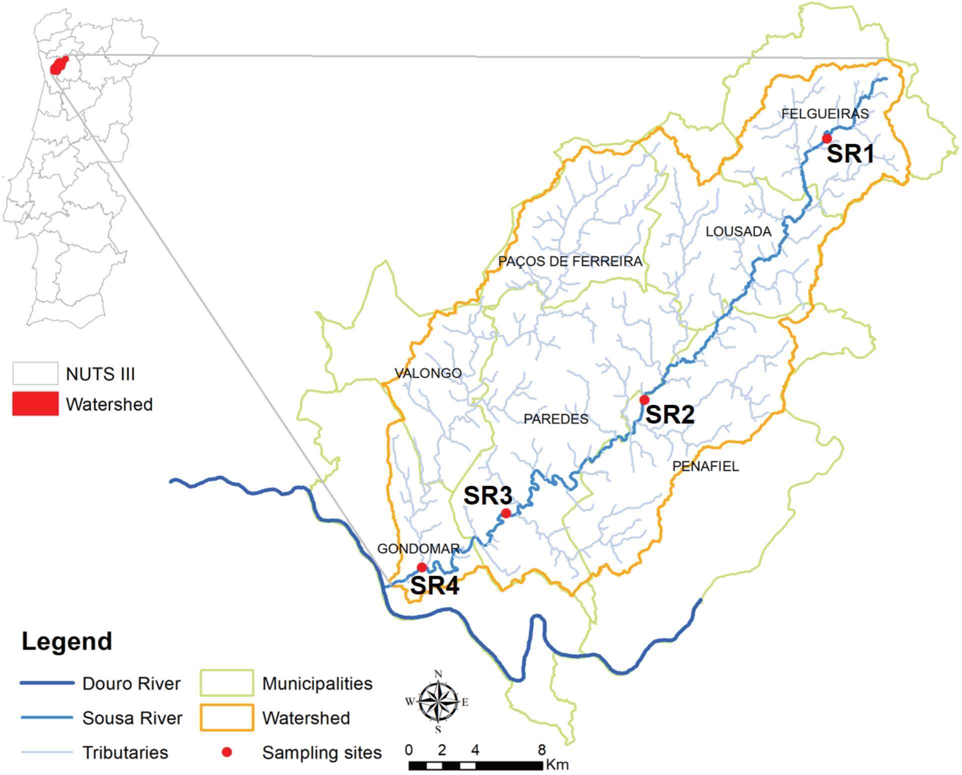


Figure 1. Location of each sampling site within Sousa River's watershed (Porto District, Portugal). *Localização de cada ponto de amostragem na bacia hidrográfica do Rio Sousa. (Porto, Portugal).*

at all sampling sites using a Hanna Instruments HI 9829 field probe. Water samples were collected in 500 mL polyethylene bottles and stored at -20 °C for further analysis. The following parameters were determined through a Hanna Instruments C 99&200 multiparametric bench photometer: ammonia (NH_3, mg/L), nitrates (NO_3^-, mg/L), nitrites (NO_2^-, mg/L), phosphates (PO_4^{3-}, mg/L), and chemical oxygen demand (COD, mg/L). Total suspended solids (TSS, mg/L) were analyzed according to APHA (1992). Given that environmental changes are not only a result of contamination impacts but also physical changes, the Visual Habitat Assessment (VHA) index (Barbour *et al.*, 1999), Riparian Forest Quality (QBR) index (Munné *et al.*, 1998), and Channel Quality Score (GQC) (Corte *et al.*, 1999) were used to evaluate the physical habitat's quality. These indices were approached as an explanatory complement of the biological elements evaluated, and not as an element of ecological quality sensu DQA.

Biological sampling

Diatoms and benthic invertebrates were seasonally monitored (Autumn: October 2016; Spring: April 2017; Summer: July 2017), except during the winter, and sampled according to national guidelines for the WFD implementation. Briefly, invertebrates were collected using a hand net (500 µm mesh) in all existing habitats and hydrodynamic conditions and fixed *in situ* with ethanol 97 % (v/v). Samples were analyzed in the laboratory, where they were sorted out, counted and identified up to the family level, except for some groups identified at a higher taxonomic level (i.e., Oligochaeta, Ostracoda, Hydracarina). Phytobenthos were sampled from randomly chosen natural surfaces (predominantly stones) in non-shaded and turbulent flow zones and fixed with Lugol's iodine 33 % (v/v). In brief, permanent slides of diatom frustules, previously digested in H_2O_2 (30 %) and H_2SO_4 (96 %) and mounted with Naphrax (Brunel Microscopes Ltd., UK; RI=1.73) were used in order to count 400 valves up to the species/variety level. Diatom species were identified using a 100× immersion objective (Zeiss Axio Scope A.1 light microscope), according to Krammer and Lange-Bertalot (1985-1991) and Lange-Bertalot (1995-2012).

Indices and taxonomical metrics

For both biological communities and for all sampling dates, richness (S), Simpson's diversity (D1) and equitability (E) indices were calculated (Simpson, 1949). The ecological status was assessed by applying the Northern Portuguese Invertebrate Index (IPtIN; EC, 2013) and the polluosensitivity index (IPS, Coste, 1982) for benthic invertebrates and phytobenthos, respectively. Values were expressed as the Ecological Quality Ratio (EQR) and given a designated quality class (I – excellent to V – bad). In addition, for benthic invertebrates the total number of individuals ($Log_{10}N$), percentage of Ephemeroptera, Plecoptera and Trichoptera (% EPT), % Oligochaeta, % Chironomidae, and the Iberian Biological Monitoring Working Party Index (IBMWP, Alba-Tercedor & Sánchez-Ortega 1988) were also determined. Finally, IPS was calculated using OMNIDIA 7® software version 5.3 (Lecointe *et al.*, 1993).

Data analysis

A Partial Least Squares (PLS) regression was performed in order to evaluate the relationship between physicochemical parameters and biological communities. Additionally, a Cluster Analysis was executed upon the sampling sites to interpret the results (Supplementary data, Table S1 and S2, available at http://www.limnetica.net/en/limnetica). The input matrix was a correlation matrix (which indicates the similarity and closeness between objects) and was converted to distances before the analysis began, i.e., all correlations were transformed as 1-Pearson *r*. Moreover, an Analysis of Variance (ANOVA) was done followed by Duncan's Multiple Range test (DMRT) to measure the specific differences between the pairs of means. For this analysis, all the species/families that did not have at least two occurrences at the same sampling site or in the same season were eliminated. All statistic tests were performed using Statistica® version 13.0, with a $p < 0.05$ when applicable.

RESULTS AND DISCUSSION

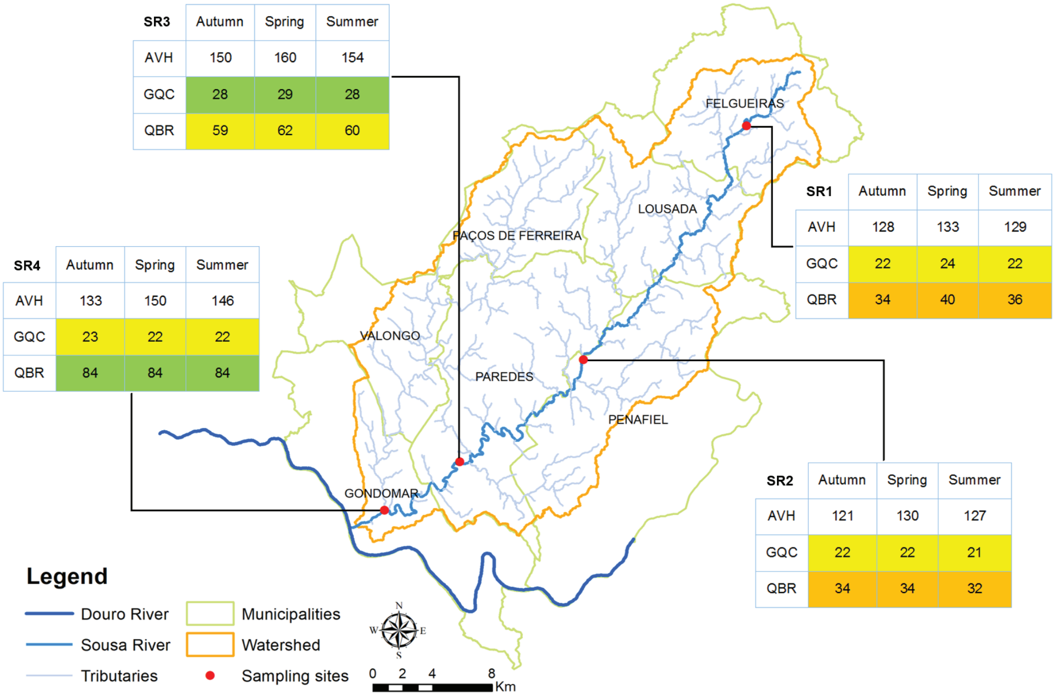
Analysis of environmental data

Conductivity, water temperature, dissolved oxygen, nitrites, phosphates and total dissolved and suspended solids showed considerable seasonal variations, with no evident upstream-downstream patterns overall (Table 1). Moreover, some of these parameters were compared to the maximum thresholds that establish the "good" chemical status in northern Portuguese rivers (Table 1).

In autumn, both SR1 and SR2 presented a "bad" chemical status due to high values of NO_3^- (≥ 25.0 mg/L) and NH_4^+ (≥ 1.0 mg/L), respectively. High concentrations of nutrients can be observed in autumn and winter due to agricultural runoffs caused by rainfall. These contribute significantly to the aquatic ecosystems degradation and are associated with negative effects (e.g., toxins created by harmful algal blooms due to eutrophication and oxygen depletion) on biological communities (Berenzen *et al.*, 2005; Moss, 2008). In summer, only SR2 presented a "bad" chemical status with values superior to 25.0 mg(NO_3^-)/L and 1.0 mg(NH_4^+)/L (Table 1). Although nitrate is an essential nutrient for plant's growth and development, in excess, it can lead to eutrophication and even hydrological problems due to fast-growing macrophytes that slow the current's velocity (EPA 2012). Overall, the sampling locations presented high values of dissolved oxygen, with the highest concentrations being observed in spring. At this time, the COD values registered were the lowest, i.e., low amounts of oxidizable organic material were found in the samples collected in spring, suggesting low disturbances in the dissolved oxygen levels (Table 1). Concerning nutrients, summer exhibited the highest values for PO_4^{3-} (2.51 mg/L), NO_3^- (27.02 mg/L), and NO_2^- (1.25 mg/L). These acute disturbances occur especially in spring and summer when an increased application of fertilizers occurs and river discharge is typically lower.

Table 1. Spatial and temporal variation of the physicochemical parameters analyzed. Note: Temp – water temperature, DO – dissolved oxygen, Cond – Conductivity, TDS – total dissolved solids, COD – chemical oxygen dissolved, PO_4^{3-} – phosphates, NO_3^- – nitrates, NO_2^- – nitrites, NH_4^+ – ammonium, TSS – total suspended solids. GCS corresponds to the maximum threshold for physicochemical parameters to establish a "Good" chemical status in rivers according to the Portuguese National Water Institute. *Variação espácio-temporal dos parâmetros físico-químicos analisados. Nota: Temp – temperatura da água, DO – oxigénio dissolvido, Cond – condutividade, TDS – sólidos totais dissolvidos, COD – carência química de oxigénio,* PO_4^{3-} *- fosfatos,* NO_3*- – nitratos,* NO_2^- *– nitritos,* NH_4^+ *– amónia, TSS – sólidos totais suspenso: GCS corresponde ao limite máximo de um parâmetro físico-químico de forma a estabelecer um "bom" estado ecológico de acordo com o Instituto Nacional da Água.*

Sampling site	Temp (°C)	pH	DO (mg/L)	Cond (µS/cm)	TDS (mg/L)	COD (mg/L)	PO_4^{3-} (mg/L)	NO_3^- (mg/L)	NO_2^- (mg/L)	NH_4^+ (mg/L)	TSS (mg/L)
Autumn											
SR1	15.50	8.08	7.25	161.00	79.00	35.00	0.22	25.30	0.16	0.50	21.00
SR2	12.30	7.16	11.06	157.00	79.00	71.30	1.04	23.50	0.20	1.50	17.20
SR3	12.45	7.20	10.18	160.00	80.00	32.00	0.32	23.90	0.26	0.59	16.20
SR4	12.30	6.36	8.60	156.00	78.00	38.00	0.15	21.30	0.10	0.33	19.00
Spring											
SR1	12.07	6.91	10.34	142.00	76.00	9.33	0.53	17.72	0.16	0.39	10.40
SR2	13.30	8.12	10.27	155.00	77.00	8.33	0.44	18.16	0.10	0.71	5.40
SR3	14.77	7.78	10.59	165.00	83.00	22.33	1.23	19.49	0.07	0.42	10.80
SR4	16.12	8.37	10.30	162.00	81.00	20.66	0.84	19.94	0.03	0.38	8.00
Summer											
SR1	19.62	6.90	8.50	165.00	83.00	31.60	0.44	18.61	0.26	0.48	5.60
SR2	22.26	6.93	7.94	227.00	113.00	35.33	1.29	27.02	1.25	1.02	6.00
SR3	23.90	7.46	8.70	209.00	127.00	46.33	2.51	22.59	0.33	0.38	7.00
SR4	23.84	7.84	8.58	239.00	119.00	49.00	1.59	14.62	0.07	0.27	8.00
GCS	–	6 – 9	≥5.0	–	–	–	–	≤25.0	–	≤1.0	–



SR3	Autumn	Spring	Summer
AVH	150	160	154
GQC	28	29	28
QBR	59	62	60

SR1	Autumn	Spring	Summer
AVH	128	133	129
GQC	22	24	22
QBR	34	40	36

SR4	Autumn	Spring	Summer
AVH	133	150	146
GQC	23	22	22
QBR	84	84	84

SR2	Autumn	Spring	Summer
AVH	121	130	127
GQC	22	22	21
QBR	34	34	32

Figure 2. Representative map of Sousa River's watershed, with the sampling sites and their respective results for the VHA (Visual Habitat Evaluation index), QBR (Riparian Forest Quality), and GQC (Channel Quality Score) indices. Colors represent quality classes. QBR – *green*: good quality; *yellow*: acceptable quality; *orange*: poor quality; GQC – *green*: channel slightly disturbed; *yellow*: channel significantly altered. *Mapa representativo da bacia hidrográfica do Rio Sousa, com os pontos de amostragem e respetivos resultados dos índices VHA, QBR, e GQC. As cores representam classes de qualidade. QBR –* verde*: boa qualidade; amarelo: qualidade aceitável;* laranja*: qualidade medíocre; GQC –* verde*: canal ligeiramente perturbado,* amarelo*: canal significativamente alterado.*

Scores and corresponding classes of the habitat quality indices (VHA, QBR, and GQC) determined at each sampling site within each season are schematically represented in figure 2.

Briefly, SR1 was found to be heavily modified, with both river banks replaced by concrete walls to support roads and small agricultural crops. In fact, different channelization processes (e.g., damming, meander shortening, bank protection, etc.) modify stream's morphology by deepening and widening the channel, with an overall increase in the flow velocity resulting in ecological implications for many aquatic organisms with specific water velocity requirements (Brooker, 1985; Landemaine *et al.*, 2014). Additionally, alterations of the sediment dynamics, due to ever increasing erosion events, lead to the degradation of the physicochemical water quality and river habitats are lost due to meander shortening (Wilcock & Essery, 1991; Landemaine *et al.*, 2014). Moreover, the presence of constant islands throughout the seasons also lead to changes in the channel's depth and current velocity. Further downstream, SR2 exhibited strong erosion signs on the margins due to deforestation of the riparian corridor in order to give place to intensive agricultural practices and small roads. Riparian corridors represent transition zones, between terrestrial and aquatic ecosystems, of extreme importance for biodiversity and ecosystem services (Sousa *et al.*, 2017). The inexistence of a stable riparian ecosystem can lead to an increase in the water's temperature, consequently, diminishing the concentration of dissolved oxygen (Whitehead *et al.*, 2009). In contrast, SR3's margins were largely occupied by forest and shrubs, although, dominated by exotic species (e.g., *Eucalyptus* sp.). The presence of eucalyptus' monocultures can dry and impoverished soils, leading to a strong reduction in the watercourses flow and, therefore, biodiversity. There are studies that demonstrate that the current changes in land use from native to exotic riparian vegetation can affect fish (Correa-Araneda *et al.*, 2015; Ferreira *et al.*, 2015, Fierro *et al.*, 2016) and macroinvertebrate communities (Abelho & Graça, 1996; Correa-Araneda *et al.*, 2015; Fierro *et al.*, 2016). Finally, SR4 presented only small signs of margin erosion with its riparian forest being mainly constituted by autochthonous species (e.g., *Quercus* spp. and *Salix* sp.). Here, in the driest seasons, an island emerges, altering the channel's depth and watercourse.

Taxonomical metrics and indices

Benthic diatoms

Throughout the study, high D1 values were registered (0.77 to 0.93; Table 2), suggesting the presence of rich diatom communities. Nevertheless, its distribution was not uniform since, in general, low values of Simpson's Evenness were verified (0.16 to 0.52; Table 2). Some researchers have found that diversity can decrease (Rott & Pfister, 1988; Sonneman *et al.*, 2001) or increase (Stevenson *et al.*, 2008; Van Dam, 1982) with pollution, and that it changes differently depending on the type of pollution (Juttner *et al.*, 1996). Patrick (1973) hypothesized that diversity assessments of pollution are ambiguous when composite indices are used since different contaminants have different effects on species richness and evenness. Moreover, some pollutants (e.g., organic pollution) can differentially stimulate some species growth and therefore, decrease evenness (Patrick, 1973). More recently Stevenson *et al.* (2008) constrained diversity to just the sensitive taxa found in reference sites. The values obtained from the IPS (Table 2) at all sampling sites, throughout the seasons, corresponded to a moderate contamination (class III). As observed, the SR4 sampling site (IPS = 3.7, out of 5.0) presented the highest value, in autumn, and SR4 (IPS = 2.91) and SR3 (IPS = 2.85, out of 5.0) the lowest in spring and summer, respectively (Table 2). According to Van Dam's *et al.* (1994) detailed checklist of freshwater diatoms and respective ecological indicator values, these last two sampling sites were dominated by nitrogen-tolerant autotrophic and facultative heterotrophic taxa (such as, *Achnanthidium*

Table 2. Spatial and temporal variation of the diatom communities' diversity and evenness indices, and the polluosensitivity index (IPS) with its respective quality classes. Note: D_1 – Simpson's diversity; E – Simpson's evenness; III – moderate quality. *Variação espácio-temporal dos índices de diversidade e equitabilidade da comunidade de diatomáceas e do índice de poluossensibilidade (IPS) com as respetivas classes de qualidade. Nota: D_1 – Diversidade de Simpson; E – Equitabilidade de Simpson; III – qualidade média.*

Sampling site	D_1	E	IPS	Quality class
Autumn				
SR1	0.87	0.28	3.57	III
SR2	0.88	0.29	3.27	III
SR3	0.81	0.19	3.26	III
SR4	0.77	0.16	3.7	III
Spring				
SR1	0.93	0.42	3.32	III
SR2	0.87	0.28	3.1	III
SR3	0.92	0.40	3.31	III
SR4	0.93	0.52	2.91	III
Summer				
SR1	0.88	0.26	3.62	III
SR2	0.93	0.51	3.1	III
SR3	0.89	0.29	2.85	III
SR4	0.88	0.34	3.56	III

Table 3. Spatial and temporal variation of various taxonomic metrics based on benthic invertebrates communities. Note: EPT – Ephemeroptera, Plecoptera, Trichoptera; D_1 – Simpson's diversity; E – Simpson's evenness. *Variação espácio-temporal de várias métricas taxonómicas com base nas comunidades de invertebrados bentônicos. Nota: EPT – Ephemeroptera, Plecoptera, Trichoptera; D_1 – Diversidade de Simpson; E – Equitabilidade de Simpson.*

Sampling site	**Chironomidae (%)**	**Oligochaeta (%)**	**EPT (%)**	**D_1**	**E**
Autumn					
SR1	30.81	1.17	35.82	0.78	0.20
SR2	57.12	1.16	22.59	0.63	0.15
SR3	15.74	11.11	56.48	0.86	0.49
SR4	44.70	3.79	40.15	0.74	0.42
Spring					
SR1	26.07	6.62	17.71	0.81	0.22
SR2	38.34	0.27	57.23	0.72	0.26
SR3	59.03	0.39	28.89	0.58	0.14
SR4	31.59	0.00	42.22	0.79	0.29
Summer					
SR1	20.81	3.35	6.06	0.72	0.21
SR2	30.33	0.55	34.15	0.76	0.33
SR3	36.36	5.59	38.46	0.76	0.38
SR4	23.53	3.92	47.06	0.79	0.43

minutissimum or *Planothidium lanceolatum*, and *Nitzschia amphibia* or *Gomphonema parvulum*, respectively) which favor moderate levels of oxygen. These species need, periodically, elevated concentrations of organically bound nitrogen, thus favoring waters with these conditions. In summer, the SR3 site also registered high values of PO_4^{3-}, which probably led to an increase in the number of cells of these tolerant species (Finenko, & Krupatkina-Akinina, 1974).

Benthic invertebrates

Taxonomic metrics based on benthic invertebrates are described in Table 3. The percentage (%) of Chironomidae was mainly influenced by season (cold *vs.* warm waters), more than by the up- to downstream gradient, except for SR3. Overall, the biomass decreased constantly between seasons. On the other hand, no evident pattern was associated with the % Oligochaeta. Concerning the % EPT and Simpson's diversity and evenness, an increase occurred in its values from up- to downstream, in summer, which could be attributed to a better overall habitat quality or an increase of the riparian corridor quality (see AVH and QBR values above). Composition measures of sensitive *taxa*, (e.g., % EPT) are often used to provide information about streams' conditions. Ephemeroptera, Plecoptera and Trichoptera orders are particularly sensitive to organic pollution within the ecosystem and can, therefore, be used to identify locally impacted regions (Herman & Nejadashemi, 2015). However, the % EPT was mainly composed of families tolerant to organic pollution (e.g., Baetidae, Caenidae, and Hydropsychidae) (Jesus, 2001; Moog *et al.*, 1997; Tachet *et al.*, 2003), with low abundances of sensitive *taxa* (e.g., Plecoptera families) being found. The diversity and evenness indices showed similar results to those found in the diatom communities, with high values of diversity and low values of equitability.

In order to have a fast and clear idea of the water's biological quality, a representative scheme of Sousa River's watershed, with the results of the biotic (IBMWP) and the multimetric (IPtIN) indices, was prepared with the corresponding colors of the biological quality classes (Fig. 3). In general, IBMWP scores decreased from up- to downstream sites, with only SR1 being classified as "slightly contaminated" in all

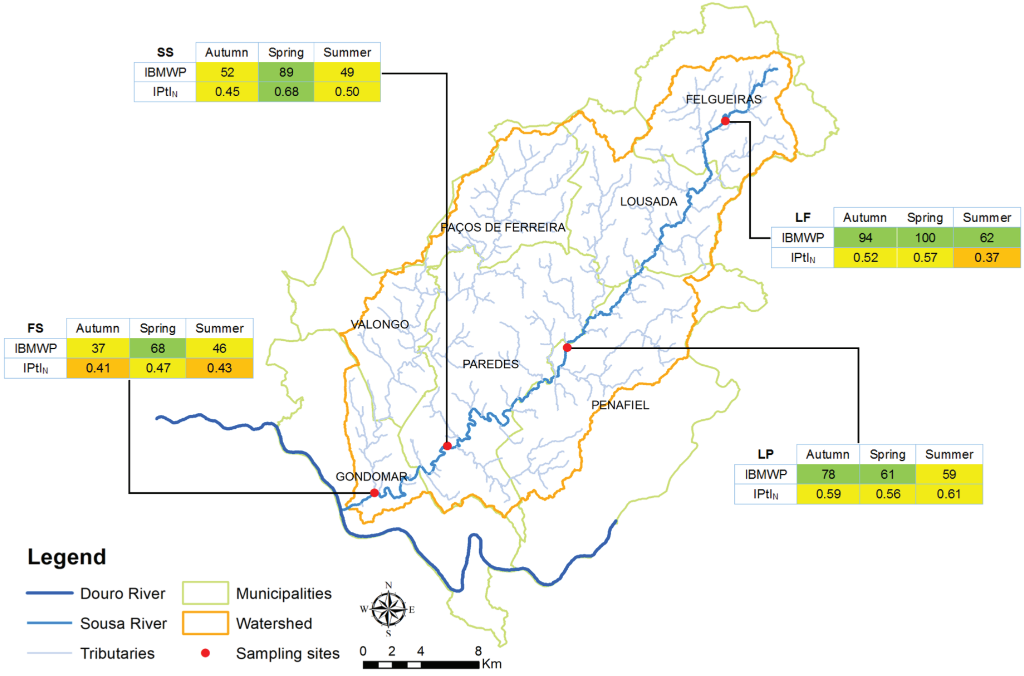


Figure 3. Representative map of Sousa River's watershed, with the sampling sites and their respective results for the IBMWP (Iberian Biological Monitoring Working Party), and IPtIN (Northern Portuguese Invertebrate Index) indices. Colors represent quality classes. IBMWP – *green*: water slightly contaminated; *yellow*: water moderately contaminated; IPtI$_N$ – *green*: good quality; *yellow*: moderate quality; *orange*: poor quality. *Mapa representativo da bacia hidrográfica do Rio Sousa, com os pontos de amostragem e respetivos resultados dos índices IBMWP e IPtI$_N$. As cores representam classes de qualidade. IBMWP –* verde*: boa qualidade;* amarelo*: qualidade aceitável;* laranja*: qualidade medíocre; GQC –* verde*: canal ligeiramente perturbado,* amarelo*: canal significativamente alterado.*

seasons (highest IBMWP score = 100). Nonetheless, low IPtI$_N$ scores classified SR1 in a "medium" to "poor" ecological status (Fig. 3). The disappearance of sensitive *taxa* (e.g., Odonata and Ephemeroptera) together with low values of evenness and diversity lead to this decrease. The remaining sampling sites were classified from "poor" to "good" ecological status according to IPtI$_N$. Overall, the values obtained from the IPtI$_N$ index followed a spatial-temporal variation pattern very similar to IBMWP since this biotic index is part of the metrics that integrate IPtI$_N$.

Interactions between physicochemical parameters and biological communities

Benthic diatoms

Throughout the study, 49 species belonging to 29 genera were identified from all four sampling sites located in Sousa River's watershed. *Navicula*, *Nitzschia* and *Pinnularia* were the genera most frequently represented, by 5 taxa each. However, for the PLS regression only 37 diatom species and eleven physicochemical parameters were considered. The first three axes accounted for 48.9 % of the cumulative variance in the diatom-physicochemical data (eigenvalues: $\lambda_1 = 8.44$, $\lambda_2 = 4.76$, and $\lambda 3 = 7.33$).

The first two factors explained 20.1 % and 11.3 % of the diatom-physicochemical data variation, respectively, as some temporal patterns were revealed (Fig. 4A). According to the bi-plots, autumn was characterized by high values of sensitive species (e.g., *Psammothidium subatomoides* (Cluster 8), *Eunotia pectinalis* (Cluster 13), *Navicula hungarica* (Cluster 14) and *Cocconeis placentula* (Cluster 15)) (Coste, 1982; Kelly *et al.*, 2005; Krammer & Lange-Bertalot, 1985-91), together with low values of tolerant taxa (e.g., *Luticola mutica* (Cluster 18) and *Nitzschia palea* (Cluster 22)). Moreover, autumn presented high values of TSS and low values of pH (Fig. 4C), explained mainly by the occurrence of soil run-offs due to casual events of precipitation. However, the inexistence of a riparian corridor could be directly affecting this phenomenon since it can act as a barrier to the entry of organic matter into the watercourse. Further, spring showed the widest distribution, with the most upstream sites (SR1 and SR2) presenting high values of *Craticula halophila* (Cluster 2), *Navicula phyllepta* (Cluster 7) and *Nitzschia pusilla* (Cluster 14) while the remaining sites exhibited high values of *Planothidium lanceolatum* (Cluster 21), *Achnanthidium affine* and *Nitzschia palea* (Cluster 22) (Coste, 1982; Kelly *et al.*, 2005; Krammer & Lange-Bertalot, 1985-91). Spring also showed high values of DO and low values of nitrogen compounds (e.g., nitrates and nitrites) as well as COD. Although low levels of nutrients were in fact registered, spring was dominated by nitrogen-tolerant autotrophic *taxa*. In summer, high values of sensitive species (e.g., Pinnulariaceae species (Cluster 17) and *Achnanthidium delicatulum* (Cluster 16)) were associated with *Nitzschia amphibia* (Cluster 19) and *Gomphonema parvulum* (Cluster 20), together with low values of *Craticula halophila* (Cluster 2) and *Psammothidium subatomoides* (Cluster 8) (Coste, 1982; Kelly *et al.*, 2005; Krammer & Lange-Bertalot, 1985-91). Summer clearly exhibited high values of conductivity, PO_4^{3-}, temperature and TDS, mainly due to the low flow rate (i.e., decreased dilution factor) and high air temperatures, commonly found in this season (Fig. 4C).

The first and third dimensions (Fig. 5) explained 20.1 % and 17.5 % of the diatom-physicochemical data variation, respectively, allowing to differentiate the SR1 sampling site from the

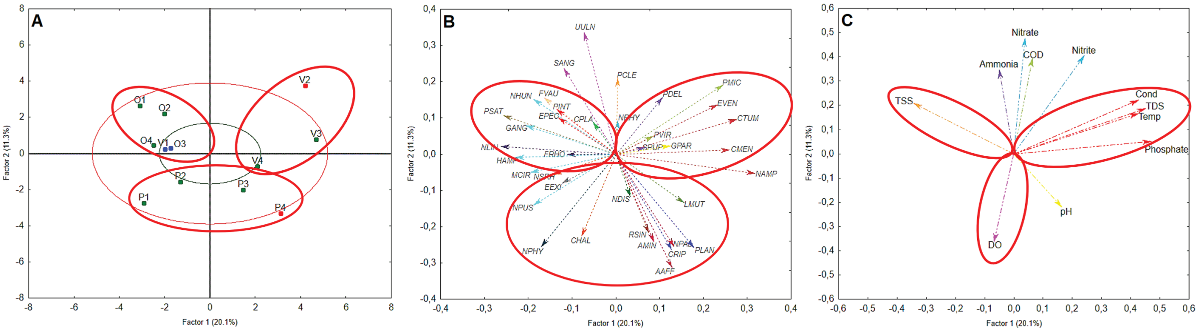


Figure 4. (A) Partial Least Squares (PLS) bi-plot (F1 *vs.* F2) of all the sampling sites throughout all the seasons. Upper limits of confidence intervals: *green*: 25 %; *red*: 75 %; Seasons: O – autumn; P – spring; V – summer; Sampling sites: 1 – SR1; 2 – SR2; 3 – SR3; 4 – SR4; (B) PLS bi-plot showing the distribution of diatom species; (C) PLS bi-plot showing the physiochemical parameters distribution. TSS – Total Suspended Soils; COD – Chemical Oxygen Demand; Cond – Conductivity; TDS – Total Dissolved Solids; Temp – Temperature; DO – Dissolved Oxygen. *(A) Bi-plot da Regressão Parcial de Mínimos Quadrados (PLS) (F1* vs. *F2) de todos os pontos de amostragem ao longo de todas as estações do ano. Limites superiores dos intervalos de confiança:* verde*: 25 %;* vermelho*: 75 %; Estações do ano: O – outono; P – primavera; V – verão; Pontos de amostragem: 1 – SR1; 2 – SR2; 3 – SR3; 4 – SR4; (B) Bi-plot da PLS a demonstrar a distribuição das espécies de diatomáceas; (C) Bi-plot da PLS a demonstrar a distribuição dos parâmetros físico-químicos. TSS – Sólidos suspensos totais; COD – carência química de oxigénio; Cond – condutividade; TDS – sólidos dissolvidos totais; Temp – temperatura da água; DO – oxigénio dissolvido.* AAFF – *Achnanthidium affine*; AMIN – *Achnanthidium minutissimum*; CHAL – *Craticula halophila*; CMEN – *Cyclotella meneghiniana*; CPLA – *Cocconeis placentula*; CRIP – *Craticula riparia*; CTUM – *Cymbella tumida*; EEXI – *Eunotia exigua*; EPEC – *Eunotia pectinalis*; EVEN – *Encyonema ventricosum*; FRHO – *Frustulia rhomboides*; FVAU – *Fragilaria vaucheriae*; GANG – *Gomphonema angustatum*; GPAR – *Gomphonema parvulum*; HAMP – *Hantzschia amphioxys*; LMUT – *Luticola mutica*; MCIR – *Meridion circulare*; NAMP – *Nitzschia amphibia*; NDIS – *Nitzschia dissipata*; NHUN – *Navicula hungarica*; NLIN – *Nitzschia linearis*; NPAL – *Nitzschia palea*; NPHY – *Navicula phyllepta*; NPUS – *Navicula pusilla*; NRHY – *Navicula rhyncocephala*; NSRH – *Navicula subrhyncocephala*; PCLE – *Placoneis clementis*; PDEL – *Planothidium delicatulum*; PINT – *Pinnularia interrupta*; PLAN – *Planothidium lanceolatum*; PMIC – *Pinnularia microstauron*; PSAT – *Psammothidium subatomoides*; PVIR – *Pinnularia viridis*; RSIN – *Reimeria sinuata*; SANG – *Surirela angusta*; SPUP – *Sellaphora pupula*; UULN – *Ulnaria ulna*.

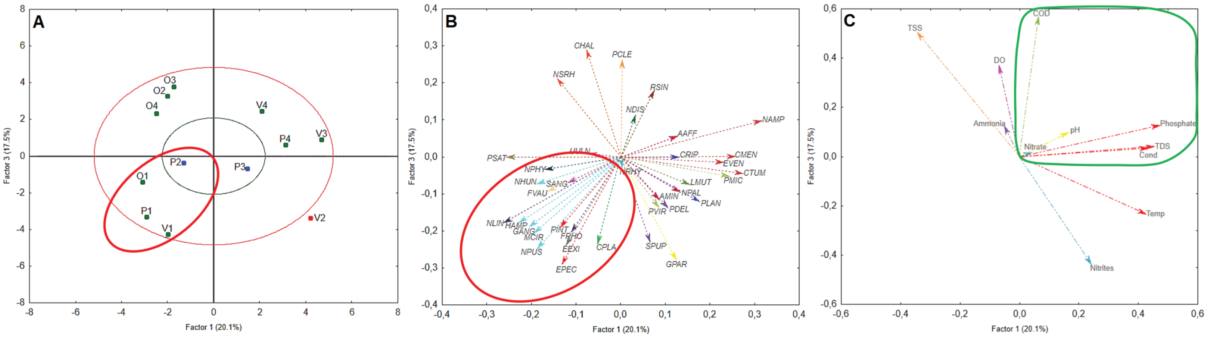


Figure 5. (A) Partial Least Squares (PLS) bi-plot (F1 *vs.* F3) of all the sampling sites throughout all the seasons; (B) PLS bi-plot showing the distribution of diatom species; (C) PLS bi-plot showing the physiochemical parameters distribution. All codes are described in figure 4. *(A) Bi-plot da Regressão Parcial de Mínimos Quadrados (PLS) (F1* vs. *F3) de todos os pontos de amostragem ao longo de todas as estações do ano; (B) Bi-plot da PLS a demonstrar a distribuição das espécies de diatomáceas; (C) Bi-plot da PLS a demonstrar a distribuição dos parâmetros físico-químicos. Todos os códigos estão descritos na figura 4.*

remaining. According to the bi-plots of figure 5C, SR1 presented low values of COD, pH, nutrients (nitrate and phosphate), conductivity and TDS. This low level of pollution is associated with significantly ($p < 0.05$) higher values of sensititve species (e.g., *Psammothidium subatomoides* (Cluster 8), *Eunotia pectinalis* (Cluster 13)), and low values of tolerant species such as, *Nitzschia amphibia* and *N. dissipata*. *Gomphonema angustatum* and *N. pusilla* (Cluster 14) also presented significantly ($p < 0.05$) higher values, when compared to the remaining sampling sites. This data shows the sensitive response that the peryphitic diatom community exhibits to nutrient enrichment, already demonstrated by other studies (Hering *et al.*, 2006, Berthon *et al.*, 2011).

Benthic invertebrates

Throughout the study, 43 families belonging to 12 taxonomic groups were identified from all four sampling sites located in Sousa River's watershed. However, for the PLS regression only 31 families and 11 physicochemical parameters were considered. The first four axes accounted for 59.1 % of the cumulative variance in the benthic invertebrate-physicochemical data. (Eigenvalues: $\lambda_1 = 8.87$, $\lambda_2 = 5.30$, $\lambda_3 = 5.24$, and $\lambda_4 = 4.85$).

The third and fourth factors explained 12.8 % and 11.8 % of the invertebrate-physicochemical data variation, respectively, (Fig. 6A and 6B), allowing the overall characterization of autumn. Although high values of individuals of tolerant families (e.g., Caenidae (Cluster 9) and Asellidae (Cluster 7)) were found, sensitive families such as, Gomphidae (Cluster 3) and Rhyacophilidae (Cluster 12) also presented high values (Alba-Tercedor & Sánchez-Ortega, 1988). On the other hand, low values of epineuston organism (e.g., Gerridae and Hydrometridae (Cluster 6)) and macrophyte-living organisms, like Physidae and Planorbidae (Cluster 5), were registered (Tachet *et al.*, 2003). The presence of a high current velocity together with the lack of shelter-like substrates could explain the low values of organisms that live at the water surface. Moreover, the low abundance, or even absence, of macrophytes and algae, in autumn, could explain the low values exhibited by families that depend of them as habitats and sources of nourishment (Tachet *et al.*, 2003). In autumn, high values of TSS, COD and NO_3^- and low values of PO_4^{3-}, temperature, conductivity, TDS and NO_2^- modulated invertebrates' diversity and biomass (Fig. 6A and 6C).

The second and third dimensions explained 12.9 % and 12.8 % of the macroinvertebrate-physicochemical data variation, respectively, allowing to characterize spring, with the exception of the SR1 sampling site (Fig. 6D and 6E). In spring, an increase in macrophytes devel-

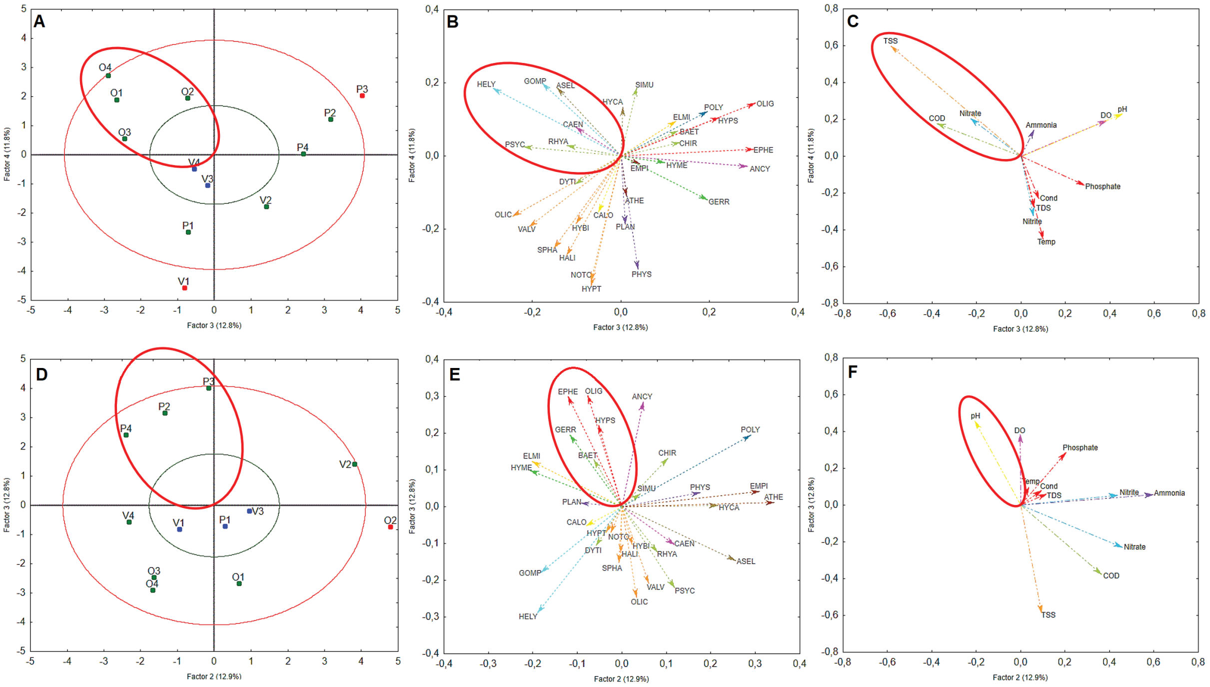


Figure 6. (A) Partial Least Squares (PLS) bi-plot (F3 *vs*. F4) of all the sampling sites throughout all the seasons. (B) PLS bi-plot (F3 *vs*. F4) showing the distribution of benthic invertebrate taxonomic groups: (C) PLS bi-plot (F3 *vs*. F4) showing the physiochemical parameters distribution; (D) Partial Least Squares (PLS) bi-plot (F2 *vs*. F3) of all the sampling sites throughout all the seasons; (E) PLS bi-plot (F2 *vs*. F3) showing the distribution of benthic invertebrate taxonomic groups; (F) PLS bi-plot (F2 *vs*. F3) showing the physiochemical parameters distribution. Upper limits of confidence intervals: *green*: 25 %; *red*: 75 %; Seasons: O – autumn; P – spring; V – summer; Sampling sites: 1 – SR1; 2 – SR2; 3 – SR3; 4 – SR4. TSS – Total Suspended Soils; COD – Chemical Oxygen Demand; Cond – Conductivity; TDS – Total Dissolved Solids; Temp – Temperature; DO – Dissolved Oxygen. *(A) Bi-plots da Regressão Parcial de Mínimos Quadrados (PLS) (F3* vs. *F4) de todos os pontos de amostragem ao longo de todas as estações do ano; (B) Bi-plots da PLS (F3* vs. *F4) a demonstrar a distribuição dos grupos taxonómicos de invertebrados bentônicos; (C) Bi-plots da PLS (F3* vs. *F4) a demonstrar a distribuição dos parâmetros físico-químicos. (D) Bi-plots da Regressão Parcial de Mínimos Quadrados (PLS) (F2* vs. *F3) de todos os pontos de amostragem ao longo de todas as estações do ano; (E) Bi-plots da PLS (F2* vs. *F3) a demonstrar a distribuição dos grupos taxonómicos de invertebrados bentônicos; (F) Bi-plots da PLS (F2* vs. *F3) a demonstrar a distribuição dos parâmetros físico-químicos. Limites superiores dos intervalos de confiança:* verde*: 25 %;* vermelho*: 75 %; Estações do ano: O – outono; P – primavera; V – verão; Pontos de amostragem: 1 – SR1; 2 – SR2; 3 – SR3; 4 – SR4. TSS – Sólidos suspensos totais; COD – carência química de oxigénio; Cond – condutividade; TDS – sólidos dissolvidos totais; Temp – temperatura da água; DO – oxigénio dissolvido.* ANCY – Ancyldiae; ASEL – Asellidae; ATHE – Athericidae; BAET – Baetidae; CAEN – Caenidae; CALO – Calopterygidae; CHIR – Chironomidae; DITY – Dytiscidae; ELMI – Elmidae; EMPI – Empididae; EPHE – Ephemereliidae; GERR – Gerridae; GOMP – Gomphidae; HALI – Haliplidae; HELY – Helycopsychidae; HYBI – Hydrobiidae; HYCA – Hydracarina; HYME – Hydrometridae; HYPS – Hydropsychidae; HYPT – Hydroptlidae; NOTO – Notonectidae; OLIC – Oligochaeta; OLIG – Oligoneuriidae; PHYS – Physidae; PLAN – Planorbidae; POLY – Polycentropodidae; PSYC – Psychodidae; RHYA – Rhyacophilidae; SIMU – Simuliidae; SPHA – Sphaeridae; VALV – Valvatidae.

opment led to a decrease in the ability of inorganic substrate to provide habitat for other species. Therefore, it was observed an increase of Ephemeropteran individuals such as, Ephemerellidae and Oligoneuriidae (Cluster 13), which rely on macrophytes to survive (Tachet *et al.*, 2003) and, on the other hand, a decrease in Rhyacophilidae (Cluster 12) and Valvatidae (Cluster 10) species. According to the bi-plots of figures 6D and 6F, spring exhibited high values of pH and DO and low values of NO_3^-, COD and TSS.

The first and fourth dimensions explained 21.6 % and 11.8 % of the benthic invertebrate-physicochemical data variation, respectively, allowing to characterize summer (Fig. 7A and 7B). Summer showed high values of organic pollution tolerant families (Alba-Tercedor & Sánchez-Ortega, 1988). The high values of planorbids and physids (Cluster 5) are due to an even greater increase of macrophytes while the presence of Gerridae and Hydrometridae (Cluster 6) could be explained by the decrease of the current velocity. This decrease could also explain the low values of Simuliidae (Cluster 12) and Hydropsychidae (Cluster 13), in this season, since these invertebrates are well adapted to high current velocities and, therefore, favor well oxygenated waters (Tachet *et al.*, 2003). According to the bi-plots of figures 7A and 7C, summer exhibited high values of NO_2^-, PO_4^{3-}, water temperature, TDS and conductivity and low values of TSS and DO.

The first and third factors explained 21.6 % and 12.8 % of the macroinvertebrate-physico-chemical data variation, respectively, allowing to characterize the sampling site SR1 (Fig. 7D and 7E). According to the bi-plots of figures 7D and 7F, SR1 exhibited low values of NH_4^+, NO_2^-, PO_4^{3-}, water temperature, TDS and conductivity, which modulated the invertebrates' diversity and biomass. Once more, the low level of pollution present in SR1 was associated with high values of sensitive families, such as Calopterygidae (Cluster 11) and Rhyacophilidae (Cluster 12) (Alba-Tercedor & Sánchez-Ortega, 1988), in comparison with other sampling sites. Also, Dytiscidae (Cluster 12) and Haliplidae (Cluster 10) presented high values; these Coleoptera favor waters abundant in macrophytes as they used them as habitat and a source of food (Tachet *et al.*, 2003). Benthic inverte-

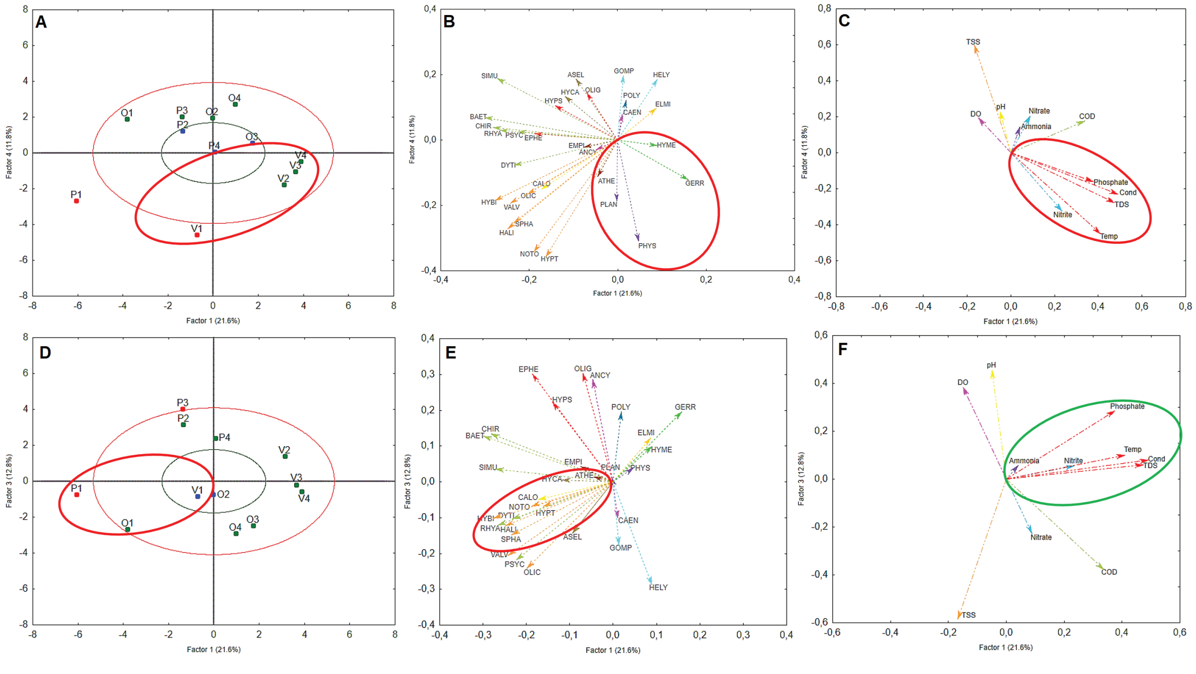


Figure 7. (A) Partial Least Squares (PLS) bi-plot (F1 *vs*. F4) of all the sampling sites throughout all the seasons. (B) PLS bi-plot (F1 *vs*. F4) showing the distribution of benthic invertebrate taxonomic groups: (C) PLS bi-plot (F1 *vs*. F4) showing the physiochemical parameters distribution; (D) Partial Least Squares (PLS) bi-plot (F1 *vs*. F3) of all the sampling sites throughout all the seasons; (E) PLS bi-plot (F1 *vs*. F3) showing the distribution of benthic invertebrate taxonomic groups; (F) PLS bi-plot (F1 *vs*. F3) showing the physiochemical parameters distribution. All codes are described in figure 6. *(A) Bi-plots da Regressão Parcial de Mínimos Quadrados (PLS) (F1* vs. *F4) de todos os pontos de amostragem ao longo de todas as estações do ano; (B) Bi-plots da PLS (F1* vs. *F4) a demonstrar a distribuição dos grupos taxonómicos de invertebrados bentônicos; (C) Bi-plots da PLS (F1* vs. *F4) a demonstrar a distribuição dos parâmetros físico-químicos. (D) Bi-plots da Regressão Parcial de Mínimos Quadrados (PLS) (F1* vs. *F3) de todos os pontos de amostragem ao longo de todas as estações do ano; (E) Bi-plots da PLS (F1* vs. *F3) a demonstrar a distribuição dos grupos taxonómicos de invertebrados bentônicos; (F) Bi-plots da PLS (F1* vs. *F3) a demonstrar a distribuição dos parâmetros físico-químicos. Todos os códigos estão descritos na figura 6.*

brates, similarly to diatoms, responded sensitively to organic pollution, although they were more associated with low values of other nitrate ions (NH_4^+ and NO_2^-).

According to the WFD's Annex V (EC, 2000), a "one-out, all-out" (OOAO) approach is generally applied when two or more BQEs are integrated into a biomonitoring assessment program. This rule means that the overall ecological status of a waterbody is determined by the lowest score obtained by any of the BQEs, and the supporting physicochemical and hydromorphological quality elements. Theoretically, the "OOAO" approach is most suitable when different stressors are responsible for the degradation of each BQEs. However, both BQEs responded sensitively to the same stressors in this study, possibly leading to serious uncertainties associated with the individual BQEs assessments (Caroni *et al.*, 2013). Moreover, its implementation was found to induce Type I errors, eventually classifying a water body as being below the "Good" ecological status, even if it was not (Borja & Rodriguez, 2010; Moe *et al.*, 2015). Several authors have discussed new alternative approaches to the "OOAO" principle, pointing out the need for more: (i) integrative methods (Borja & Rodriguez, 2010; Voulvoulis *et al.*, 2017); (ii) emphasis in reporting progress on individual BQEs; and (iii) pressure-specific weight of evidence approaches (Carvalho *et al.*, 2019).

CONCLUSION

In conclusion, the present study revealed that assessing through a combination of different biological indicators can be much more informative than physicochemical parameters and biological indices from only one aquatic community. Results showed that Sousa River was throughout all year in a "bad" to "medium" ecological status, with benthic invertebrates being more descriptive of the ecological conditions than the diatom communities. However, both biological communities responded similiarly to the same disturbances, which could be a problem in the light of the "one-out, all-out" principle, leading to over-precautionary results with more sites failing to achieve the "Good" status than should. Thus, the authors suggest that more studies applying an integrative approach are essential.

Additionally, the river habitat evaluation exposed profound transformations at the SR1 and SR2 sites. SR3 was the only site presenting a good channel quality, although transformations in its riparian zone were clear, debilitated by deforestation and the presence of exotic species. Indeed, a further step is necessary to continue biomonitoring programs throughout the years and even after restoration programs are finished in order to cope with the objectives proposed by the Water Framework Directive until 2027.

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