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# **Diatom communities and ecological status classification in the upper Po River basin**

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## **ABSTRACT**

One of the main challenges in river management is the setting of nutrient thresholds that support good ecological status, which is the main objective to achieve for the European member states. This is a complex process, which needs an accurate analysis of the data collected so far for the ecological classification of rivers belonging to different typologies. We analysed the data of the multiannual monitoring concerning diatoms and nutrients in the upper Po River (NW Italy) with the aim of exploring the response of diatom community in terms of species composition, ecological guilds and indices. We considered data of 390 samples, of which 2/3 belonging to the “Central macrotype” (i.e. lowland stretches) and 1/3 to “Alpine siliceous”. We performed a Principal Coordinate Analysis to detect community patterns with respect to water chemical classification and macrotypes highlighting species and ecological guilds characteristic of samples along a water quality gradient. We then performed a partial RDA to focus on the role of environmental and spatial factors in shaping the

diatom community in each of the two macrotypes. Finally, we investigated the concordance between the Italian normative indices ICMi (for diatoms) and LIMECO (a chemical index of water quality). We found significant differences in the diatom communities of the two macrotypes and in their response to water quality and to spatial factors. Communities resulted as much more uniform in sites with a low water quality, with characteristic species such as *Navicula gregaria*, *Nitzschia palea* and *Sellaphora nigri*. On the other hands, moderately disturbed sites (in terms of trophic level) were characterised by the highest guild diversity. The RDA confirmed the importance of spatial factors in shaping the diatom assemblages, especially in Alpine streams where the physical barriers may condition species dispersion. The comparison between the two normative indices highlights that the correspondence in the classification is achieved in the 57% (Alpine macrotype) and 43% (Central macrotype) of samples. According to our findings, we suggest the revision of the ICMi, both class boundaries and reference value. In addition, we recommend to lower LIMECO threshold for total phosphorus: indeed, several studies have shown significant changes in the diatom community composition starting from very low values (below the current LIMECO threshold, i.e. 50  $\mu\text{gL}^{-1}$ ). Moreover, the extension of our study to the whole Po River basin will complete our knowledge of species not yet included in the diatom indices and of the community response to nutrient levels also in other macrotypes.

## INTRODUCTION

Benthic diatoms are recognised worldwide as a reliable indicator of ecological health in rivers and streams. Indeed, they own most of the desirable attributes of the “perfect ecological indicator”, such as wide distribution, presence in all seasons, taxon-specific response to multiple stresses, key-role in the river trophic chain. For these reasons, many countries such as the United States and the European Union (EU) members have adopted standard methods and specific diatom-based metrics in their monitoring programs. In the EU the Water Framework Directive 2000/60/EC (European Commission, 2000) has led to the intercalibration of a large number of diatom metrics given their contribution to the ecological assessment of water bodies. In Italy, a new index derived from the intercalibration exercise has been adopted as the standard for the ecological classification of rivers, the Intercalibration Common Metrics Index ICMi (Mancini and Sollazzo, 2009) which is calculated as the mean of the Ecological Quality Ratio (EQR) of two existing indices, IPS (CEMAGREF, 1982) and TI (Rott *et al.*, 1999).

The WFD aims at achieving at least the good ecological status for all the rivers whilst a recent survey highlighted that less than half of the rivers met this objective (Kristensen *et al.*, 2018). To improve

the current ecological status of rivers it is crucial to identify the causes of degradation by relating appropriate biological metrics to environmental stressors. Among the various stressors to which diatoms respond, nutrients (i.e. phosphorus and nitrogen) enrichment is, without doubt, one of the most important, because of its role in regulating algal growth. Though, the quantitative relationship between nutrient concentration and benthic algae growth is not so clear for rivers, while it has been investigated in deep by limnologists for lakes, starting with the pivotal works of Vollenweider (1968; 1976) who found a clear relationship between phosphorus and chlorophyll-a concentration. Nevertheless, excessive nutrients are still considered as one of the most broadly problematic chemical stressors in rivers and streams (U.S. Environmental Protection Agency 2016; Kristensen *et al.*, 2018). Recent studies have attempted to establish nutrient thresholds driven by ecological criteria also for rivers (see Poikane *et al.*, 2021 for an overview), using a wide range of diatom indices and community metrics. A wide variety of thresholds has been suggested, this variety being strongly related to the variables (both biological and chemical) analysed, to the method applied, and to the type of river.

In Italy the application of diatom indices for the river classification has many distinctive aspects: 1) data concerning diatom flora is scattered and relatively recent compared to other close countries such as France and Germany. Published studies on the Italian flora regard in particular the Apennines starting from 1999 and confined to single Apennine streams (Dell'Uomo *et al.*, 1999; Torrisi and Dell'Uomo, 2006; Dell'Uomo and Torrisi, 2011), Eastern Alps from 1998 (Cantonati, 1998) and Western Alps from 2004 (Battezzore *et al.*, 2004); 2) the river network is very diverse encompassing 24 hydroecoregions; 3) Italy has adopted a new index for the WFD application that has very little been considered in the scientific literature, except for the intercalibration exercise proposed by Almeida *et al.*, 2014 for the Mediterranean river type.

For these reasons, before the establishment of national diatom-based nutrient thresholds, it is necessary to analyse extensive databases concerning diatom communities collected following the application of the WFD.

This is the first study specifically focused on the analysis of data collected in the most representative macrotypes of Northern Italy (namely Alpine siliceous, R-A2 and Central, R-C). The aim is to explore the relationships of nutrients and diatoms in rivers considering, besides the normative diatom metrics, also the taxonomical and functional composition. Results of this analysis could be the initial step towards the setting of nutrient thresholds consistent with good ecological status, according to the objectives of the WFD. To achieve these objectives, we analysed the dataset regarding diatom community composition and nutrient concentrations coming from the WFD monitoring programme performed by the Regional Environmental Protection Agency (hereafter, ARPA) of Piedmont (NW Italy) from 2009 to 2016 in the upper part of the Po river basin, the longest river in Italy.

## **METHODS**

### **Study area**

The study area is the upper catchment of the Po River comprised in the Piedmont region, NW Italy. This basin represents a hydrogeological system of European relevance (De Luca *et al.*, 2020). Piedmont has a high climatic, lithological and orographic variability: the Po drains the semicircle formed by the Alps and Apennines, which surround the region on three sides. The Alpine section includes peaks over 4000 m asl such as Monte Rosa and Gran Paradiso but other features of this region are the damp rice paddies, the hillsides of Langhe, Roero, Monferrato and the western part of the Padana plain. This landscape diversity reflects the presence of five river macrotypes, among which the most represented are the R-A2 (Alpine siliceous) and the R-C (Central, corresponding to lowland stretches). The upland river basins are located in the Alps, with altitudes ranging between 300 and >4000 m asl and maximum precipitation mostly recorded in spring and autumn. The hydrological regime shifts from nival (i.e., high flow season in late spring and low flows mainly during winter) to pluvio-nival (i.e., two periods of high flows, in spring and autumn, and two periods of low flows, in summer and winter) as the altitude decreases. In these catchments, water abstractions and morphological alterations currently represent the most significant pressures. Lowland river basins within the Piedmont borders are located in the Po Plain at low altitudes (below 300 m asl) and are characterised by the pluvio-nival regime. In these areas, the catchments are modified by human activities, especially intensive agriculture and animal farming, urbanization and industry.

### **Data source**

We analysed diatom and water chemical data of the R-A2 and R-C river types (Fig. 1) collected by ARPA and available at the website <https://webgis.arpa.piemonte.it> (retrieved October 2020). The available data range is from 2009 to 2016. Sampling for chemical analysis has a higher frequency than biological quality elements, we selected the samples collected in the closest date possible to the biological ones.

The total number of samples was 390, collected in 139 sites, of which 50 belong to R-A2 (122 samples) and 89 (268 samples) to R-C river type. Sampling frequency was twice per year, in most cases in spring and autumn.

Diatom data were available as relative abundance of each taxon. Diatom samples were collected and processed by ARPA following the Italian standard method (ISPRA, 2014), based on the European standards UNI EN 13946:2005 (European Committee for Standardization, 2005) and UNI EN 14407:2004 (European Committee for Standardization, 2004) and following updates.

We selected water chemical data corresponding to the schedule of the biological samples to obtain a correspondence between the two matrices (i.e. biological and environmental). We considered the following water parameters: ammonium (NH<sub>4</sub>-N), Biological Oxygen Demand (BOD), conductivity (COND), dissolved oxygen (DO), nitrates (NO<sub>3</sub>-N), soluble reactive phosphorus (SRP), pH, total nitrogen (TN), total phosphorus (TP). For statistical analysis purposes, values below the detection limit were split in half.

## **Database preparation**

Diatom taxonomic list was revised and updated following the most recent literature papers and taxa names were uniformed to obtain comparable inventories over the whole surveillance period. Each taxon was assigned to one of the four ecological guilds described by Rimet and Bouchez (2012): low profile (small, fast-growing diatoms), high profile (large or colonial diatoms), motile (fast-moving diatoms), and planktic (diatoms adapted to lentic environment). Diatom inventories were then inserted in the OMNIDIA software version 6.0.8 for the calculation of the quality indices. The use of the most updated version of this software allowed comparable results over the whole sampling period. For each inventory we calculated IPS (Indice de Polluo-Sensibilité Spécifique; CEMAGREF, 1982) and TI (Trophic Index; Rott *et al.*, 1999) values. We then calculated ICMi according to Mancini and Sollazzo (2009). First, for each sample, we calculated Ecological Quality Ratios (EQRs) between observed and reference IPS and TI values proposed by Mancini and Sollazzo (2009). Second, we calculated the mean between IPS EQRs and TI EQRs, obtaining the ICMi final values.

As regards chemical data, we calculated for each sample the Italian normative LIMECO index (Government of Italy, 2010), which is based on four chemical parameters (namely ammonium, nitrate, TP and DO). LIMECO is an Italian synthetic index used to summarise the chemical quality and classify rivers into 5 classes, from *high* (1<sup>st</sup> class) to *bad* (5<sup>th</sup> class). LIMECO contributes to the river ecological classification according to the Italian legislation. Finally, we prepared a spatial matrix based on the geographical coordinates of the study sites.

## **Statistical analyses**

### **Water quality - Differences between macrotypes**

Possible differences in terms of water parameters between R-A2 and R-C were tested through t-test in R 3.6.0.

### **Diatom composition according to macrotypes and LIMECO index**

We performed a Principal Coordinate Analysis (PCoA) to detect possible differences in terms of taxonomic composition (Bray-Curtis distance) among diatom samples collected in sites belonging to different macrotypes (R-A2 and R-C) or LIMECO quality classes. Using R package *vegan* (Oksanen et al. 2020), we plotted the results as an ordination diagram and we connected the points for sites to the centroids calculated for the different macrotypes (Fig. 2a) and LIMECO classes (Fig. 2b). Possible dissimilarity in taxonomical matrices of diatoms collected in R-A2 and R-C and LIMECO were tested through a Two-Way PERMANOVA (Anderson, 2001). Similarity Percentage Analysis (SIMPER) (Clarke, 1993) was used to determine the contribution of each species to the average Bray–Curtis dissimilarity between the two macrotypes (R-A2 and R-C) and LIMECO quality classes. The PCoA, the Two-Way PERMANOVA and the SIMPER analyses were performed in PAST 4.03 (Hammer *et al.*, 2001).

The set of ecological guilds and relative abundances extrapolated from the functional matrix were subjected to a non-parametric Mann-Whitney U test, performed in PAST 4.03, to check for differences 1) between R-A2 and R-C sites and 2) among the LIMECO water quality classes.

### **Influence of environmental and geographical variables on diatom community**

We then investigated the role of water parameters in shaping the diatom community in both R-A2 and R-C sites. To achieve this goal, for each macrotype we performed a partial-RDA (Peres-Neto *et al.*, 2006; De Bie *et al.*, 2012) built up on two explanatory matrices, to focus on physical-chemical variables, partialling out the spatial component of the taxonomic variation.

A physical-chemical matrix [C] included all the 9 parameters cited above, and a spatial matrix [S] included a set of orthogonal spatial variables derived from the geographical coordinates of the study sites. By a Moran's Eigenvector Maps analysis (MEM, Dray *et al.*, 2006), we partitioned the spatial information into the spatial variables. These variables represent the potential autocorrelation between spatial points at different scales and they can model coarse patterns in the community data and then progressively represent finer-scale patterns (Borcard *et al.*, 2004). Each spatial variable corresponds to a specific spatial structure and scale. For both matrices, we applied a forward selection to obtain a parsimonious combination of variables, i.e., including only variables with a significant relationship with the community matrix. We separately tested the [C] and [S] matrices against the taxonomic matrix and we decomposed total community variation into pure components and their intersections. We tested the significance of the chemical component using a Monte Carlo test with 1000

permutations. The partial RDA analyses were performed with the R package *vegan* (Oksanen *et al.*, 2020).

## RESULTS

### Sampling sites – water quality

Physical and chemical parameters detected over the whole survey are summarised in Tab. 1. We detected significant differences between R-A2 and R-C for all the considered parameters, except for ammonia and pH. As expected, R-A2 samples showed lower nutrient concentrations, BOD and conductivity than R-C sites. On the other hand, we noticed the highest dissolved oxygen values in R-C with maximum values reaching 184% and 18.3 mg L<sup>-1</sup>, probably due to algae blooms in some sampling sites. Samples belonging to R-A2 were mostly classified as high based on the LIMECO classification (88.5%); about 6% were classified as good; about 5% as moderate and only one site fell into the worst quality class of LIMECO. No site was classified into the 4<sup>th</sup> LIMECO quality class (poor). On the other hand, 51% of the samples belonging to R-C were classified as high based on the LIMECO classification; 25% fall into the second quality class; 16% into the third; ca. 6% into the fourth class and 7 sites out of 268 were classified into the worst class.

### Diatom communities: comparison among macrotypes and water quality classes

A total of 338 diatom taxa were detected in the study area (207 in R-A2, 304 in R-C). For statistical analyses, rare taxa (i.e. never exceeding 2% of relative abundance and/or recorded in less than 10 samplings over 390) were excluded. The final biological matrix accounted for 163 diatom species. The visual inspection of the PCoA, which ordered samples according to their community composition, highlighted that R-A2 sites are mostly placed on the left side of the diagram while R-C are mainly distributed on the right side, with a clear separation of the two spider graphs (Fig.2a). According to LIMECO, we also noticed a rather high dispersion within the sites classified as high or good (i.e. blue and green in Fig.2b) which denotes high heterogeneity among samples in terms of diatom species composition. On the contrary, samples classified from moderate to bad (i.e. yellow, orange and red) were very close, and consequently similar to each other, pointing out a lower heterogeneity in terms of species composition. The centroids of “high” and “good” samples are well separated from the three other LIMECO categories, which are very close among them.

Results of the Two-Way PERMANOVA performed on the taxonomic matrix confirmed the differences among species composition according to both macrotypes ( $F=7.6727$ ;  $p=0.0001$ ) and LIMECO quality class ( $F=2.0856$ ;  $p=0.0001$ ), but not to their interaction ( $F=-56.313$ ;  $p=0.9672$ ).



To single out the contribution of each species to the average Bray–Curtis dissimilarity between macrotypes and LIMECO quality classes, we performed a Similarity Percentage Analysis (SIMPER) on the taxonomic matrix. As shown in Tab. 2, several species contributed to the differences between the two macrotypes. As expected, in R-A2 the species contributing most to this difference were mainly sensitive taxa, widely distributed in oligo-mesotrophic streams and were, accordingly, typical of mountain streams. These taxa were, in order of importance: *Achnantheidium minutissimum* (Kützing) Czarnecki, *Achnantheidium pyrenaicum* (Hustedt) Kobayasi, *Cocconeis lineata* C.G. Ehrenberg, *Achnantheidium gracillimum* (Meister) Lange-Bertalot, *Gomphonema elegantissimum* Reichardt & Lange-Bertalot in Hofmann & al. and *Encyonema silesiacum* (Bleisch in Rabh.) D.G. Mann. On the contrary, species contributing most to the differences between macrotypes and typically recorded in R-C were ubiquitous taxa, widely distributed in lowland rivers with meso-eutrophic status: *Sellaphora nigri* (De Not.) C.E. Wetzel et Ector comb. nov. emend., *Reimeria sinuata* (Gregory) Kociolek & Stoermer, *Cocconeis euglypta* C.G. Ehrenberg, *Nitzschia fonticola* Grunow in Cleve et Möller and *Nitzschia dissipata* (Kützing) Grunow.

The same analysis was performed on LIMECO quality classes (Tab. 3). In general, *Achnantheidium* species were typical of the 1<sup>st</sup> quality class, but well represented up to the 3<sup>rd</sup> one denoting a wide ecological tolerance. On the contrary, species generally found in eutrophic lowland rivers, such as *Mayamaea permitis* (Hustedt) Bruder & Medlin, *Navicula gregaria* Donkin and *Craticula subminuscula* C.E. Wetzel & Ector were, accordingly, found in the 4<sup>th</sup> water quality class. *Sellaphora nigri* (De Not.) C.E. Wetzel et Ector comb. nov. emend. resulted as the most tolerant taxa, with a mean relative abundance of ca. 11% in the worse quality class.

The compositional differences between the two macrotypes and the LIMECO quality classes were confirmed by the analysis of the ecological guilds. Planktic taxa were excluded from the analysis because they accounted on average for less than 1% of the community. The ternary plot of Fig. 3a shows that samples belonging to the R-A2 macrotype were mainly composed of low profile and high profile taxa, while a higher functional heterogeneity characterised samples belonging to the R-C macrotype (Fig. 3b), that were composed of all the ecological guilds (i.e. low profile, high profile and motile). Moreover, R-C macrotype was characterized by a significantly higher abundance of motile taxa than R-A2 (Tab. 4). This difference was confirmed through the non-parametric Mann-Whitney U test, which detected also significant differences among R-A2 and R-C in terms of low profile and high profile guilds.

Also the LIMECO class influenced diatom functional composition (Fig. 3). Low profile species were significantly more abundant in the high water quality class and their abundance gradually decreased

up to the 4<sup>th</sup> LIMECO class (Tab.4). Unexpectedly, the 5<sup>th</sup> water quality class sheltered a higher median value in terms of low profile taxa than the 4<sup>th</sup> quality class, however, this difference could be due to the low number of observations included in this category (n=7) and to the biofilm stage of development (see comments in the following sections). High profile guild showed the same pattern as low profile with a gradual decrease of abundance from the 1<sup>st</sup> to the 5<sup>th</sup> LIMECO water quality class. Finally, the motile guild showed exactly the opposite pattern in comparison to low and high profile guilds, with increasing abundances towards the poorest water quality class. However, despite median values being significantly lower in the first LIMECO class in comparison with the others, we noticed a high dispersion of data denoting that the presence of motile taxa was recorded also in good or high-quality sites.

### **Diatom communities: water parameters vs spatial component factors**

We then analysed whether diatom communities in the two macrotypes were driven by spatial factors or physical chemical parameters. The partial RDA performed on samples collected in the Alpine macrotype (Fig. 4a) highlighted the strong dominance of the spatial matrix, which included 17 PCNM vectors referring to both coarse and fine-scale spatial autocorrelation after forward selection, in comparison to the chemical one. The spatial component represented the key driver in explaining diatom composition, accounting for 15% of the total variance. The spatially structured environmental parameters represented 7% of the total explained variance. On the other hand, chemical parameters just explained 3% of the total explained variation. In this case, TP and SRP were not significantly related to the community matrix and they were excluded from the ordination diagram (Fig. 4a) where nitrate and TN were negatively correlated with both RDA axes. Conductivity and pH were negatively correlated with RDA1 but positively correlated with RDA2.

The partial RDA performed on samples collected in the Central macrotype (Fig. 4b) highlighted again a strong dominant role of the spatial component in explaining diatom composition. Indeed, this matrix alone explains 11% of the total variance. Chemical and spatially structured environmental parameters were less important in shaping the communities of the R-C macrotype, both of them explaining 3% of the total variance. Five chemical parameters (namely pH, nitrate, conductivity, TP, SRP) were included in the ordination represented in Fig. 4b, where RDA1 was mainly correlated with nutrient concentrations, while RDA2 represented pH and conductivity.

## **Diatom indices vs LIMECO**

Most of the samples collected in the Alpine macrotype were classified as at least good basing on diatom indices (in detail 91% basing on IPS, 75% basing on TI and 83% basing on ICMi). In the Central macrotype we observed higher discordance among diatom indices classification with TI being more severe (with only 23% of the samples classified as at least good), followed by IPS (65% of the samples classified as at least good) and ICMi (71% of the samples classified as at least good). The pattern of diatom indices (IPS, TI and ICMi) along an environmental gradient represented by the LIMECO water quality classes is shown in the boxplots of Fig. 5. As expected, all the diatom indices here considered tend to worsen going from the 1<sup>st</sup> to the last LIMECO class, for both macrotypes. However, samples classified as “high quality” according to LIMECO show a rather wide range of diatom indices values, Indeed, IPS and TI show median values falling into the first/second quality class and raw values reached even the 4<sup>th</sup> class. As expected, TI worked better in the Alpine than in R-C, where values and quality judgment underestimated water quality (see high/good LIMECO quality class in comparison to TI values). In many cases (i.e. 21.8% of the whole database), ICMi exceeded 1.

In the Alpine macrotype, we always noticed an important difference, in terms of diatom indices values, between the high LIMECO quality class and the lower ones. On the contrary, diatom indices values were quite comparable between 2<sup>nd</sup> and 3<sup>rd</sup> LIMECO water quality class, as indices were not able to discern good/moderate quality status.

In R-C, we observed a more gradual decrease of the diatom indices values, which generally followed water quality depletion. The only exception was represented by the 5<sup>th</sup> water quality class. In this case, IPS and ICMi showed higher median values than those observed in the 4<sup>th</sup> LIMECO class. This increase was also slightly observed when calculating TI. However, we also noticed a high data dispersion for this category, with IPS data ranging from the 2<sup>nd</sup> to the 5<sup>th</sup> quality class and from the 2<sup>nd</sup> to the 4<sup>th</sup> concerning the ICMi index. As already noticed before, this could be due to the low number of samples included in this category (n=6) for the R-C macrotype.

## **DISCUSSION**

To our knowledge, this is the first study to examine an extensive database of a significant portion of the Po river containing diatom and environmental data collected after the application of the WFD in Italy. We investigated the relationship between diatom communities and WFD related index to the water chemistry as a preliminary step towards the establishment of diatom-base nutrient thresholds.

Our findings shed light on the difference between the two prevailing macrotypes in the study area in terms of diatom community (taxonomical and functional composition) and their response to water quality. We confirmed the importance of spatial factors and highlighted concordance and drawbacks of the normative indices currently used in Italy.

### **Diatom communities and water quality in the two macrotypes**

Rivers included in the study area as resulted from the synthetic index LIMECO are in general of good quality. In R-A2 sites, the mean LIMECO value was  $0.855 \pm 0.168$  corresponding to the high status, in R-C the mean was  $0.638 \pm 0.214$ , corresponding to the good status. It should be remarked that the study area lies in the initial portion of the Po river basin where rural landscape is still predominating and the only cities with a population over 100,000 are Turin (nearly 850,000) and Novara (nearly 102,000). Nevertheless, the river catchment is affected by a wide range of human pressures and this is reflected by the nearly 25% of sites classified as “not good” in the Central river types.

Diatom assemblages show significant differences according to water quality (evaluated by the LIMECO index) and river macrotype. Our data showed a strong homogenization of diatom species composition along the increasing disturbance gradient represented by the LIMECO classification (see the PCoA), confirming results obtained by Pillsbury *et al.* (2019). Recently, homogenization of diatom communities has also been observed in response to hydrological alterations and urbanization in Mediterranean rivers which also led to significant species loss at both local and regional scales (Falasco *et al.*, 2021a) and a reduced role of diatoms in the total benthic chlorophyll-a (Piano *et al.*, 2016). Moreover, the present study confirmed the high trophic value of some species, mainly collected in the R-C macrotype, such as *N. gregaria* (Hicks and Taylor, 2019; Hausmann *et al.*, 2016; Szczepocka *et al.*, 2016), *Nitzschia palea* (Licursi *et al.*, 2016; Szczepocka *et al.*, 2016; Yang *et al.*, 2015), *Gomphonema parvulum* (Lu *et al.*, 2020; Szczepocka *et al.*, 2016) and *C. euglypta* (Trábert *et al.*, 2020). *S. nigri* was confirmed as one of the most tolerant diatom species (see Hausmann *et al.*, 2016 as *Eolimna minima*; Yang *et al.*, 2015). On the contrary, other taxa such as *A. minutissimum*, *A. pyrenaicum* and *E. silesiacum* mainly preferred waters confirming previous observations (Çelekli *et al.*, 2019; Pillsbury *et al.*, 2019; Hausmann *et al.*, 2016; Yang *et al.*, 2015; Black *et al.*, 2011). From a functional point of view, as expected, Alpine streams belonging to R-A2 were characterized by low profile taxa confirming the adaptation of this guild to the resource limitations (Passy, 2007; Berthon *et al.*, 2011; Novais *et al.*, 2014; Stenger-Kovács *et al.*, 2020) and its resistance to physical disturbance (i.e. the high turbulence characterizing the Alpine stretches). In these rivers, low nutrient and light availability limit the development of a mature three-dimensional biofilm favouring the dominance of low profile taxa. On the other hand, higher light availability, ion and nutrient concentration of the

macrotype R-C enhanced the development of a more complex biofilm structure characterised by motile taxa (Berthon *et al.*, 2011; Licursi *et al.*, 2016; Stenger-Kovács *et al.*, 2020). This guild is known to be composed of mostly eutrophic and pollution tolerant species (Passy, 2007), probably due to their ability to absorb and store nutrients (Berthon *et al.*, 2011). We found a weak relationship between the high profile guild and nutrient concentration, as already observed by Passy (2007). Intermediate LIMECO categories were also characterised by the highest guild diversity confirming the hypothesis that thick biofilms host strong resource gradients that can be exploited by different guilds in different ways (Stevenson *et al.*, 1996). Once more, the functional analysis of the diatom community provided important and interesting results, which improved the information provided by the taxonomic composition analysis (Falasco *et al.*, 2020; 2021a).

### **The influence of physical chemical and spatial factors on diatom communities**

In recent years, many studies demonstrated that not only environmental local factors but also spatial ones may play an important role in diatom species distribution and abundance. Indeed, the spatial component has been recognised as one of the most important drivers for diatom communities especially in mountain areas, where geographical and topographical processes may influence the species dispersal (Dong *et al.*, 2016; Piano *et al.*, 2017; Falasco *et al.*, 2019). Although the influence of spatial factors increases with geographical distance (Heino *et al.*, 2010), it can be high also at the regional scale (Bottin *et al.*, 2014). Our study corroborates these findings and showed that, especially in our study area, the effect of spatial processes is very important also due to its strong spatial organization and can soften the response of diatom to water chemistry. Among the spatial processes involved we can mention geographical barriers (i.e., spatial distance between sites), topographical and geomorphological constraints that limit the diatom dispersion. Among the chemical parameters, the ion content (through conductivity and pH) was significantly related to the diatom matrix while the only significant nutrient seemed to be nitrogen (as TN and nitrate). Phosphorus was excluded from the ordination diagram not being correlated with the community composition. Results obtained in the partial RDA applied to R-C macrotype confirm the importance of spatial factors but a lower influence of spatially structured environmental parameters. Among the latter, TP and SRP were included in the ordination diagram, unlike the diagram obtained for the Alpine macrotype. The higher variance explained by the two shared components in more pristine sites agrees with our previous findings in the Eastern Alps (Falasco *et al.*, 2019), while the different role of phosphorus between the two macrotypes can be explained by the high occurrence of low values that is particularly marked in R-A2.

## **The ecological status of the upper Po Basin: issues emerging from the comparison between diatom and chemical indices**

The comparison between ICMi and LIMECO shows a correspondence of 57% (in R-A2) and 43% (in R-C) in terms of water quality classes. Indeed, in R-A2 the ICMi diatom index underestimated the ecological quality in comparison to LIMECO in 41% of the cases, while in the remaining 2% the quality class expressed by ICMi was higher than the LIMECO one. In R-C, the trend is quite different since ICMi overestimated the ecological status in ca. 22% of the samples when compared with LIMECO; contrarily, ICMi underestimated water quality in comparison to chemical classification in 35% of the cases. The lack of agreement between the two normative indices is consistent with the findings of the partial RDA, where three LIMECO parameters out of four do not result as significant in shaping the diatom communities. The higher severity of the ICMi could be mainly due to the effects of other impacts, such as the hydromorphological alterations in the Alpine zone, or the pollution generated by herbicides in the lowland stretches, which lies outside the LIMECO calculation but indirectly included in the ICMi calculation (teratological forms considered in the IPS). On the contrary, high values of ICMi in poor LIMECO water quality sites may correspond to conditions of functional alterations of the diatom community that the diatom index does not consider. In many cases, by analysing those sites characterized by higher values of ICMi in comparison to the LIMECO classes detected, we observed undeveloped communities composed of low profile and pioneer taxa (such as *A. minutissimum*, *A. pediculus* and *C. euglypta*) which could denote that sampled biofilms were probably in their first stage of recolonization.

The species just mentioned are considered as sensitive to trophic pollution by most of the diatom indices, and this could be the reason for the overestimation of the ICMi vs LIMECO. We observed that diatom indices discriminate better among high/good water quality classes than over moderate-poor status, confirming previous observations (Pan *et al.*, 1996; Ponander *et al.*, 2007). From the analysis of the whole database, we highlighted that in almost 22% of the samples ICMi exceeded the value 1, meaning that it is higher than the mean reference value. The WFD bases the ecological status classification on a reference approach, where observed values of a given water body have to be compared with reference values for the same river typology. Reference conditions can be set in different ways: if reference sites and consequent reference values can be selected for typologies (i.e. Alpine ones) that include a good number of pristine sites, this is much challenging for Central or Mediterranean rivers where reference conditions are much more difficult to attain.

The relatively high occurrence of values  $>1$  for this index confirms what has been found in Mediterranean rivers by Falasco *et al.* (2012; 2016) and in Po tributaries located in Lombardy by Salmaso *et al.* (2019). As pointed out by these authors, the reference values currently available for

the calculation of EQRs are on average too low and need to be revised to increase the reliability of this index. For instance, in the R-C typology, the IPS reference value proposed is 16.7 corresponding to a mesotrophic environment, while 2.4 for the TI, corresponding to eutrophic conditions. To confirm this, a recent report of ARPA and Regione Piemonte (2020) points out that ICMi is the index that by far classifies rivers in higher quality classes than other biological quality elements (namely macroinvertebrates and macrophytes) and LIMECO. The apparent conclusion that could be drawn is that diatoms have a low discriminating power between quality classes. It is therefore essential to consolidate the process of revision of the thresholds between classes and of the reference values already initiated in the EU and at national level.

Another important drawback that should be improved is the number of species considered in the diatom indices calculation, since 19% of taxa included in our database is not considered in the TI calculation (corresponding to 85 taxa). For instance, in the R-A2 typology the lowest observed value was detected in the Bogna river where even though 76% of the species was included in the TI floristic list, only 26% of the individuals contained in the inventory contributed to the index calculation. Again, in the R-C typology we observed a similar situation in the Polonghera river, with 61% of the taxa included in the TI floristic list and only 32% of the individuals used to calculate it. Among the taxa missing in the TI floristic list for the index calculation we can mention some widely distributed and abundant species in NW-Italy, among the most sensitive are *Gomphonema elegantissimum*, *Nitzschia puriformis* and *Nitzschia alicae*. Moreover, tolerant species such as *Achnanthydium eutrophilum*, or taxa considered as allochthonous but nowadays spread in our rivers, such as *Achnanthydium subhudsonis* or *Cymbella tropica* (Falasco and Bona, 2013) are still non included. Finally, yet importantly, all the teratological forms are not considered in the TI calculation, even though the detection of these forms, especially in the R-C typology, can provide important information concerning the presence of toxicants in the water column (Falasco *et al.*, 2021b).

As it is well known in biomonitoring, there are several aspects to evaluate in the study of the relationship between biological quality elements and chemical parameters of aquatic environments. Firstly, the concentrations of chemicals detected at a certain moment are a "snapshot" of the water quality and do not take into account the high temporal dynamism of river processes. Conversely, biological indicators provide an integrated response to the alterations that occur over longer time frames. In the case of nutrients, the choice of which chemical parameter to consider is not so easy. As related to phosphorus, the choice of dissolved (SRP) versus total forms (TP) is still controversial (Wagenhoff *et al.*, 2017), although the recent study of Poikane *et al.* (2021) found a significant relationship between diatoms (and macrophytes) with SRP and not TP. In Italy, only TP is considered in the calculation of the synthetic index LIMECO that is used for the ecological classification of

rivers. Another issue of LIMECO is that the TP threshold between the first and the second class is very high ( $0.05 \text{ mg L}^{-1}$ ) when referring to the response of benthic diatoms. Pillsbury *et al.* (2019) cited several studies in which the greatest changes in diatom species composition occurred in the range of  $10\text{--}60 \text{ }\mu\text{g TP L}^{-1}$ . Moreover, in pristine Alpine streams of the study area it is quite frequent to find phosphorus concentrations even lower than  $10 \text{ }\mu\text{g L}^{-1}$  (Falasco and Bona, 2011).

Regarding nitrogen, most studies examined the response of diatoms to total nitrogen TN (Poikane *et al.*, 2021) while LIMECO considers nitrates and ammonia.

## CONCLUSIONS

As regards the need to set national management targets for nutrients in rivers, the main technical reference on the procedure is the EC report by Phillips *et al.* (2018) that suggests the statistical approach (mainly regression techniques between EQR and nutrient concentration) for singling out the nutrient concentrations suitable to support the good ecological status. This approach has already been tested in Central Europe (Poikane *et al.* 2019) while in other European regions, including Italy, we are still far from an “ecology-based” set up of nutrients thresholds. According to our findings, the implementation of such an approach should consider the necessity to revise the class boundaries and reference values for diatom indices. At the same time, we also pointed out the need for available data of nutrients that include TN and P concentration lower than  $50 \text{ }\mu\text{g L}^{-1}$ .

The extension of our study to the whole Po river catchment would represent an excellent opportunity to deepen some important aspects highlighted by our study. In particular, considering other macrotypes and an even greater geographical variability will increase the knowledge of the autecology of the species not yet considered by the currently applied indices and the community response to nutrient concentrations.

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**Tab. 1.** Average (mean), standard deviation (ds), minimum (min) and maximum (max) value of physical and chemical parameters observed in the two macrotypes R-A2 and R-C. Significantly different parameters between the two macrotypes are highlighted in bold.

Macrotype	Value	N-NH4 (mg L <sup>-1</sup> )	N-NO3 (mg L <sup>-1</sup> )***	TN (mg L <sup>-1</sup> )***	TP (mg L <sup>-1</sup> )**	SRP (mg L <sup>-1</sup> )*	BOD (mg L <sup>-1</sup> )*	COND (μS cm <sup>-1</sup> )***	DO (mg L <sup>-1</sup> )***	DO (%) *	pH	LIMECO***
R-A2	mean	0.117	0.770	1.63	0.035	0.026	2.17	144	10.9	97.3	7.58	0.855
	SD	0.611	0.674	1.29	0.066	0.008	3.39	118	2.13	14.2	0.423	0.168
	min	0.015	0.050	0.500	<0.050	<0.050	1.00	23.0	7.10	59.0	6.60	0.125
	max	5.30	4.10	8.70	0.730	0.090	18.0	724	24.3	172	8.60	1.00
R-C	mean	0.390	2.01	3.74	0.115	0.084	3.89	360	10.2	100	7.55	0.638
	SD	2.69	1.39	4.22	0.481	0.451	11.9	301	1.98	18.8	0.502	0.214
	min	0.015	0.050	0.500	0.025	0.025	1.00	40.0	4.80	47.0	5.20	0.063
	max	36.2	7.50	60.0	6.90	6.80	190	3820	18.3	184	8.65	1.00

N-NH<sub>4</sub>, ammonia; N-NO<sub>3</sub>, nitrates; TP, total phosphorus; TN, total nitrogen; SRP, soluble reactive phosphorus; BOD, biochemical oxygen demand; COND, conductivity; DO, dissolved oxygen; DO (%SAT), dissolved oxygen (% saturation); LIMECO, chemical index; \*p<0.05; \*\*p<0.01; \*\*\*p<0.001.

**Tab. 2.** Summary of the SIMPER analysis showing species contributing more (up to 50% of the cumulative percentage) to the compositional differences between macrotypes (R-A2 and R-C). Percentage contribution (Contrib. %) of the species to the average community dissimilarity, cumulative percentage (Cum. %) and the mean abundance of taxa in the groups (i.e., R-A2 vs R-C).

<b>Taxon</b>	<b>Contrib. %</b>	<b>Cum. %</b>	<b>A2</b>	<b>C</b>
<i>Achnanthydium minutissimum</i> (Kützing) Czarnecki	12.88	12.88	0.221	0.106
<i>Achnanthydium pyrenaicum</i> (Hustedt) Kobayasi	11.03	23.92	0.150	0.098
<i>Sellaphora nigri</i> (De Not.) C.E. Wetzel et Ector	3.878	27.79	0.033	0.036
<i>Cocconeis lineata</i> Ehrenberg	3.133	30.93	0.040	0.020
<i>Reimeria sinuata</i> (Gregory) Kociolek & Stoermer	3.071	34.00	0.020	0.046
<i>Achnanthydium gracillimum</i> (Meister)Lange-Bertalot	2.885	36.88	0.044	0.003
<i>Cocconeis euglypta</i> Ehrenberg	2.858	39.74	0.018	0.037
<i>Nitzschia fonticola</i> Grunow	2.737	42.48	0.021	0.032
<i>Gomphonema elegantissimum</i> Reichardt & Lange-Bertalot	2.519	45.00	0.036	0.009
<i>Nitzschia dissipata</i> (Kützing) Grunow	2.507	47.50	0.006	0.039
<i>Encyonema silesiacum</i> (Bleisch) Mann	2.388	49.89	0.032	0.017

**Tab. 3.** Summary of the SIMPER analysis showing species contributing more (up to 50% of the cumulative percentage) to the compositional differences between LIMECO quality classes. Percentage contribution (Contrib. %) of the species to the average community dissimilarity, cumulative percentage (Cum. %) and the mean abundance of taxa in the groups (i.e., LIMECO1, LIMECO2, LIMECO3, LIMECO4 and LIMECO5).

Taxon	Contrib. %	Cum. %	Mean abundance				
			LIMECO 1	LIMECO 2	LIMECO 3	LIMECO 4	LIMECO 5
<i>Achnantheidium minutissimum</i> (Kützing) Czarnecki	10.08	10.08	0.183	0.073	0.094	0.024	0.053
<i>Achnantheidium pyrenaicum</i> (Hustedt) Kobayasi	9.506	19.59	0.138	0.090	0.069	0.023	0.055
<i>Sellaphora nigri</i> (De Not.) C.E. Wetzel et Ector	4.177	23.77	0.029	0.030	0.054	0.071	0.106
<i>Mayamaea permitis</i> (Hustedt) Bruder & Medlin	3.472	27.24	0.010	0.028	0.061	0.074	0.058
<i>Reimeria sinuata</i> (Gregory) Kociolek & Stoermer	3.433	30.67	0.033	0.063	0.032	0.019	0.036
<i>Nitzschia dissipata</i> (Kützing) Grunow	3.410	34.08	0.019	0.066	0.025	0.024	0.013
<i>Cocconeis euglypta</i> Ehrenberg	3.128	37.21	0.029	0.037	0.035	0.030	0.038
<i>Nitzschia fonticola</i> Grunow	2.863	40.07	0.026	0.029	0.039	0.026	0.016
<i>Cocconeis lineata</i> Ehrenberg	2.828	42.9	0.025	0.028	0.030	0.000	0.051
<i>Navicula gregaria</i> Donkin	2.583	45.48	0.009	0.017	0.050	0.068	0.034
<i>Craticula subminuscula</i> (Mang) Wetzel & Ector	2.211	47.69	0.007	0.011	0.044	0.066	0.018
<i>Gomphonema parvulum</i> (Kützing) Kützing	2.207	49.9	0.016	0.024	0.026	0.048	0.029

**Tab. 4.** Median abundance values for the guilds in each river type/ LIMECO quality class and results of the Mann-Whitney U test. Letters in superscript indicate significant differences among the two river types and between LIMECO quality class.

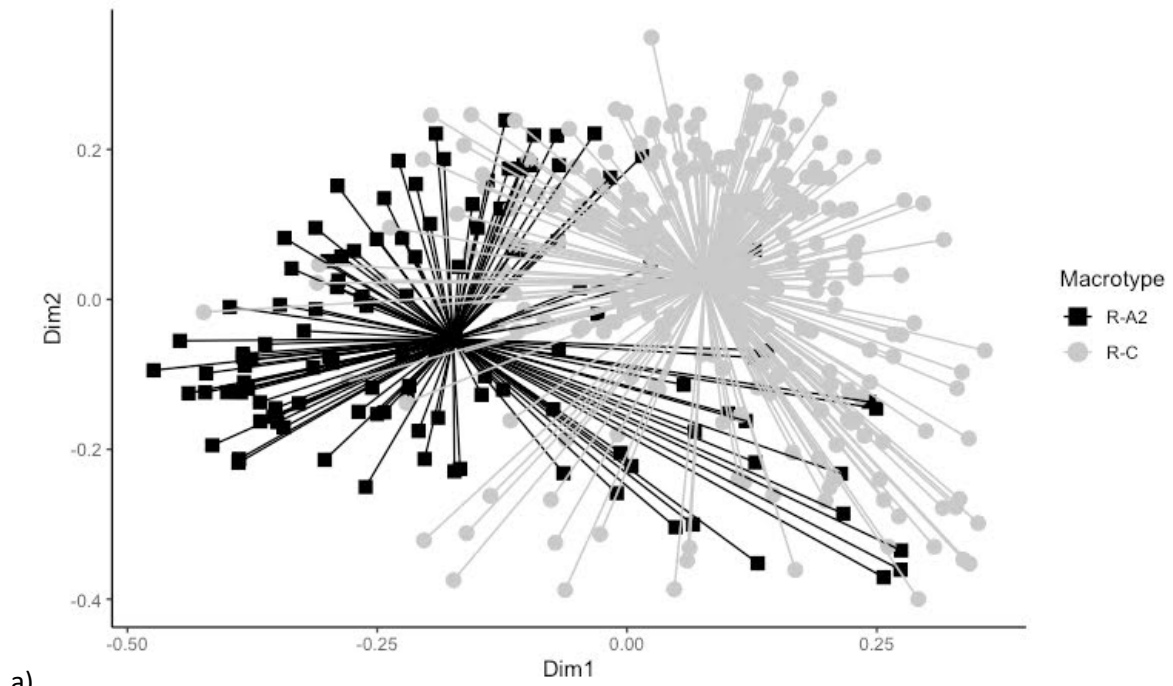
	Macrotype		LIMECO quality class				
	R-A2	R-C	1	2	3	4	5
Low profile	66% <sup>a</sup>	34% <sup>a</sup>	57% <sup>d,e,f,g</sup>	33% <sup>d,h</sup>	28% <sup>e</sup>	18% <sup>f,h</sup>	40% <sup>g</sup>
High profile	17% <sup>b</sup>	13% <sup>b</sup>	16% <sup>i</sup>	13%	11% <sup>i</sup>	9%	10%
Motile	6% <sup>c</sup>	41% <sup>c</sup>	15% <sup>j,k,l,m</sup>	36% <sup>j,n,o</sup>	54% <sup>k,n</sup>	68% <sup>l,o</sup>	48% <sup>m</sup>

<sup>a,c,d,e,f,j,k,l</sup>p<0.001; <sup>b,h,m,o</sup>p<0.01; <sup>g,i,n</sup>p<0.05.

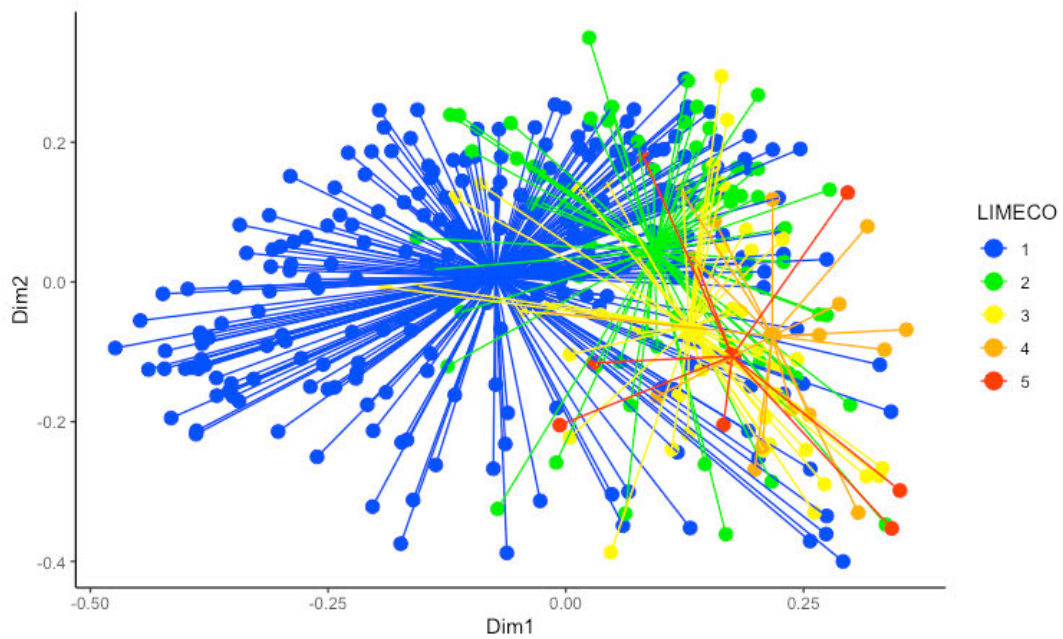




**Fig. 1.** Sampling site location collected in the two river macrotypes. Black filled squares, R-A2; grey filled circle, R-C.

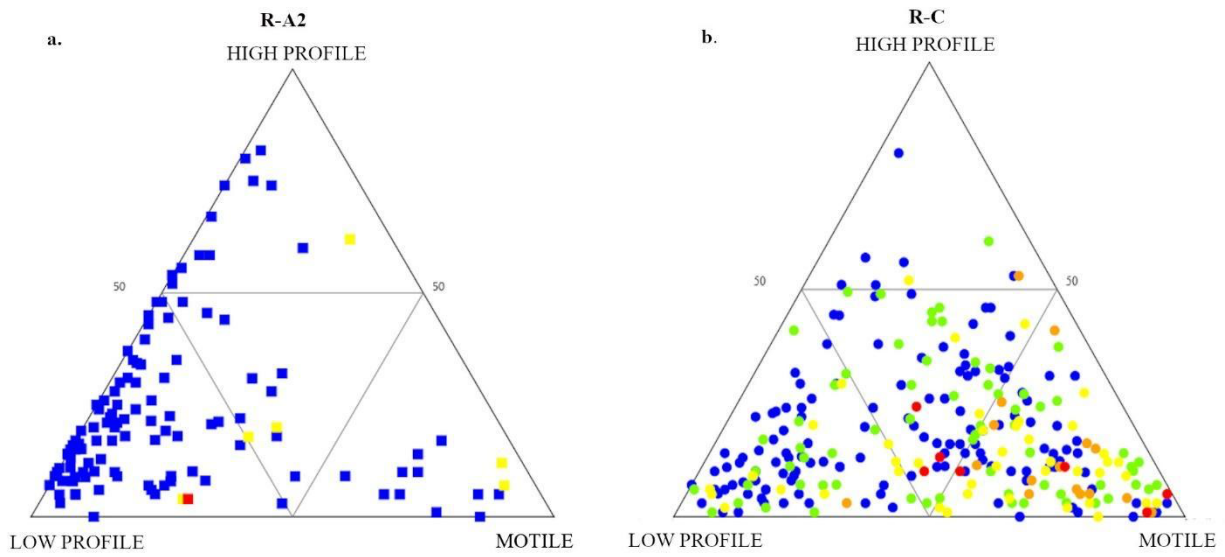


a)

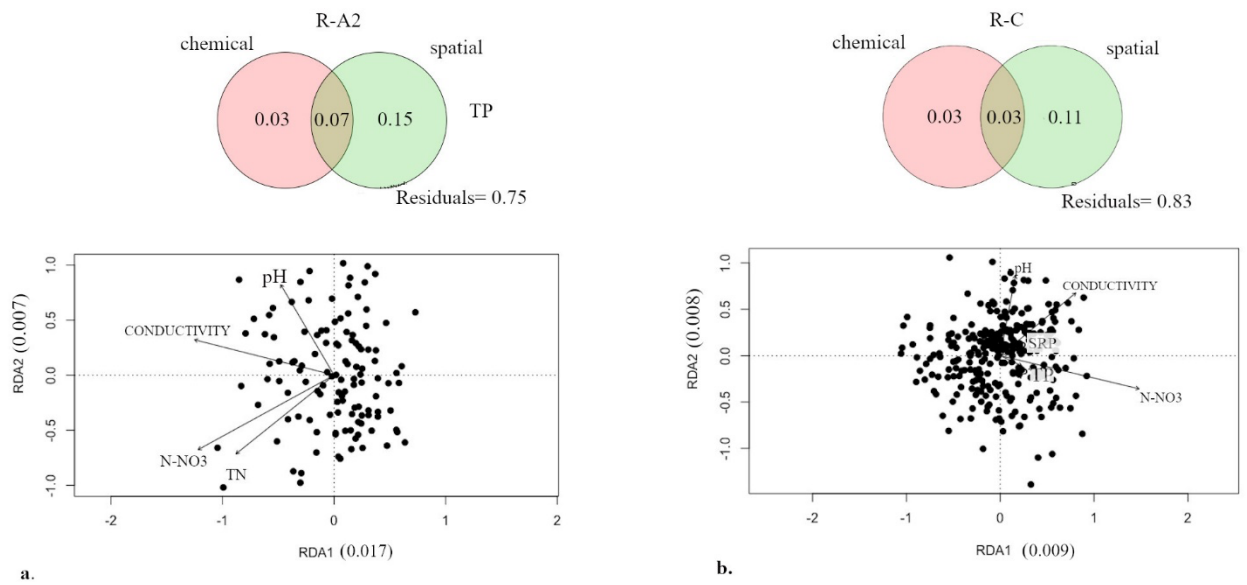


b)

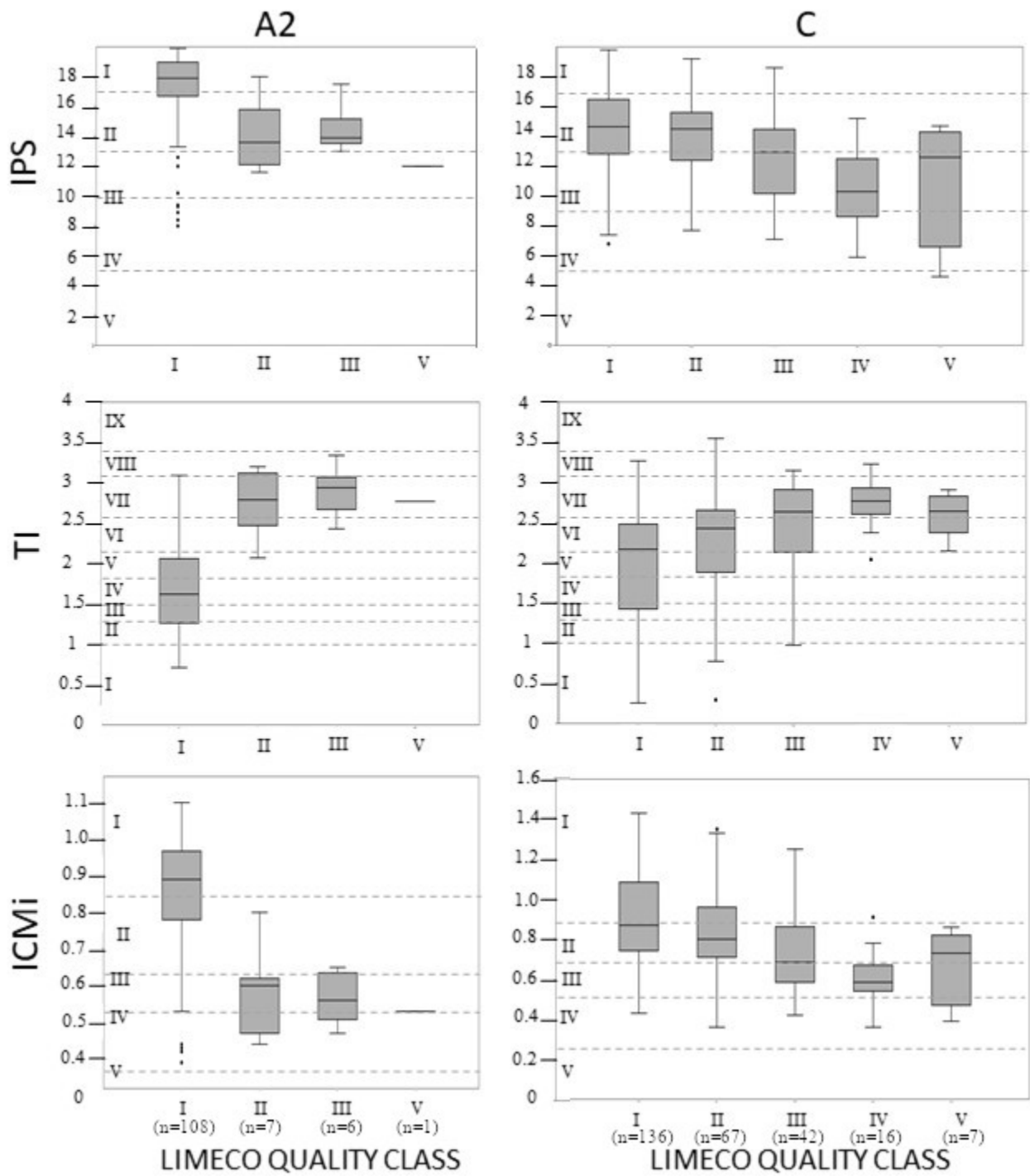
**Fig. 2.** Graphical result of the Principal Coordinate Analysis (PCoA), displaying samples based on the taxonomical matrix. In a) spider graphs are distinguished according to the river type (black squares=R-A2; grey circles: R-C); in b) each spider graph refers to a LIMECO class (blue, high; green, good; yellow, moderate; orange, poor; red, bad).



**Fig. 3.** Ternary plot showing diatom guilds composition (i.e., low profile, high profile and motile) in the two macrotypes: R-A2 (a) and R-C (b). Colours represent the water quality classification of LIMECO (blue, high; green, good; yellow, moderate; orange, poor; red, bad).



**Fig. 4.** Results from variation partitioning (partial RDA) showing the relative contributions (% of explanation) of chemical and spatial variables, as well as the shared components explaining variation in diatom communities in R-A2 (a) and R-C (b) sites. The ordination diagrams show the distribution of sampling sites according to their correlations with the significant chemical parameters. the eigenvalues are given in brackets on the two axes.



**Fig. 5.** Box plots representing the pattern of IPS, TI and ICMi values in the R-A2 (left panel) and R-C (right panel) according to LIMECO water quality classes.

**Annex 1.** Taxonomical list with guild assignment