DEFINING BENCHMARK VALUES FOR NUTRIENTS UNDER THE WATER FRAMEWORK DIRECTIVE: APPLICATION IN TWELVE PORTUGUESE ESTUARIES

Miguel Caetano^{a,b,*}, Joana Raimundo^{a,b}, Marta Nogueira^a, Maria Santos^a, Mário Mil-Homens^{a,b}, Ricardo Prego^c, Carlos Vale^b

^a IPMA, Portuguese Institute of Sea and Atmosphere, Av. Brasília, 1449-006 Lisboa, Portugal

^b CIIMAR, Marine and Environmental Research Centre, Rua dos Bragas, 289, 4050-123 Porto, Portugal

^c CSIC - Marine Research Institute, 36208 Vigo, Spain

* Corresponding author: mcaetano@ipma.pt

Abstract

The Water Framework Directive requires the assessment of physicochemical parameters supporting the biological quality elements. To achieve this evaluation, water samples were collected in 89 sites located at 42 water bodies (WB) from 12 transitional systems in Portugal. Nitrate, nitrite, phosphate, ammonium and silicate concentrations were measured in surface and near-bottom water samples collected at high- and low-tide. The influence of salinity, biological consumption, morphology and river basin inputs was examined. Results showed that nutrient variability is estuary and site specific. To overcome this inter-specificity complexity, benchmark values for nutrients were estimated based on Portuguese estuaries previously classified with good ecological status for the phytoplankton quality element. This methodology allowed classifying the chemical status for nutrients in the WBs of the surveyed systems. Water bodies from Cávado, Ave, Douro, Tagus and Sado showed "Low" status due to ammonium that was often above the benchmark value. Comparatively, nutrient concentrations in all WBs of Minho, Ria of Aveiro, Mira and Guadiana were lower than the benchmark values and therefore a classification of High was given.

Keywords: Nutrients, transitional waters, benchmark values, Water Framework Directive

1. Introduction

Estuaries are ecosystems where freshwater, including dissolved substances and suspended particles from natural processes and anthropogenic activities meet seawater. The fate of solutes and particles is driven mainly by the water circulation and biogeochemical processes. Understanding the nutrient dynamics is of primary importance because they may determine estuarine systems ecology (e.g. Hilton et al., 2006; Mainstone, 2010). Commonly, mixing diagrams are used to assess the relationship of nutrients with salinity. The linear decrease of nutrient concentration with salinity indicates that dilution

is the driving factor. Otherwise, negative or positive shifts suggest that biological consumption, internal inputs or regeneration superimpose to dilution (Olausson and Cato, 1980). Excess of nutrients may have indirect effects on water quality, biodiversity and ecosystem services (Brito et al., 2012). In UK, freshwater nutrient concentrations were considered the major cause of failures to achieve European legislative goals (Carvalho and Moss, 1995).

The application of the Water Framework Directive (WFD; Directive 2000/60/EC) requires the assessment of the chemical status of water bodies using nutrients among other parameters. The threshold values for nutrients in transitional waters have not been considered in this directive and are established by each member state (Devlin et al., 2011). The distinction of the anthropogenic pressures from the natural signal is crucial for the implementation of measures that prevent deterioration of water quality. However, no standard procedure is established for the determination of background levels (Tueros et al. 2008). According to these authors, two main approaches have been adopted: (i) sampling of pristine areas; and (ii) long-term monitoring programs or sampling at a large number of sites, then applying statistical analysis to establish a benchmark value or range (Bettencourt et al., 2004; Tueros et al., 2008).

The first approach it is difficult to apply since the uncontaminated areas with different water typologies do not exist across Europe. Although the second has the advantage of broad application no general agreement exists on the statistical methodologies to be applied. Several water modeling systems have been proposed for Portuguese estuaries (e.g. Saraiva et al., 2007; Simas and Ferreira, 2007), but they have restrictions associated with short-term variations in estuary topography, changes on velocity currents, dimension of intertidal areas and hydrodynamics in low depth areas (0.2-1m). Furthermore, the variability of light penetration in the water column and nutrient exchange across the sediment-water interface associated with advection and seepage processes in intertidal areas are not usually considered. Moreover, data of nutrients and primary production in Portuguese estuarine systems is scattered and derived mainly from research projects or short-term monitoring programs performed in few estuaries. The 90th percentile estimates the benchmark value for chlorophyll *a* (Borja et al., 2011; OSPAR, 2002, ECOSTAT, 2003). The same methodology is proposed for nutrients since the percentile-based approach will provide a more robust analysis of variability. Exceptionally high and low nutrient concentrations may occur occasionally but they are not considered using this approach. This means that there is less risk of a system being wrongly classified due to outliers. To respond for WFD demands a wide study was performed in the main Portuguese estuaries. Thus, the variability of nutrient concentrations in the water column of 12 estuaries was investigated. Nitrate, nitrite, ammonium, phosphate and silicate concentrations were measured in 42 water bodies of single-channel to broad inner-bay systems. A methodology for nutrient benchmark values was established based on the 90th percentile and applied to all estuarine water bodies. This methodology was used to classify the chemical status of nutrients on the 42 water bodies of the surveyed transitional waters.

2. Material and Methods

2.1 General description of study areas

The main estuaries from continental Portugal, classified as transitional waters according to the WFD, were surveyed (Figure 1). Accordingly to their typology the selected estuaries are characterized as (i) narrow channel (Minho, Lima, Cávado, Ave, Douro, Mondego, Mira, Arade and Guadiana); (ii) coastal-lagoon type estuary (Ria of Aveiro, hereafter named Aveiro); and (iii) semi-enclosed estuarine areas (Tagus and Sado). Table 1 presents the main water basin characteristics of these estuaries. Estuarine systems located in the south of Portugal (Tagus, Sado, Mira, Arade and Guadiana) are submitted to prolonged dry seasons and freshwater discharges occurred in short periods during winter. All watersheds are dominated by agriculture activities and forest. Industrial areas are mainly located in the basins of Tagus, Sado, Aveiro, Ave and Douro. Major cities located around the estuaries (e.g. Lisbon, Porto, Setúbal and Aveiro) discharge their effluents through submarine outfalls inside the estuaries or to adjacent coastal areas (Pereira et al., 2009; Quintino et al., 2001).

2.2. Sampling strategy

Temperature, salinity, dissolved oxygen and pH were measured *in situ*. Water samples were collected using Niskin bottles at surface (0.2 m) and near bottom (1 m above sediment) in 89 sites (geographical coordinates given as supplementary material, table S1). In each campaign, every site was visited in low and high tide, when feasible. Whenever possible, two campaigns were performed in autumn/winter and spring/summer of 2009 and 2010. Sampling sites covered all the water bodies (42) defined by Portugal in the context of WFD for the twelve transitional water systems (estuaries) according to Ferreira et al. (2006), who proposed the number and the shape of water bodies in each estuary. Sampling was performed to cover the salinity gradient: (i) tidal freshwater zone (TFZ); and (ii) mixing zone (MZ). The freshwater zone classification was consistent with the WFD where salinity was almost inexistent but the tidal effect was present. The mixing zone covers the salinity gradient. This approach derived from the wide variation of ionic strength on a minute scale over a semi-diurnal meso-tidal cycle. The salinity in a water body closer to estuaries mouth may vary up to 20 units over a tidal cycle. In general, the TFZ and MZ occupies up to 90% of the total estuarine area of the Portuguese systems.

2.3. Analytical methodologies

The multi-parametric probe YSI, model 650, was used for *in situ* physicochemical measurements. Temperature was measured with a thermistor of sintered metallic oxide. Salinity was determined from temperature corrected conductivity using Ni electrodes, calibrated with standard solutions. The pH was measured with a combined glass electrode with an Ag/AgCl reference electrode, calibrated previously with buffer solutions of pH 4, 7 and 10. Oxygen was determined with an YSI Rapid Pulse system with Clark-type sensor. Calibration was performed with aqueous sulfite solution for anoxic water and based

in water-saturated air as the medium for 100% saturation. Water samples were sub-divided to various vials according to the analytical specifications and transported to the laboratory for filtration and preservation. Suspended particulate matter (SPM) was obtained by filtering 250 mL of water through cellulose acetate membranes (0.45 μm) and the amount determined gravimetrically. Samples for the determination of dissolved inorganic nutrients were filtered through MSI AcetatePlus filters (0.45 μm), and analysis carried out with separate lines for nitrate+nitrite ($\text{NO}_3^- + \text{NO}_2^-$), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate (Si(OH)_4) using an autoanalyzer Skalar “SanPlus” (Hansen and Koroleff, 1999). Concentrations were determined using the interpolation method in a previously designed calibration curve. Procedural blanks were run every five samples and results blank-corrected. The detection limits were 0.02 μM for $\text{NO}_2^- + \text{NO}_3^-$, 0.07 μM for NH_4^+ , 0.03 μM for PO_4^{3-} and 0.02 μM for Si(OH)_4 . Certified standards (WAKO, CSK Standard Solution) used to ensure the accuracy of the procedures and precision was found to be $\pm 1.0\%$ for $\text{NO}_2^- + \text{NO}_3^-$, $\pm 2.0\%$ for NH_4^+ , $\pm 1.9\%$ for PO_4^{3-} and $\pm 1.1\%$ for Si(OH)_4 .

2.4. Data analysis

The non-parametric (quartiles, median, 10th and 90th percentile) statistics was calculated. Multivariate analysis (Principal Component Analysis, PCA) was applied to all data to identify the major drivers of system variability. Hydro-morphological characteristics and physic-chemical parameters were considered for PCA test. Additionally, Kruskal-Wallis H (KW-H) and Mann-Whitney (U) non-parametric tests were used to evaluate differences among estuaries for the different environmental parameters (salinity, temperature, O_2 , pH, SPM, $\text{NO}_3^- + \text{NO}_2^-$, NH_4^+ , PO_4^{3-} and Si(OH)_4). These tests were used due to the non-compliance with parametric ANOVA assumptions. The significance level for statistical analyses used was $\alpha=0.05$. The statistical analyses were performed using STATISTICA software 6 (Statsoft).

3. Results

3.1. Water characteristics

Data of salinity, temperature, dissolved oxygen, pH and suspended particulate matter (SPM) in the 12 survey estuaries are given as supplementary material (Table S2). In general, salinity ranged in broad intervals in all estuarine systems indicating a wide variability from freshwater conditions to seawater. A clear tidal freshwater zone (TFZ) was not detected in Ave, Aveiro and Sado estuaries. Water bodies located in the mixing zone (MZ) showed salinity variation of up to 20 units over a tidal cycle indicating high variability of ionic strength. The pH intervals (10th - 90th percentiles) in Lima (6.7-8.6), Mira (7.3-8.1) and Guadiana (7.8-9.4) were broader than in the other surveyed estuaries. The 10th percentile of dissolved oxygen was only found below 75% saturation for some samples of Cávado, Ave, Douro, Mondego, Arade and Guadiana. Lower values of dissolved oxygen in Cávado and Ave were found during low tide in bottom water samples in a WB recently dredged. The spatial distribution of SPM content

differed among the surveyed systems. The intervals 10th - 90th percentiles of SPM concentration were broader in Tagus (6.0-113 mg L⁻¹), Cávado (2.6-42 mg L⁻¹), Arade (5.3-61 mg L⁻¹) and Aveiro (6.3-58 mg L⁻¹). Whereas in the Tagus SPM decreased with the salinity, in Minho and Guadiana values were relatively uniform although with enhancements near the upper salinity limit zone in April (Minho) and September (Guadiana). SPM concentrations in Lima, Cávado, Douro, Mondego and Arade were also relatively uniform along the estuary, but with increased values in near urban areas. Ave, Aveiro and Sado estuaries showed broad variations of SPM without a spatial pattern.

3.2. Tidal freshwater zones (TFZ)

The median, 10th and 90th percentiles of nitrate+nitrite, ammonium, phosphate and silicate concentrations of seven tidal freshwater zones are presented in Figure 2. Since only one or two samples were collected in freshwater zones of Ave, Douro, Aveiro, Mondego and Sado, no statistical evaluation was performed. The median concentrations of nitrate+nitrite were significantly higher in Tagus (81 µM) and Lima (61 µM) than in Guadiana (43 µM), Minho, Cávado, Mira and Arade (< 27 µM). Broader intervals (10th - 90th percentiles) were found for Guadiana (13-71 µM) and Lima (51-104 µM). Ammonium concentrations were lower than nitrate+nitrite values, except in Cávado that showed similar medians, 26 and 27 µM, respectively. The median concentration of phosphate was higher in Tagus (4.8 µM) than in the other systems (< 1.7 µM). The silicates were significantly lower in Minho (26 µM) than in the other tidal freshwater systems (58- 94 µM).

3.2.1 Nutrient ratios

Median ratios of DIN:P found in Cávado (28), Tagus (16), Arade (38) and Guadiana (33) were comparable or above the Redfield value (16). Higher ratios were found in Minho (62), Lima (271) and Mira (77). In general, median ratios of DIN:Si were closer to the unit, although lower values were registered in Minho (0.37), Mira (0.26) and Arade (0.31). The Guadiana TFZ showed the highest variability of this ratio. The DIN:P ratios were much higher in May 2010 (median 382) than in November 2009 (22) and September 2010 (27), while significantly lower ($p<0.05$) DIN:Si ratios were found in September 2010 (0.75) than in November 2009 (1.0) and May 2010 (1.6).

3.3. Mixing zones (MZ)

The median, 10th and 90th percentiles of nitrate+nitrite, ammonium, phosphate and silicate concentrations in the mixing zones (MZ) of the twelve surveyed estuaries are presented in Figure 4. Box-plots indicate that nutrient concentrations in the MZ of each estuary varied by up to one order of magnitude. Despite this variability, the 10th - 90th percentiles range differed among systems. For example, the interval for nitrate+nitrite in Minho (0.87-6.6 µM) and Sado (0.42-7.0 µM) contain values below those registered in Ave (4.2-68 µM) and Tagus (7.6-53 µM). Ammonium concentrations in Minho

(0.21-1.6 μM) and Lima (0.41-2.6 μM) were much lower than in Douro (3.3-30 μM) and Mondego (5.5-26 μM). Intermediate situations were found for Aveiro (0.36-4.8 μM), Sado (0.19-5.0 μM) and Guadiana (0.36-4.3 μM). The phosphates in Minho (0.22-0.56 μM), Lima (0.05-0.22 μM), Aveiro (0.34-0.53 μM) and Sado (0.30-0.49 μM) were below those observed in Cávado (1.2-4.4 μM), Ave (0.84-2.5 μM), Douro (0.89-1.9 μM) and Tagus (0.55-4.0 μM). Low silicate concentration in Minho (0.85-16 μM) and Sado (0.27-8.4 μM) contrasts with high values in Lima (4.7-72 μM) and Aveiro (3.8-28 μM). Nutrient concentrations were, in general, higher in TFZ than in the MZ.

3.3.1 Nutrient-salinity relationships

Among the surveyed systems only Tagus and Guadiana presented nutrient-salinity relationships (Figure 5). In Tagus, nutrient concentrations in April were lower than in October and February. Linear relationships were obtained in April for nitrate+nitrite ($r=0.804$, $p<0.001$), phosphate ($r=0.870$, $p<0.001$) and silicate ($r=0.531$, $p<0.05$). In autumn and winter, nutrient concentrations showed broader differences between upper and lower estuary. Furthermore, most of nitrate-nitrite, phosphate and silicate concentrations in intermediate salinities are projected above the straight line connecting freshwater and seawater end-members (black dashed line in Figure 5). In Guadiana, the best fitting line for nitrate+nitrite versus salinity included data from the three surveys ($r=0.796$, $p<0.001$). Two relationships were discerned in spring and autumn/winter for phosphate ($r=0.544$, $p<0.05$ and $r=0.577$, $p<0.001$, respectively) and silicate ($r=0.549$, $p<0.05$ and $r=0.797$, $p<0.001$, respectively). Both Tagus and Guadiana estuaries showed increased ammonium concentrations at high salinity zones, mainly in winter. Poor nutrient-salinity relationships were obtained for Minho, Lima, Cávado, Ave, Douro, Aveiro, Sado and Arade.

3.3.2 Nutrient ratios

The molar ratios DIN:P in the MZ were up to three times the Redfield value (16). The broadest interval of DIN:P ratio was found in Lima and Mondego, with a maximum in the mid-salinity zone (715) and high-salinity zone (401), respectively. Most of the estuarine systems presented median DIN:Si ratios close to the unit, being higher in Douro (2.6) and lower in Mira (0.58). The highest variability of this ratio was found at Sado where the 10th and 90th percentiles were 0.20 and 18.5.

4. Discussion

Differences between data presented in this study and high nutrient concentrations reported in other studies (Table 2) may be explained by distinct sampling strategies. Data of exceptional periods of rainfall and consequent sharp increase of river flows, which usually corresponds to a pulse of nutrient input into the estuaries (Falcão, 1996), were not considered. Moreover, highly impacted areas have not been considered. Sampling sites were selected to represent the average conditions of the water bodies in the

12 estuaries. Despite the exclusion of extreme situations, nutrients in low- and high-tide of surface and bottom waters still presented considerable differences on concentration and spatial distribution among the studied estuaries. Principal component analysis (PCA) was used to identify the major drivers of system variability, though with no statistical relevance (Figure 6). Ordination based on estuary hydromorphology and physicochemical parameters separated nitrate+nitrite and silicate from salinity along the first axis accounting only for 21.5% of the variability. No clear influence of the other parameters was found with the second axis justifying 15.8% of the variability. Furthermore, no separation was observed between estuarine systems. Although no major driver was found for nutrient variability the specificity of the surveyed estuaries with respect to the distribution of nutrients might reflect different combinations of nutrient inputs and internal cycling, hydrology, morphology and biological consumption.

4.1. Nutrient inputs to river basin

The assessment impact of nutrients enrichment in estuaries may use nutrient load or sets of nutrient concentrations depending on data availability (Devlin et al., 2011). Nevertheless, all of the methodologies agree on the establishment of reference conditions to evaluate deviations and consequent impacts on water chemistry. For some of the studied estuaries an estimate nutrient loading is available, but reports specify the lack of information on various calculation elements, which leads to high level of uncertainty (<http://www.apambiente.pt>). Moreover, most of the river-estuarine systems have several dams and impoundments along the basin that retain solutes as nutrients at an unknown scale. On this basis, it was optioned to evaluate the variability of nutrients in the tidal freshwater zone (TFZ) that will reflect natural weathering, extension and practices of agriculture and discharges of urban effluents in the river drainage basin. These processes may contribute, although at different proportions, to the enhancement of nutrient concentrations in the river end-member. In fact, nutrient concentrations in TFZ were higher than in the MZ. The elevated values of nitrate+nitrite in freshwater that reaches the upper estuaries of Lima, Tagus and Guadiana (Figure 3) is most likely attributed to agricultural activities in the large river basins or discharges from cities located nearby the margins (Domingues et al 2005; Azevedo et al., 2013). Elevated ammonium concentrations and low oxygenation in Cávado appear to indicate input of organic matter associated with general anthropogenic activities (Peixoto, 2008) and results from dredging operations. The excess of DIN relatively to P in freshwater entering the MZ of Minho, Lima and Mira is probably indicative of human activities in the river basins (Azevedo et al., 2013; Medeiros et al., 2012). The proportion of cultivated lands in river basins and the lack of large industry complexes suggest that excess of DIN should be associated with agriculture.

4.2. Nutrient-salinity relationships in the mixing zone (MZ)

Nutrient concentrations and their variation along salinity gradients in estuaries are highly influenced by inputs from weathering and diffuse sources in the watershed, anthropogenic sources and tributaries discharging directly into the estuaries and internal regeneration. These processes may be counter-balanced entirely or partially by biological consumption of nutrients and dilution with seawater (Painting et al., 2007). Nutrients are often related to salinity that is the most conservative constituent of transitional waters to examine physical mixing *versus* nutrient consumption and inputs. Most of the surveyed estuaries showed no nutrient-salinity correlations or only exhibited weak relationships in certain sampling periods. Thus, the use of salinity gradients to divide zones of the estuary seems inadequate. In fact, only the freshwater end-member was clearly identified. This conclusion reinforces the sampling strategy in considering just a TFZ and a MZ. When mixing of freshwater and seawater was confined to a restricted section of the estuary, nutrient-salinity relationships were poorly defined. Mixing in Minho, Cávado and Ave takes place near the estuary mouth, being density stratification constantly modified by the highly dynamic exchanges with the sea (Table S2). In contrast, the mixing zone in Sado is confined to the upper channel as result of the low river discharge (Ferreira et al, 2003). Under those conditions, nutrient salinity relationships were useless to elucidate consumption or input of nutrients. Another example of weak nutrient-salinity relationship was observed at Aveiro, a lagoon-type estuary with a narrow connection to the sea and several inner branches (Rodrigues et al., 2012). The Vouga River enters in a confined branch of the estuary and the mixing of fresh and seawater differed among the other branches (Lopes et al., 2007). As a result nutrient concentrations at intermediate salinity values differed considerably, probably reflecting regeneration of nutrients, exchanges with extensive intertidal flat areas and local sources. Linear relationships were found in Tagus (nitrate+nitrite, phosphate and silicate), Guadiana (nitrate+nitrite and silicate), Mondego and Mira (silicate) but not for all sampling periods. Most likely, biological consumption compensated eventual regeneration of nutrients, exchanges with marsh areas and anthropogenic inputs (Cabrita et al., 1999; Caetano et al., 2012). Otherwise, it appears that high biological consumption of nitrate and silicate may explain data below the dilution line found for Mondego, and for phosphate in Guadiana. The DIN:P ratio in the mid-salinity zone of Lima and in the high salinity zone of Mondego reaching 40 times the Redfield ratio highlights the nitrogen enrichment relatively to phosphorus. Either localized sources, related to the urban and industrial effluents or agriculture possibly explain those enrichments. Polynomial convex curves (not shown) were found in Lima (nitrate+nitrite and silicate) and Tagus (nitrate+nitrite, phosphate and silicate) pointing to addition of nutrients to the estuary, either through tributaries and urban effluents ending in the estuary, or eventually exchanges with salt marsh areas adjacent to the water bodies.

4.3. Differences on hydrology and morphology

The studied estuaries are systems of narrow to wide morphologies, freshwater discharges with different regimes and temperature differs with the latitude (Ferreira et al., 2003). North narrow estuaries, such as Minho, Lima and Douro receive high river inputs, while south narrow estuaries (Guadiana and Mira) showed pronounced differences on freshwater discharges between dry and wet seasons. According to Saraiva et al. (2007) in estuarine systems with short residence-time (e.g. Douro) a reduction in nutrient load will only produce a decrease in nutrient transit and will not affect the system's global ecological status. Waters in the broad estuaries of Tagus and Sado have longer residence times, which may have influence phytoplankton communities. Brito et al. (2012) suggest that microalgae growth on semi-enclosed estuaries is limited to confined areas. This finding is in line with the large proportion of water volume exchanged on daily to fortnight scales between the estuaries and the adjacent sea, which is not in favor of the accumulation of microalgae biomass.

4.4. Influence of suspended and bottom sediments

Turbidity is a limiting factor to the nutrient consumption by estuarine primary producers (Olausson and Cato, 1980; Gameiro et al., 2011). The suspended particulate matter (SPM) content measured in low and high tide varied among the surveyed systems, with Tagus showing the highest values (Figure 2). Concentration of SPM increased during flood and ebb periods of strong tidal currents that erode the topmost sediments (Vale and Sundby, 1987; Duarte and Caçador, 2012). The increase of SPM may limit the phytoplankton growth, although in an intermittent way since SPM concentrations vary over short-time scales (Vale and Sundby, 1987; Ferreira et al., 2003). Besides the tidal currents, strong winds in shallow waters may also contribute to the erosion of topmost sediments enhancing the amount of suspended particles in the water column (Simon, 1989). The increasing temperature in summer coupled with changes in the sediment redox status also plays an important role in the exchange of nutrients, namely ammonium and phosphate, across the sediment-water interface (Cabrita et al., 1999; Caetano et al., 1997). These factors are more relevant in estuarine systems like Aveiro and Tagus characterized by shallow waters (<2m) and extensive intertidal areas. Caetano et al. (2012) showed that the efflux of nitrogen compounds and phosphate from Tagus salt marsh sediments due to tidal advection was a relevant source of nutrients to the water column. Furthermore, the linkage of Fe, Mn, nitrogen and phosphorus biogeochemical cycles also play a major rule in export/retention of ammonium, nitrate and phosphate from sediments (e.g. Mackin and Aller, 1984; Krom and Berner, 1981).

4.5. Biological consumption of nutrients

Phytoplankton biomass commonly assessed as chlorophyll *a*, is a simple and integrative indicator of microalgae response to nutrient enrichment (Devlin et al., 2007a, b). Brito et al. (2012) used historical data of chlorophyll *a* and classified as "high" or "good ecological status" the Minho, Lima, Cávado, Ave, Douro, Mondego, Tagus and Mira water bodies. The worst classification was proposed to the upper part

of Sado (moderate to poor) and Guadiana (high to poor status) estuaries. The relatively low values of chlorophyll *a* could be interpreted as weak response of the microalgae biomass to nutrient availability. The pronounced decrease of nutrients registered in Mondego and Tagus in the spring and summer surveys, relatively to the winter campaign, points to the nutrient consumption by phytoplankton and possibly other primary producers such as macroalgae, seagrass and salt marsh plants covering the extensive intertidal areas of these estuaries (Martins et al., 2007, Lillebø et al., 2005). Other processes, like denitrification due to decrease of oxygen and changes on the phosphorus sorption may change the nutrient availability on a daily to tidal scale (e.g. Mackin and Aller, 1984). Changes in the nutrient proportions may induce alteration in phytoplankton assemblages (Turner and Rabalais, 1994; Domingues et al., 2005; 2012). The DIN:Si ratios in freshwaters point to adequate growing conditions for diatoms that are single-celled algae encased in a silica cell wall. Ratios lower than the unit found in Minho, Lima and Arade promotes the competition in favor of other algae with no Si requirement like picoplankton cyanobacteria. The large variability of DIN:P and DIN:Si ratios in the MZ of all estuaries points to the existence of nutrient conditions to grow several algae species.

4.6. Benchmark values

To manage the water quality of transitional water bodies (WB) the WFD stated that "at high status the 'general physicochemical' elements must be within their natural background ranges" while at "good ecological status nutrient concentrations do not exceed the levels established to ensure the functioning of the ecosystem and the achievement of the values specified for the biological quality elements". According to Devlin et al. (2011), each member state can establish their threshold values for nutrients. OSPAR (2003) indicated that the nutrient enrichment is assessed estimating the average value of winter concentrations and compare with the regionally specific reference concentrations (Devlin et al., 2007b). Classification of WB is based on non-exceedance of predetermined thresholds for nutrients (Devlin et al., 2007b). This metric ensures some degree of alignment between previous directives and WFD assessments. The approach adopted by UK was to focus on sites that achieved Class A and B in the general quality assessment that is routinely undertaken, as these classes are equivalent to "good quality" sites under the WFD (UKTAG, 2006). Concentrations of solutes (e.g. nutrients) were then collated for these sites, and the 90th percentile concentration was calculated. Taking the 90th percentile is, therefore, a precautionary approach for establishing the maximum concentration of a nutrient that is still consistent with a site achieving "good quality" (Crane et al., 2007). Recently, Cabral et al. (2012) evaluate the ecological quality status of Portuguese estuaries based on the quality element fish. Results showed that all transitional waters presented "Good" status with exception of Douro in 2009. By using, the phytoplankton quality element Brito et al. (2012) showed that among the evaluated estuaries, Minho, Lima, Cávado, Douro, Tagus and Mira were classified as "Good". Thus, in the absence of pristine sites or long term historical datasets, the benchmark values for each nutrient was defined as the

concentration established by the 90th percentile using all data from Portuguese estuaries pre-classified as "Good". Silicate benchmark values were not calculated because this nutrient is less influenced by anthropogenic activities (OSPAR, 2005). Although usually monitored, it is not widely used in assessments. The use of 90th percentile for nitrate+nitrite, ammonium and phosphate also allows accounting the natural variability. To test the hypothesis that benchmarks calculated for TFZ and MZ (Group 1) are adequate for the 12 studied estuaries, complementary calculations were made for: (i) Group 2 - WFD salinity intervals (<1, 1-25 and >25) and; (ii) Group 3 - within the classic salinity gradient approach <1 (freshwater), 1-6 (oligohaline), 6-20 (mesohaline), 20-30 (polyhaline), and >30 (euhaline). The estimated nutrient benchmark for the different salinity groups are presented in Table 3. High benchmark values of nitrate+nitrite in the lower salinity of the 3 groups may be attributed to inputs from rivers associated mainly with watershed runoff. These values decreased with salinity intervals being gradual in the Group 3. Otherwise, ammonium and phosphate showed small differences between freshwater (salinity <1) and the different salinity classes of each group. For these nutrients neither group showed a clearly advantage of benchmark estimation for narrow salinity intervals. Variations were within 20%, which may be explained by exchanges with the suspended sediments or inputs from intertidal and salt marsh areas (Caetano et al., 2012). Taken into consideration the hydrological characteristics of the Portuguese estuaries where salinity of aWB may change by up to 20 units over a tidal cycle it is more adequate use only two intervals (salinity <1-TFZ and 1-35 MZ). This approach was also considered the more precautionary for nitrate+nitrite taken into account that freshwater is the main source of this nutrient (Figure S1). The proposed approach was not tested for coastal lagoons or estuaries with minor freshwater input where the seawater component may play a relevant role. Therefore, the benchmark values from Group 1 are proposed as the adequate benchmark to be used for management proposes in the Portuguese estuaries.

4.7. Water bodies' classification

For each sample the ratio of nutrient concentrations to the corresponding benchmark value (salinity <1-TFZ and 1-35 MZ) was calculated as [nutrient]/benchmark. Ratios ≤ 1.0 mean that nutrient concentration does not exceed benchmark value, and consequently the nutrient condition leads to a "High" score. Ratios >1.0 and ≤ 2.0 are proposed as "Medium" classification (or in risk). Values above 2.0 are classified as "Low" since the benchmark is clearly surpassed with consequences for the water body chemical status. On this basis, all ratios for each WB of the 12 studied estuaries were calculated. Then, the 90th percentile of all ratios of each WB was calculated and the final classification given by the "worst" score. By using this methodology, the natural variability is included and does not mask higher scores. An advantage of this methodology is the applicability with limited number of data avoiding the "negative" effect of outliers. Table 4 gives the scores for the WBs of the 12 estuaries, indicating the parameters responsible the "Medium" and "Low" scores. Nutrients in all WBs of Minho, Aveiro, Mira and Guadiana

were lower than the benchmark values and therefore a classification of "High" is proposed. Thus, concentrations of nutrients in these estuaries do not put at risk the chemical status of water bodies. Some WBs of Lima, Mondego and Arade presented "Medium" status indicating that variability of nutrients overlap slightly the benchmark values. Local sources of nutrients or morphologic characteristics may contribute to the nutrient increase. In these cases, it is proposed the intensification of monitoring programs in time and space. Water bodies of Cávado, Ave, Douro, Tagus and Sado showed "Low" score, and ammonium was the nutrient more often found above the corresponding benchmark value. Human activities are probably the main reason for the deviation. For the Tagus estuary the high turbidity may also prevent the biological consumption of nutrients in the water column (Gameiro et al., 2011). Although Cávado, Douro and Tagus were classified in "good ecological status" using the phytoplankton quality element (Brito et al., 2012), levels of nutrients were clearly high indicating minor sensitivity of this biological indicator. The increase of nutrients apparently did not lead to eutrophication symptoms like shading of seagrass and benthic algae due to the overgrowth of some fast growing of opportunistic groups. In those estuaries the nutrient sources should be identified and mitigation measures taken to decrease their load. Monitoring programs should be designed for the specificities of each estuarine system.

5. Conclusions

Nutrient concentrations in transitional waters vary due to inputs from weathering in the drainage basins and anthropogenic activities, estuarine processes and biological consumption. To overcome the inter-specificity aspects of surveyed estuaries an integrated methodology to establish nutrient benchmark values is proposed. These values allowed classifying the chemical status for nutrients in 42 WB of transitional waters in 12 Portuguese estuaries. Different measures were proposed for WB with "Medium" and "Low" scores. The proposed methodology is a straightforward tool to for the application of the Water Framework Directive and may support management actions that might lead to changes in the classification. Priority work should be focus on the improvement of knowledge in estuarine systems with scattered or poor data availability.

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Figure Captions

Figure 1 – Location of the twelve studied estuaries (Minho, Lima, Cávado, Ave, Douro, Ria of Aveiro, Mondego, Tagus, Sado, Mira, Arade and Guadiana) in the Iberian Peninsula (Southwest Europe). Limits of the drainage basins are represented by confined areas.

605 **Figure 2** – Box and whisker plots of temperature, dissolved oxygen, pH and concentration of suspended particulate matter (SPM) in Minho, Lima, Cávado, Ave,
606 Douro, Aveiro, Mondego, Tagus, Sado, Mira, Arade and Guadiana. Horizontal lines correspond to the median and the edges of the box the 10th and 90th
607 percentiles.

608
 609 **Figure 3** – Box and whisker plots of nitrate+nitrite, ammonium, phosphate and silicate concentrations in
 610 the tidal freshwater zones of seven estuaries. Horizontal lines correspond to the median and the edges
 611 of the box the 10th and 90th percentiles.

Figure 4 – Box and whisker plots of nitrate+nitrite, ammonium, phosphate and silicate concentrations in the mixing zone of Minho, Lima, Cávado, Ave, Douro, Aveiro, Mondego, Tagus, Sado, Mira, Arade and Guadiana. Horizontal lines correspond to the median and the edges of the box the 10th and 90th percentiles.

Figure 5 – Plots of nutrients (nitrate+nitrite, phosphate and silicate) concentration versus salinity in mixing zones of Tagus and Guadiana estuaries. Black dashed line represents the conservative nutrient mixing - dilution line.

628

629 **Figure 6** – Principal component analysis of hydromorphology characteristics (Morphology, Mean River

630 Flux) of the 12 surveyed estuaries (Minho-MN, Lima-LM, Cávado-CV, Ave-AV, Douro-DO, Aveiro-RA,

631 Mondego-MO, Tagus-TE, Sado-SA, Mira-MR, Arade-AR and Guadiana-GU) and physic-chemical

632 parameters (Temp., pH, O₂, Salinity, SPM, nitrate+nitrite, phosphate, ammonium and silicate) measured

633 in waters samples at surface, bottom, low, high tide, autumn/winter and spring/summer (Season).

Table 1 - Main water basin characteristics of Portuguese rivers. Estuarine systems are grouped in a grey scale accordingly to their morphology

	Morphology ^a	Annual mean precipitation (mm) ^b	Annual mean river flow (m ³ s ⁻¹) ^b	Estuarine volume (10 ⁶ m ³) ^a	Estuarine area (km ²) ^a	Watershed area (10 ³ km ²) ^a	Watershed population (10 ⁶) ^a
Minho	Narrow channel	1708	294	67	23	17	1000
Lima	Narrow channel	1780	64	19	5	2.5	80
Cávado	Narrow channel	1637	71	ne	ne	1.6	ne
Ave	Narrow channel	1522	31	ne	0.17	1.4	ne
Douro	Narrow channel	908	903	65	6	97.6	4123
Ria of Aveiro	Coastal lagoon	1501	32	84	60	3.4	700
Mondego	Narrow channel	1132	80	21	9	6.7	66
Tagus	Semi-enclosed estuary	799	315	2200	330	80	9030
Sado	Semi-enclosed estuary	669	8.7	770	160	7.7	270
Mira	Narrow channel	668	2.9	17	3	1.6	26
Arade	Narrow channel	691	ne	ne	2.1	0.97	Ne
Guadiana	Narrow channel	597	91	96	1.8	66.8	1900

^a Ferreira et al. (2003); ^b <http://snirh.pt/> and Cabral et al. (2011); ne - not estimated

Table 2 - Concentration ranges of dissolved inorganic nitrogen (DIN), phosphate (PO_4^{3-}) and silicate (Si(OH)_4) in Minho, Lima, Cávado, Ave Douro, Ria of Aveiro, Mondego, Tejo, Sado, Mira, Arade and Guadiana obtained in the present study and in other studies.

Estuarine system	DIN (μM)	PO_4^{3-} (μM)	Si(OH)_4 (μM)	References
Minho	1.3-40	0.18-2.5	0.85-85	Present study
	2.1-782	0.32-45	-	Sousa et al. (2008)
	3.1-156	0.11-11	-	Sousa et al. (2007a)
Lima	3.8-172	0.05-1.9	4.7-72	Present study
	168-589	0.97-8.7	59-77	Azevedo et al. (2013)
Cávado	7.2-134	1.1-4.7	2.9-93	Present study
	71-1600	6.4-39	-	Peixoto (2008)
	57-159	-	-	Teixeira et al. (2008)
Ave	5.6-88	0.70-2.7	2.4-93	Present study
	380-1321	2.9-31	-	Pascoal et al. (2003)
	187-280	-	-	Teixeira et al. (2013)
Douro	6.4-179	0.72-3.3	0.38-36	Present study
	66-111	1.1-1.6	39.8-68.6	Azevedo et al. (2006)
	1.6-657	0.2-6.8	0.9-257	Azevedo et al. (2008)
Ria of Aveiro	1.6-21	0.27-0.53	1.7-28	Present study
	3.0-856	0.5-137	10-144	Lopes et al. (2007)
	1.4-413	0.2-11	1.8-284	Rodrigues et al. (2012)
Mondego	5.0-61	0.090-1.0	0.79-78	Present study
	1.9-51	0.2-3.0	-	Libellø et al. (2005)
	11-40	0.77-1.4	-	Sousa et al. (2007b)
Tagus	3.8-92	0.25-7.5	48-124	Present study
	45-93	2.3-3.7	35-116	Brogueira and Cabeçadas (2006)
	0.20-182	1.4-19	3.7-258	Gameiro et al. (2007)
Sado	0.31-52	0.24-1.5	0.10-18	Present study
	3-152	1.2-2.2	7-180	Cabeçadas et al. (1999)
	14-457	16-103	-	Rocha et al. (2013)
Mira	3.5-56	0.18-0.89	1.5-87	Present study
	8-40	0.25-0.70	-	Medeiros et al. (2012)
Arade	0.41-148	0.12-1.8	0.16-90	Present study
Guadiana	0.57-79	0.06-3.4	0.98-78	Present study
	8-47	0.86-3.7	2.2-6.23	Chícharo et al. (2001)
	4.6-100	1.3-3.1	1.3-87	Domingues et al. (2005)

647 **Table 3** - 90th percentile of nitrate+nitrite, ammonium and phosphate concentration (μM) in the
648 three groups of salinity classes.

	Group 1 Salinity classes		Group 2 Salinity classes			Group 3 Salinity classes				
	<1	1-35	<1	1-25	>25	<1	1-6	6-20	20-30	>30
NO_3^- + NO_2^-	89	47	89	63	30	89	73	53	43	22
NH_4^+	18	18	18	20	15	18	18	20	24	14
PO_4^{3-}	2.8	3.4	2.8	3.9	2.4	2.8	4.2	3.6	3.7	2.1

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Table 4 - Score of the water bodies (WBs) for the 12 Portuguese estuaries. The parameters responsible for scores are H (high-green), M (medium-yellow) and L (low-red). Attributed colours are conditioned by the worst score.

Estuarine system	WB1	WB2	WB3	WB4	WB5	WB6
Minho	H	H	H	H	H	-
Lima	H	M (NO_3^-)	M (NO_3^-)	-	-	-
Cávado	M (PO_4^{3-}) L (NH_4^+)	M (PO_4^{3-}) L (NH_4^+)	-	-	-	-
Ave	M (NO_3^-) NH ₄ ⁺ L (PO_4^{3-})	-	-	-	-	-
Douro	H	M (NH_4^+)	M (NO_3^-) L (NH_4^+)	-	-	-
Ria of Aveiro	H	H	H	H	H	-
Mondego	M (NH_4^+)	M (NH_4^+)	H	-	-	-
Tagus	H	M ($\text{NO}_3^- \text{PO}_4^{3-}$)	M ($\text{NH}_4^+ \text{NO}_3^- \text{PO}_4^{3-}$)	M (NO_3^-) L (PO_4^{3-})	-	-
Sado	M (PO_4^{3-})	H	H	H	L (PO_4^{3-})	M (PO_4^{3-})
Mira	H	H	H	-	-	-
Arade	H	M (NO_3^-)	-	-	-	-
Guadiana	H	H	H	H	-	-

Figure S1 - Variability of nutrients with salinity for the Minho, Lima, Cávado, Douro, Tagus and Mira estuaries. Horizontal lines represent the benchmark values for TFZ (Transitional Freshwater Zone) and MZ (Mixing Zone).