Universidade de Aveiro Departamento de Biologia
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Bacterial Community as a Complementary Tool to the Water Directive Framework in Ecological Quality Assessment of Caima River

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Tânia Silva Vidal e co-orientação da Doutora Helena Correia de Oliveira, Investigadoras em Pós-Doutoramento do Departamento de Biologia e CESAM, e co-orientação científica do Professor Doutor Mário Verde Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

Aos meus pais e irmão

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Palavras-chave

Diretiva Quadro da Água, estado ecológico de ecossistemas de água doce, parâmetros físico-químicos, metais, macroinvertebrados, perifiton, comunidade bacteriana, citometria de fluxo

Resumo

Os sistemas aquáticos de água doce têm vindo a sofrer uma severa degradação e perda de biodiversidade, derivado de atividades humanas como a agricultura, indústria, atividades mineiras desenvolvimento urbano e alterações climáticas. Assim, a União Europeia implementou a Diretiva Quadro da Água (DQA), com o principal objetivo de atingir o bom estado ecológico em todas as massas de água. No entanto, a DQA revelou ser bastante complexa, com metodologias muito morosas e dispendiosas. Com este estudo, pretende-se desenvolver uma metodologia rápida e económica, estudando a composição da comunidade bacteriana por citometria de fluxo, como ferramenta complementar à DQA. Para a concretização deste trabalho, foram estudados 3 locais do rio Caima com diferentes tipos de impactos: a nascente – local de referência; Bustelo - a jusante de uma estação de tratamento de águas residuais e o Palhal - com escorrências provenientes de uma mina desativada, no inverno, primavera e verão aplicando a metodologia estabelecidas pela DQA usando os macroinvertebrados e perifiton como comunidades biológicas estudadas. Adicionalmente foi aplicada a análise multivariada aos dados recolhidos por citometria de fluxo à comunidade de bactérias da coluna de água e dos elutriados dos sedimentos e aos resultados das comunidades de macroinvertebrados e perifiton obtidos da DQA. No geral, os parâmetros físico-químicos, e as quantificações de metais mostraram valores mais elevados nos elutriados dos sedimentos do rio, do que na coluna de água mostrando a importância da análise desta matriz que não está contemplada na DQA. Resultados sensu DQA mostraram que nem sempre as comunidades de macroinvertebrados e perifiton foram concordantes na resposta aos diferentes tipos de impactos e que a qualidade ecológica dos locais avaliados foi melhor do que era expectável. Por outro lado, a análise multivariada das comunidades de macroinvertebrados e perifiton foi bastante discriminatória, associando elevados níveis de nutrientes e metais com organismos mais tolerantes, que se encontram em locais mais impactados, e organismos sensíveis com altos níveis de oxigénio dissolvido em locais mais pristinos. A análise da comunidade bacteriana revelou uma distinta separação entre bactérias LNA e HNA nos sedimentos, de acordo com os diferentes stresses ambientais, sendo HNA, nos sedimentos, um ótimo indicador de contaminação. Estes resultados revelam que a comunidade bacteriana oferece uma boa resolução de locais contaminados usando a citometria de fluxo como metodologia rápida de avaliação complementar à avaliação do estado ecológico sensu DQA sendo, no entanto, necessárias mais estudos aplicados a outras tipologias de rios e outros tipos de impactos para confirmar a validade desta nova metodologia.

Keywords

Water Framework Directive, ecological status of freshwater ecosystems, physicochemical parameters, metals, macroinvertebrates, periphyton, bacterial community, flow cytometry

Abstract

Freshwater ecosystems have been suffering severe degradation and loss of biodiversity, caused by human disturbances such as agriculture, industry, mining, urban development and climate changes. Therefore, the European Union reached an agreement and implemented the Water Framework Directive (WFD), with the main goal of reach a good ecological status in all water bodies. However, WFD is very complex, methodologies are time-consuming and costly. Thus, the main objective of this study is to develop a rapid and cost-effective approach, by studying the bacterial community composition by flow cytometry, as a complementary methodology to WFD. To achieve this, we study 3 sampling sites at Caima River along the seasons (winter, spring and summer), with different levels of environmental impacts (Nascente- river source- with little impact, Bustelo- downstream WWTP and Palhal- exposed to mine drainage), applying first the WFD criteria and then multivariate analysis for macroinvertebrate, periphyton and bacteria communities. Physico-chemical, metals and bacteria samples were collected from the water column and sediment river bottom, showing that in all the parameters (with some exceptions) and metals the concentrations were higher in sediments.

Results showed that not always the macroinvertebrate and periphyton communities were sensitive to an increased nutrient input, resulting in an ecological status higher than expected. On the other hand, community structure analysis for macroinvertebrates and periphyton was very discriminatory, associating high levels of nutrients and metals with more tolerant organisms in impacted sites, and sensitive organisms with high levels of dissolved oxygen corresponding to pristine environments. Bacteria community analysis revealed a clear separation of LNA and HNA bacteria in sediment according to the different environmental stress, being possible to dissociate the majority of the impacted sites from the clean sites, being HNA a good indicator of contamination. These results revealed that bacteria community in sediments has more reliable data about the impacts that a freshwater ecosystem can suffer. The discriminating power of bacteria community analyzed by FCM provided good responses, although, further investigations are needed to confirm the feasibility of this new method, as a complementary tool in the water quality assessment.

INDEX

Chapter 1 - General introduction and objectives

1.1. Hydric resources: importance and monitoring

Water is acknowledged as the basis for all existing life on the planet, acting as the cornerstone of human society's existence and development. Although 2/3 of our planet is composed of water, its major part is found in oceans, and only 1% is found in rivers and lakes. Curiously, rivers and lakes are more diversified and contain 12% of animal species while oceans contain only 7%. Many freshwater habitats and their biota are being rapidly destroyed without the possibility of being studied and protected (Gleick, 1993).

The global population growth and the increasing of the industrialization level have been creating an enormous pressure on the aquatic resources, since these are increasingly used by humans, for both industrial, agricultural and energy purposes and navigation (Maksimovic *et al*., 1996). These practices require an overexploitation of this resource, resulting in long-term consequences, such as freshwater systems degradation and contamination, affecting both its quantity and quality (WHO, 1992). The runoff of agricultural fertilizers and industrial waste that result in nitrates and toxic chemicals contamination, mine drainage that generate contamination by metal and discharges of urban effluents that originate contamination by organic matter are the main factors for the insufficient quality and quantity of freshwater (Maksimovic *et al*., 1996; Gleick, 1993; Biswas & Tortajada, 2016). Nowadays, the climate changes are an alarming issue, since they have a negative impact in the ecosystems and biodiversity, affecting them directly by temperature and flow patterns variations, and indirectly in many aspects of the lotic systems functioning (Allan & Castillo, 2007). Thus, is essential to plan and to manage the water resources in order to ensure a good ecological quality, as well as the habitats conservation as defended by the implementation of Water Framework Directive (WFD Directive 2000/60/CE) in 2000 in Europe (Altenburger *et al*., 2015). Until the early 1990s, the freshwater monitoring was based mostly on physical and chemical parameters and classified accordingly as fit or not for human consumption. WFD requires both chemical and biological analysis for an improved assessment and holistic integration on the

ecological status of freshwater ecosystems (Chaves *et al*., 2006; Hering *et al*., 2003). Therefore, the monitoring of bioindicator freshwater communities is a sensitive tool to detect stress and contamination that may occur during a period of time and to detect differences from one place to another in rivers and small catchments (Li *et al*., 2010).

1.2. Water Framework Directive

1.2.1. Assessment of water quality in rivers

The Water Framework Directive (WFD) is the main legislation regarding the protection and sustainable utilization of European hydric resources, and it established that Member-States must protect, improve and recover all the water bodies, in order to reach good water quality by 2027 (Arle *et al*., 2016; Poikane *et al*., 2014). The criteria for their classification plays a key role in the WFD implementation process, defining the ecological state of a water body from the following elements of water quality that presents worse classification:

- **Biological quality elements** (macroinvertebrates and periphyton);

Bioindicators are organisms, chemical markers or biological processes that when altered, indicate environmental changes. These changes allow us to evaluate the influence of environmental stress on the composition and functioning of ecosystems and to study trends, by monitoring them with repeated measurements over time (Markert *et al*., 2003).

Macroinvertebrates are used since the creation of the Saprobiensystem in 1908 (for organic pollution detection) as a bioindicator community. This methodology created for water quality evaluation developed the first biotic indices, specially designed for organic contamination, making use of indicator organism concept, in which an organism can indicate clean or polluted conditions. Macroinvertebrates have been used worldwide by environmental agencies in bioassessment and were modified and improved especially within the AQUEM and STAR project developing alternative approaches to assess different types of stressors by defining type-specific multimetric indices. The advantages of macroinvertebrates use are the sensitivity to pollution and rapid response to external disturbances, relatively long lifes, having the capacity to integrate the effects of the

environmental variations in the short-term, providing information to understand the cumulative effects (Cummings, 1996; Sharma & Rawat, 2009). Another community widely used for freshwater environments monitoring is periphyton, and especially diatoms, which are considered the best bioindicators for having a fast response to environmental changes and for integrating environmental conditions better than any other organism. Because they have short life cycles and high reproduction rates, they reflect short-term impacts and sudden variations in the environment. As organisms that usually cling to the substrate, their growth and development can directly respond to physical, chemical and biological variations (INAG, 2009; Giorgio *et al*., 2016; Li *et al*., 2010). In spite of not being present in the Portuguese WFD, other types of bioindicator communities like fishes and macrophytes were proposed for water quality assessment in the European WFD. But unfortunately, European states members did not reach a consensus yet about their use and the major obstacles like fish mobile behavior towards contamination scenarios and species-poor plant standards, besides the high number of metrics (Birk *et al*., 2012).

- **Chemical and physico-chemical elements supporting the biological elements including general physico-chemical quality elements, and specific pollutants**;

According to the WFD, these parameters are essential for the ecosystem balance and water quality maintenance. These parameters ensure the water quality for human consumption, industrial and irrigation, but also represent an important role in life support, creating an integral part of the metabolic processes involved in the development of biological activities. Abiotic chemical and physico-chemical elements are used to define the stream types which were an essential basis for the development of assessing systems. Stream types might serve as units which shows a certain biotic and abiotic discontinuity in comparison to neighboring entities. The most important abiotic factors are stream morphology, geochemistry, altitude, stream size and hydrology that are used to define stream typology which in turn are used to define more than 100 stream types across Europe. Regarding the values of specific pollutants and priority substances (metals, pesticides), they are published in European Commission chemical status documents. In the case of priority substances whenever the values quantified were above threshold

recommended it was immediately considered as bad quality (European Commission, 2000; Patil *et al*., 2012).

- **Hydromorphological elements supporting the biological elements**;

This criteria is evaluated by the hydrological regime, which is defined by variation of the seasonal distributions of the watercourses and reflect the regional climatic patterns, river continuity and morphological conditions, which are related to the variation of river depth and width, riverbed structure and substrate as well as the composition and structure of the riparian zone.

All these parameters are important to assure an abiotic support essential for the establishment of numerous species. Dramatic changes of these features can cause significant losses in stability and diversity of biological communities, as well as the depletion of the structure and functionality of these ecosystems (INAG, 2009; Quercus, 2016). The River Habitat Survey was the method chosen for Europe-wide application and support the collection of a large amount of qualitative and quantitative geomorphological data on 500 meters alongside the river sample unit surveyed. Summarizing complex information, the Habitat Quality Assessment (HQA) and Habitat Modification Scores (HQS) indices quantify physical habitat quality and richness and the degree of morphological degradation (Szoszkiewicz *et al*., 2006).

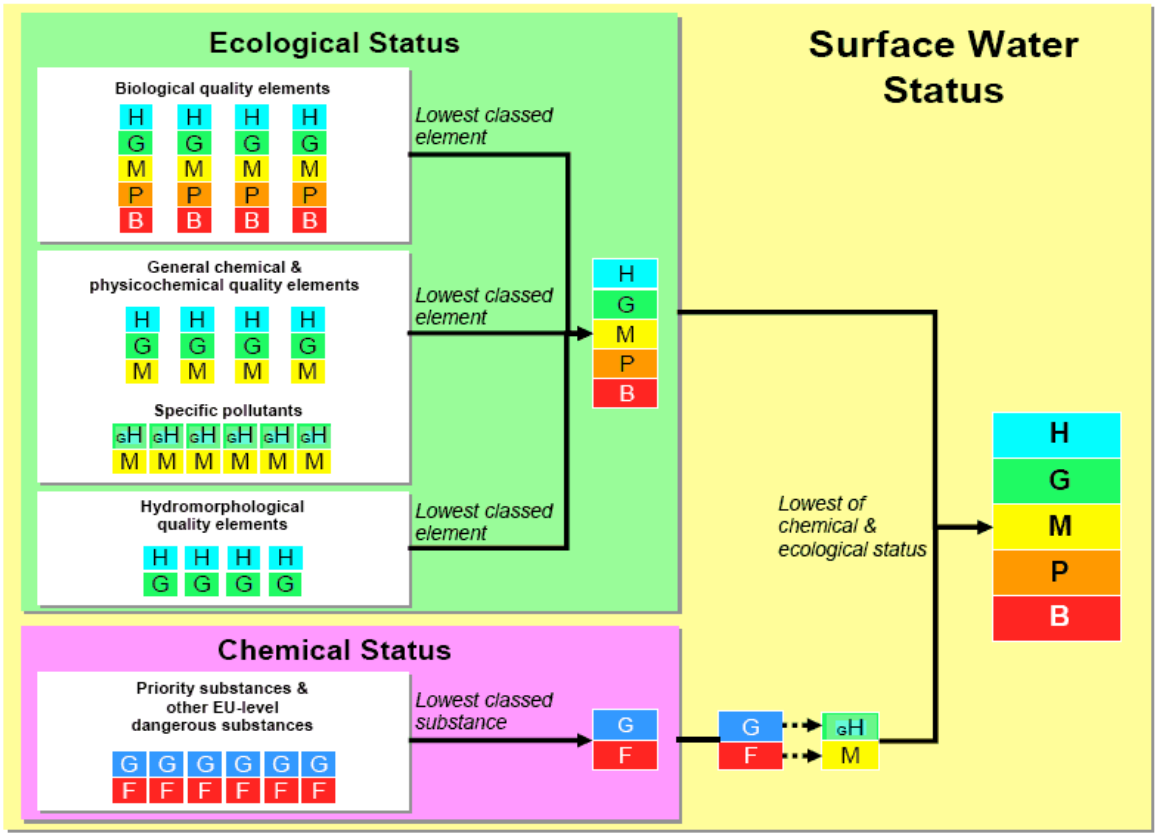

Figure 1-Scheme of the classification system under Water Framework Directive/Water law (Devlin *et al*., 2013).

After the evaluation of the biological, chemical and hydromorphological, the lowest classed elements overlap higher class maximizing the protection of the most sensitive community of freshwater environment (one out all out) (Fig 1). Generally, the site being evaluated is compared against the reference status (for the same water mass typology) based on the Ecological Quality Ratio, which is a ratio between reference conditions and the measured status of the biological quality elements and are given in five classes: high status (no difference to reference conditions), good status (slight differences), moderate status (moderate differences); poor and bad status (major differences from reference conditions). Good ecological status are the target value that all surface water bodies have to achieve in the near future. The establishment of the reference conditions for each typology is essential for the ecological status evaluation. Reference conditions must reflect totally or nearly undisturbed conditions for hydromorphological elements, general physical

and chemical elements and biological quality elements; concentrations of specific synthetic pollutants should be close to zero or below the detection limit of most advanced analytical techniques in general use and concentration of specific non-pollutants, should remain within the range normally associated with background levels. In many rivers and streams, a true reference condition cannot be found. It was suggested modeling, find historical data on old archives, near sites with the same typology to complete the information about the sampling sites (Nijboer *et al*., 2004).

Finally, the WFD is mandatory for all water bodies, rivers, lakes, transitional waters and coastal waters. WFD and the ecological status evaluation are, nowadays, challenged to deal with new sources of pollution that becomes more widespread and complex due to the effects of combined climate change and stressors (Lücke & Johnson, 2009).

1.2.2. Criticism to the WFD methodology

The WFD has been a huge change in the paradigm in the biomonitoring of European aquatic ecosystems. It has changed the management objectives from merely pollution control to ensure a more holistic perspective of protection of ecosystem integrity. Deterioration and improvement of ecological status are defined by the response of the biota, rather than by changes in environmental parameters. However, the methods has been more complex, Member States have spent resources and considerable time to develop tools to prepare river basin management plans (Hering *et al*., 2010). As an obligatory measure to WFD, biological communities has been widely studied, being its identification/classification both difficult and time consuming, while requires also very specialized work to sample (macroinvertebrates and diatoms communities) (Bertrand *et al*., 2006).

In Europe, bioassessment methods differ geographically, from region to region, different species may occur; relevant stressors may differ and applicable taxonomic resolution also may vary according to the knowledge of the regional fauna and flora. Additionally, each EU-member preferred developing country-specific methods, either to continue using existing times series by adapting their national methods to the WFD or to

regard for specific ecoregional and biogeographic situation; therefore a multitude of methods result instead of a handful of methods applicable Europe-wide (Birk *et al*., 2012). Moreover, not all assessment methods have been harmonized yet, since the information gathered could be done in countless ways: sampling can be performed with different equipment, data can be collected with different methods, identification of organisms can be to different taxonomic levels and based on different keys and comparison of methods hampered by completely different metrics and/or assessment concepts, making difficult the procedures for class boundary setting. In Europe, the bioassessment methods also differ geographically, as organism response to stress may vary by region and ecosystem type, different species may occur, relevant stressors may differ and applicable taxonomic resolution may vary (Poikane *et al*., 2014). The free access to chemicals products and licensing of new chemical products with multiple usages and little knowledge of impacts in terms of chemicals mixtures to the biological communities, was another important criticism to WFD (Altenburger *et al*., 2015). The current regulation of environmental quality is mostly based on a limited number of single chemicals leaving unprotected the ecosystems of interactions among chemicals lowering significantly the safety threshold values known and threatening water systems and the biological communities. As an example, more than 100 000 chemicals are registered in EU, where 30 000 to 70 000 are in daily use (Loos *et al*., 2008) not to mention the transformation products and the products that will enlarge the number chemicals products. It's expected that a fraction of those will be found in the environment and water systems. Therefore, to safeguard the environment protection and biodiversity the exposure to chemicals and chemicals mixtures must be minimized and efforts must be engaged in assessment and management of risky mixtures. Risk assessment approaches based on bioassays, biomarkers are integrative techniques for response assessment that allow diagnosis of the degree of impact of toxic chemicals. The bioassay integration in regulatory water quality monitoring is recommended and supported by the fact that many of these methods were published as harmonized standard protocols as OECD guidelines and ISO standards. *In vitro* and *in vivo* bioassays and biomarkers have been successfully used in monitoring programs by OSPAR (Oslo-Paris Commission) for marine and estuarine environments (Brack *et al*., 2017). The use of ecotoxicological tools in toxicity

profiling is a first tier approach for screening the hazards of complex environmental mixtures knowing or without knowing the active constituents. The toxicity profiles can be used for prioritization of sampling locations and for establishing cause-effect relationships by identifying the pollutants responsible for the observed toxicity closing the gap between ecology and chemistry (Brack *et al*., 2017; Martinez-Haro *et al*., 2015). This was the major criticism towards the WFD, not knowing exactly what caused the changes translated into the biological communities response. Several authors in literature, already suggest other types of bioassays to evaluate: the function of ecosystems (leaf litter bioassays e.g. Pascoal *et al*., 2003; Young & Collier, 2009); daphnia *in situ* bioassays and biomarkers (Damasio *et al*., 2008); biomarkers with macroinvertebrates caddisfly *Hydropsyche exocellata* (Prat *et al*., 2013).

In Portugal, the main difficulties are caused by the absence of simultaneous monitoring for biological elements and physicochemical parameters, the lack of long-term monitoring data that allow distinguishing natural changes from anthropogenic changes, the scarcity of standardized and systematized data for biological elements and the nonexistence of qualitative and quantitative monitoring networks with adequate spatial representativeness (Mendes & Ribeiro, 2014).

1.3. Bacterial communities in freshwater ecosystems as potential bioindicator?

The biogeochemical importance of bacteria in freshwater ecosystems was first recognized in the 1940s by Lindeman (1942). Since this early acknowledgment of the critical role of bacteria in regenerating and mobilizing nutrients in freshwater food webs, it has become clear that aquatic bacteria drive transformations and the cycling of most biologically active elements in these ecosystems (Newton *et al*., 2011). Furthermore, bacterial communities play an important role as principal degraders and remineralizers of organic compounds to their inorganic constituents; they contribute to the breakdown/transformation of organic material, the recycling of several key elements as nitrogen, phosphorous, and sulphur and they are also responsible for breakdown and detoxification of a variety of pollutants (Sigee, 2004; Allan & Castillo, 2007). These

transformations form a crucial relation within ecosystems as they serve as an important food source for higher trophic level organisms (e.g. aquatic invertebrates) (Pernthaler & Amann, 2005; Findlay, 2010).

In pursuit for an alternative biological community for WFD ecological quality assessment and rapid methodology to analyze its results, this Thesis is devoted in trying to use bacterioplankton and bacteria biofilm, in sediment of river bottom, as bioindicator of changes in water quality using a rapid screening methodology. As already mentioned bioindicators used to assess the biodiversity of freshwater ecosystems have been mostly confined to macro-organisms such as benthic macroinvertebrates (Klemm *et al*., 2002), algae (Omar, 2010), fishes, amphibians and periphyton (Li *et al*., 2010). The majority of microbial studies in aquatic ecosystems have focused on indicator bacteria related to fecal and organic pollution but is essential a profound understanding of microbial community composition, seasonal dynamics and the influence of environmental factors to support the restoration and conservation of water quality and ecosystem health (Wang *et al.*, 2016; Zhang *et al*., 2012).

Bacteria have short life cycles and are very sensitive to changes in the environment and so they have the potential to offer an early indication of shifts in the ecosystem before macro-organisms respond (Peter *et al*., 2011; Pernthaler, 2013). Factors such as temperature (Adamczuk *et al*., 2015), nutrient concentrations (Wakelin *et al*., 2008), salinity (Dai *et al*., 2013) and heavy metals (Yang *et al*., 2013) have been found to modify bacterial communities in river water.

Recent advances in molecular biology and the development of new tools and techniques allowed the improvement of new insights through the application of fingerprinting methods as DNA based methods, using molecular 16S rRNA-based cultivation-independent approaches e.g. denaturing gradient gel electrophoresis (DGGE) (De Figueiredo *et al*., 2012a), sequencing (Xie *et al*., 2016), pyrosequencing (Kaevska *et al*., 2016) and other techniques such as fluorescent in situ hybridization (FISH) (Lupini *et al*., 2011) and flow cytometry (FCM) (Elhadidy *et al*., 2016), that today are crucial to study bacterial communities composition in riverine water.

Despite the recent efforts to study these communities, the specific factors that drive temporal and spatial variations in bacterial community structure are poorly understood because different influencing factors are observed in different studies.

In Europe, most of the published work focuses on identifying the bacterial community composition (BCC) and its relationship to physico-chemical and spatial variables. For example, (Boi *et al*., 2016) studied the BCC along a river impacted by different sources of pollutants concluding that although bacterioplankton abundance varies among the seasons, the differences along the flow path have shown to be more effective to explain the abiotic and biotic variability of the riverine water quality. Another study conducted by Read *et al*., (2015) aimed to understand how BCC responds to anthropogenic pressures across a major river basin. Their results revealed that BCC was more related to spatial parameters than physical and chemical variables since they were unable to identify a physico-chemical parameter that suggests that the most polluted rivers shared relatively similar communities. Other studies address the question of how wastewater treatment plants (WWTP) affects the bacterial communities, suggesting that microbial diversity may be affected by nutrients, ammonium and phosphorus concentrations, organic matter content and by the degree of pollution (Haller *et al*., 2011; Perujo *et al*., 2016; García-Armisen *et al*., 2014).

In Portugal, to the best of our knowledge, only two published works report the study of bacterial community composition relative to water quality. According to de Figueiredo *et al*., (2012a), the spatial variation of BCC along the Cértima River (a small Portuguese river markedly impacted by agriculture) depends mostly on parameters such as total suspended solids (TSS), total organic carbon, organic matter, electrical conductivity and HCO_3^-. Another work aimed to discover if there was a biogeographical pattern for BCC of 20 Portuguese water bodies under a severe summer drought. They found bacterial phylotypes common to several water bodies, suggesting a transversal persistence over the country, under severe drought conditions (De Figueiredo *et al*., 2012).

Since there is very little knowledge about the bacterial communities composition in Portuguese rivers, we decided to make a generalist approach to study the composition and distribution of bacterial communities in river bottom sediment biofilms and water column

by FCM and DGGE. These techniques were chosen for being relatively inexpensive, sensitive and simple to study bacterial communities in the water column and sediment biofilm. Both water and sediments microorganisms perform key ecological functions, however, microorganisms suspended in currents are only an instantaneous indicator of water quality whereas biofilm communities are relatively sessile and therefore are more likely to be an efficient indicative of local conditions (Ibekwe *et al*., 2016; Beier *et al*., 2008).

1.4. Study of bacterial communities by Flow Cytometry

Flow Cytometry (FCM) was developed in the 1960s and was first applied to mammalian cell counting and analysis. It was only in the late 1970s that this technique began to be used by microbiologists (Bailey *et al*., 1977), being limited due to the non-specific binding of fluorescent dyes and low instrumental sensitivity. The development of molecular techniques such as PCR and DNA sequencing has led to the use of independent culture methods for the evaluation of microbial communities as well as the FCM which provides detection and analysis of uncultured cells with high precision (Wang *et al*., 2010; Hammes & Egli, 2010).

FCM is a technique that allows counting, examining and classifying microscopic particles suspended in a liquid medium, which aligned will intersect a light beam, usually a laser, that will allow the particles (cells) to be analyzed according to the light scattering in different angles (Figure 2) (Paul, 2001).

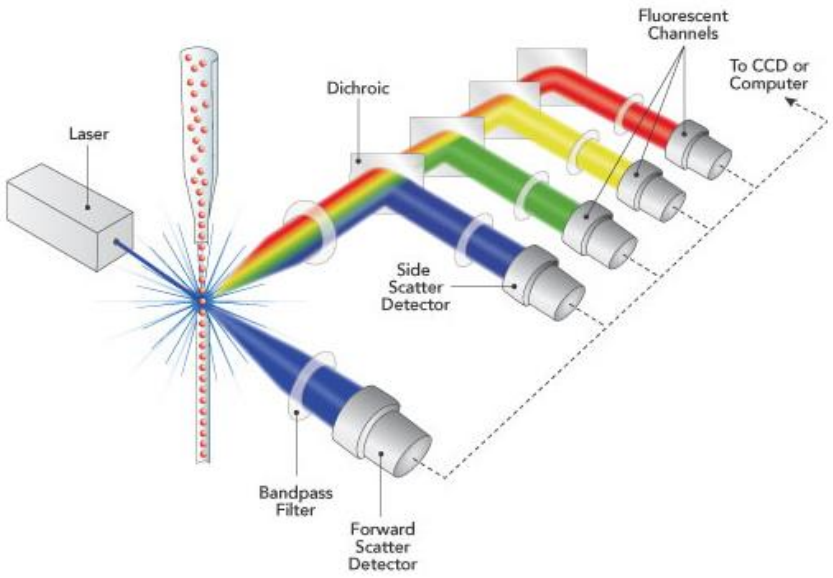

Figure 2- The basic components of a flow cytometer. Retrieved from https://www.sem-rock.com/flow-cytometry.aspx

When the cell suspension is injected, it crosses the flow sheath, cell by cell through the beam that is perpendicular to the flow. The single passage of the cells is obtained by hydrodynamic focusing of the sample stream and is injected into a buffer solution which, by encountering different pressure and velocity of the sample allows the flow to proceed under a laminar regime. When intercepting the cell, the light beam can undergo forward scattering (FSC), side scattering (SSC) and excitation of the fluorochromes. The FSC corresponds to the particle size and is detected by a set of photomultiplier tubes or photodiodes when the light is deviated up to 20˚ from the laser axis. Typically, the larger the cell, more light will be forward scattered. The side dispersion of light is obtained when the radiation is diverted to 90˚ by lenses, dichroic mirrors and optical filters and sent to photomultipliers. The SSC provides information on complexity, such as granularity and internal structures of cells. The photon flux from the photomultipliers is converted into an electrical signal and then analyzed with appropriate software (Paul, 2001; Hammes & Egli, 2010; Koch *et al*., 2014).

When the objective is the detection of specific target cells it is necessary to use fluorescent dyes that are excited by the light beam emitting fluorescent light (Givan, 2001; Wang *et al*., 2010). FCM is able to measure thousands of particles per second, while producing multi-parametric data related to light dispersion properties and fluorescence. Some fluorescent stains such as SYBR® Green I and SYTO 9, 13 binds preferentially to nucleic acids (Vives-Rego, 2000; Zipper *et al*., 2004; Falcioni *et al*., 2006), making it possible for FCM to measure bacterial concentrations. Moreover, the fluorescence intensity of such stain is directly related to the amount of nucleic acids present in the treated sample, i.e, fluorescence intensity recorded for one labeled bacterial cell should be directly related to its nucleic acid content, which is dependent on both the type of bacteria as well as its physiological state (Günther *et al*., 2008; Prest *et al*., 2013; Liu *et al*., 2016). Based on the clearly different fluorescence intensity and SSC signals detected by FCM in combination with nucleic acid stains, bacteria have been broadly classified into two groups: low nucleic acid content (LNA) bacteria and high nucleic acid content (HNA) bacteria, thus creating a bacterial community "fingerprint" (Liu *et al*., 2013; De Roy et al., 2012; Liu *et al*., 2016; Romdhane *et al*., 2014). Thereby, FCM fingerprints provide information on the bacterial community characteristics and are a sensitive method for detecting small changes and shifts within the bacterial community, that are not reflected in cell concentration measures (Van Nevel *et al*., 2017; Prest *et al*., 2014).

FCM has been shown to be a potential tool for monitoring and rapid assessment of water quality due to their numerous advantages and applications. The main advantages of FCM are fast analysis (50000 cell/s), high accuracy (<5% instrumentation error), no DNA extraction needed, sensitivity (detection as low as 100 cells per milliliter), multi-parameter analysis and compatibility with a diversity of staining and labelling methods providing broad information at the single-cell level (Wang *et al*., 2010; Chantzoura & Kaji, 2017; Hammes & Egli, 2010; Prest *et al*., 2013). However, FCM also has limitations such as being restricted to liquid sample analysis, while soil and sediment samples require special pretreatment (e.g. suspension in the liquid phase, sonication and permeabilization), sophisticated data analysis and relatively high detection limit for certain bacteria *(*Wang *et al*., 2010; Hammes & Egli, 2010).

In this work, we used a Bacterial Counting Kit (Molecular Probes™, Invitrogen) which includes an SYTO® BC dye, a high-affinity nucleic acid stain that easily penetrates both gram-positive and gram-negative bacteria as well as a calibrated suspension of polystyrene microspheres that serve as a reference standard to indicate sample volume. The collected samples were then subjected to a bacterial DNA extraction procedure analyzed by denaturing gradient gel electrophoresis (DGGE).

1.5. Study of bacterial communities by Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA gene

DGGE tool was used to compare and complement the bacterial community analysis obtained by flow cytometry. Denaturing gel electrophoresis applied to Microbiology is studied since the publications of the pioneering works of (Muyzer *et al*., 1993).

The development and application of molecular techniques based on PCR, such as the establishment of clone libraries and DGGE of 16S rRNA gene sequences, revealed that the bacterioplankton community is constituted of many bacteria that had not been detected by culture-based techniques (Muyzer *et al*., 1993; Muyzer, 2000). This fingerprinting method has been applied to environmental samples over the last decades, (Araya *et al*., 2003; Selje *et al*., 2005; de Figueiredo *et al*., 2012; Ke *et al*., 2015) being now widely adopted in the field of bacterial ecology, enabling the simultaneous analysis of numerous samples and to compare temporal and spatial patterns (Wang *et al*., 2016; de Figueiredo *et al*., 2012).

DGGE allows the separation of small polymerase chain reaction products, up to 400-500 bp. This technique is based on the extraction of total genomic DNA directly from the sample and amplification by PCR of a variable zone of the gene encoding the RNA of the subunit bacterial ribosome (16S rRNA), using universal primers for conserved areas of this gene (Muyzer, 1999; Fromin *et al*., 2002). Because these products all have the same size, they are separated according to their melting temperatures that can be achieved in polyacrylamide gels containing a gradient of DNA denaturants, typically a mixture of urea and formamide. PCR products enter the gel as a double-stranded molecule, as they proceed through the gel, the denaturing conditions progressively become stronger. PCR products

with different sequences, therefore, start melting at different positions in the gel due to their G + C content and distribution in the DNA sequences (e.g. GC-rich domains melt at higher temperatures). Melting proceeds in so-called melting domains. Once a domain with the lowest melting temperature reaches its melting temperature at a particular position, a transition from a double-stranded to a partially melted/dissociated molecule occurs and migration of the molecule will practically halt. However, the presence of a high melting domain (a GC clamp added to one primer) prevents the complete strand separation (Top, 1992). The final result is a gel with a pattern of bands which is a visual profile of the most abundant species in the studied microbial community. These communities profiles can be further analyzed with statistical methods. This approach permits the monitoring of changes in microbial communities over time and/or in response to changes in environmental conditions and it can be used also as a semi-quantitative method to estimate species abundance and richness (Paul, 2001; Fromin *et al*., 2002; Marzorati *et al*., 2008).

The major advantages of DGGE over other profiling techniques is that it is possible to excise band from gel for amplification and sequencing and also analyze a large number of samples simultaneously. On the other hand, it is difficult to compare between gels, it is time-consuming, does not allow direct taxonomic identification and different DNA sequences of different bacteria can display the same separation as a result of the same GC contents (El Sheikha *et al*., 2012; Douterelo *et al*., 2014; Tabit, 2016).

1.6. Objectives and structure of the dissertation

The present work intends to test if the bacterioplankton and the river bottom sediment bacteria of lotic freshwater environments could be used in bioassessment of ecological status within the WFD using flow cytometry and DDGE analysis. In order to achieve that several other goals, more specific, related with the work were developed:

i. To search for 3 sampling sites that combine the conditions requested to perform the study.
ii. To sample and analyze the macroinvertebrate and periphyton communities on 3 sampling sites selected from Caima River during winter, spring and summer following WFD analysis.

iii. To compare the results obtained from the river water and bottom river sediment bacteria community by flow cytometry and DDGE analysis, on 3 sampling sites of Caima River.

iv. To compare all information from macroinvertebrate, periphyton and bacterial information to infer the suitability of the last as bioindicator of ecological status.

The first mentioned objective was related to the careful search of the sampling site in order to full address the purpose of the study. Some literature and field work were requested to search for the ideal combination of sampling sites that provide the gradient of increase of bacteria community that will allow validating our theory. After the choice of the 3 sampling sites, sampling started (ii) at winter season following the Portuguese WFD methodology and the macroinvertebrates and periphyton communities were sampled. The same procedure was repeated in spring and summer (autumn was collected but there was no time to process all the samples and having the data available on time to compare with the other seasons). The WFD analysis allowed the classification of the ecological status of sampling sites chosen. The objective iii) was related with the novelty of this work by applying flow cytometry to study of bacterial community present in the river water column (bacterioplankton) and also bacteria in the river sediments. Furthermore, the results were compared with the DGGE analysis of the same communities from the 3 sampling site during the 3 seasons. This study will enable to understand if the bacterial community will be influenced by seasonality and exactly how it affected the bacterial communities. Regarding the last objective iv) all the information gathered will be analyzed by multivariate analysis (CANOCO 4.5 software – Scientia Software) in order to extract relationship among the environmental variables and the biological communities (macroinvertebrates, periphyton and bacterioplankton) along the sampling sites.

In order to address the objectives described, the dissertation was divided into **three chapters**:

The present chapter (**chapter 1**) is essentially a literature review covering all the requested information to understand the purpose of the work developed. It started with the presentation of the importance of the WFD, in Europe, and the need in protect and

maintain the good ecological status of all lotic, lenthic and transitional environments. It explains generally how the evaluation was done and the biotic communities employed and some criticism pointed as all the detailed information on the two techniques used to study the bacterial community as a bioindicator of ecological status. **Chapter 2** present the integrated results of the WFD and bacteria community analysis by multivariate analysis of the data obtained. This chapter was built following the specific layout usually used in journal articles, with the purpose of submitting this work to a specialized international journal. At last, **chapter 3** consists of the final remarks and integrative discussion of all the results.

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Chapter 2 – Spatio-temporal variation of bacteria content using flow cytometry as a complementary tool to Water Framework Directive assessment of Caima River

2.1. Introduction

Freshwater environments are hotspots of biodiversity, containing 6-10% of all species and one-third of all invertebrates species worldwide. Freshwater ecosystems have been suffering severe degradation and loss of biodiversity due to the overexploitation of this resource, caused by human disturbances such as agriculture, industry, mining, urban development and climate changes making them prone to degradation (Maksimovic *et al*., 1996; Allan & Castillo, 2007). Freshwater resources are essential for sustaining human existence and the alterations of river, lakes and wetlands have defined the economic development for centuries (Pander & Geist, 2013). The European Union reacted to the predicted loss of biodiversity and decreasing of human well-being by approving the Water Framework Directive (WFD) in 2000. WFD was probably one of most significant and ambitious legislative instrument in the water field on an international basis for all European countries. Its main goal was to achieve sustainable water resources management and development across national and regional borders and achieve the "good ecological status" for all water bodies (watercourses, lakes, coastal waters, groundwater) (Albrecht, 2013) by integrating both chemical, biological and hydromorphological quality elements. A key component of WFD legislation was the definition of "good ecological status" that has to be reached by 2015 by all water bodies, except "heavily modified" (Erba *et al.,* 2009). However by the end of 2015 47% of EU surface waters did not reach "good ecological status" (Birk *et al*., 2012) and Member states availed themselves to extend beyond 2015 to achieve all WFD environmental objectives by the end of 2027 (Voulvoukis *et al*., 2017). This has led to the WFD effectiveness questioning as a policy tool with many reviews highlighting drawback and weaknesses (Birk *et al*., 2012; Altenburger *et al*., 2015; Brack *et al*., 2017). The major criticism was related to the multitude of methods used instead of a handful of methods used Europe-wide. The lack of cause-effect relationship in ecological quality assessment within the WFD improving the capacity of ascertaining the causes that

produced the unwanted changes in the biological communities closing the gap between ecology and chemistry (Martinéz-haro *et al*., 2015). WFD would benefit from the multidisciplinary approaches integrating multiple lines of evidence, as an example of ecotoxicological line of evidence, in biological approach (Vidal *et al*., 2012; Birk *et al*., 2012; Martinéz-Haro *et al*., 2015). Another criticism to the WFD was the requirement of highly specialized technicians, elevated costs, time-consuming methodologies and historical data of freshwater environments. In Portugal, like in many other Mediterranean countries, the lack of historical data to describe the reference conditions which are fundamental for WFD, in ecological status evaluation, since Ecological Quality Ratios (EQR) are used to compare the results obtained with pristine/reference conditions for the river typology, in evaluation. The human impacts are so intense and widespread that reference sites satisfying the criteria for minimal disturbance do not exist (Chaves *et al*., 2006). In order to overcome some difficulties of WFD concerned with time-consuming and elevated costs, we propose a rapid evaluation of bacteriological community in the water column and river bottom sediment, using complementary methodologies based on flow cytometry (FCM) and denaturing gradient gel electrophoresis (DGGE) analysis. Bacterioplankton plays a critical role in the ecological function regulating a broad array of chemical transformations (e.g. decomposition of organic matter) which sustain the balance in aquatic ecosystems. Bacterioplankton are highly dynamic in composition and structure and respond quickly to different environmental gradients across ecosystems which can influence the water quality (Sun *et al*., 2017). This approach used a rapid and fast response technique that will allow starting the characterization of the bacteria communities samples from Portuguese rivers, posteriorly complemented with DGGE analysis and its potential biological indicator value and compare it with the commonly sampled macroinvertebrate and periphyton communities. If this methodology were successfully validated and a correspondence between bacterial communities present and ecological status were established, it will allow a first general picture of the ecological status of the studied water bodies, in about one or two days, from the sampling moment. FCM and DGGE are becoming more and more affordable and frequent in use, for rapid characterization of water bacterial assemblages,

requiring equally specialized technicians to operate those equipments but require less time and money.

Caima River was chosen as case study because it is recipient of point source and diffuse contamination by organic compounds and metals (Nunes *et al*., 2003; Vidal *et al*., 2012) being an important tributary of the Vouga River in Vouga River basin (central western Portugal) which is an important source of drinking water and irrigation in the region (Nunes, 2007). Specifically, Caima River passes through urban and agricultural areas being affected by diffuse and organic pollution in its upper section and wastewater from WWTP and metal-rich run-off from deactivated Palhal mine in the lower section. The main metals founded in this mine was copper and lead (Nunes *et al*., 2003; Vidal *et al*., 2012). Vidal *et al.,* (2012) showed that sediment elutriates obtained from the sediments river bottom of Palhal sampling site are rich in lead, cadmium, zinc and copper and affect standard organisms in ecotoxicological tests. The present study intends to evaluate the ecological status of three Caima River sampling sites by using the WFD approach based on macroinvertebrates, periphyton and additionally bacteria communities. Bacterioplankton and bacteria from river sediments were collected simultaneously and were analyzed by FCM and DGGE analysis to evaluate the possibility of bacteria community being a rapid bioassessment tool to complement the WFD methodology working as a bioindicator of ecological status and to compare the information generated. Samples were collected seasonally (winter, spring, summer) to evaluate not only the capability to respond to different stressors but also to evaluate how bacterial communities changes with seasons and different physico-chemical characteristics of the sampling sites.

2.2. Material and methods

2.2.1. Study area and collection of samples

Caima River is a tributary of Vouga River with approximately 50 km long. The river source is at Serra da Freita (Northern Portugal), located in Albergaria da Serra at 900 m of altitude, and flows to the right riverside of Vouga River, located in Albergaria-a-Velha. Caima River has some sections of unmodified margins in forested paths and

modified/heavily modified when the river crosses the villages. The riverbed at the source was composed mainly of very large rocks and hence more different than the other two sampling sites being mostly composed of boulders, cobble, pebble and some gravel. Site 1 was located near the river source-Albergaria da Serra (40˚52ˈ3.734ˈˈN, 8˚16ˈ22.199ˈˈW) and has minimum human disturbance. Between the time that the sampling start to be visited and the start of sampling, strong raining events dragged ashes from summer fires into the river course. Site 2 was located strategically after the effluent from a WWTP – in Bustelo do Caima where domestic and industrial waste (40˚48ˈ21.258ˈˈN, 8˚26ˈ44.581ˈˈW) were dumped into the river. Site 3 was located bordering a deactivated mine- Mina do Palhal (40˚44ˈ31.132ˈˈN, 8˚27ˈ16.222ˈˈW - inactive since the 1920s) that still drains-metal rich effluent into the river in raining season. Sampling was carried out in January (winter); April (spring); September (summer) of 2017. Their codes and location are shown in Fig 3.

General chemical and physical characterization was carried out *in situ* for each sampling site: pH, temperature (˚C), conductivity (µS cm^{-1}) and dissolved oxygen (% and mg L^{-1}) using multiparameter water quality probe Aquaprobe AP-2000 (Aquaread®). Six L were collected from surface water, 3L of them were used for further characterization (see Laboratory analysis) and the other 3L were collected in specific pre-treated water bottles for DGGE analysis. Additionally, 3 sterilized urine containers (60ml) were used to collect the water samples, in triplicate, for FCM analysis. Sediments were collected, from each river site, constituting composed samples whenever as possible from sediments of the river bed. The sediments were collected from the upper layers of river bed into plastic (about 3 kg) airtight bag after collection and used for elutriate production (see Elutriates production) and posterior physico-chemical characterization and metal analysis. Both water and sediment samples were transported to the laboratory in the dark at 4˚C.

Macroinvertebrates sampling was done as recommended by Portuguese Water Institute (INAG, 2008) according to the presence of microhabitats. Benthic macroinvertebrates were obtained using a hand net (500 µm pore size; square frame 0,30 × 0,30 m), by kick-sampling small transect covering similar area and sampling effort across sites the substrate so that the macroinvertebrates detached and entered the net by the

action of the stream water. Collected samples were placed into plastic containers and preserved with 96% of ethanol (Hauer & Resh, 1996).

Periphyton sampling was done as recommended by INAG (2008a). Small-sized rocks were scraped with a hard toothbrush for removal of the top diatoms biofilm. They must be sampled at least 5 stones to represent an area of approximately 100 cm^2. The samples were preserved with Lugol solution and transported to the laboratory at 4˚C.

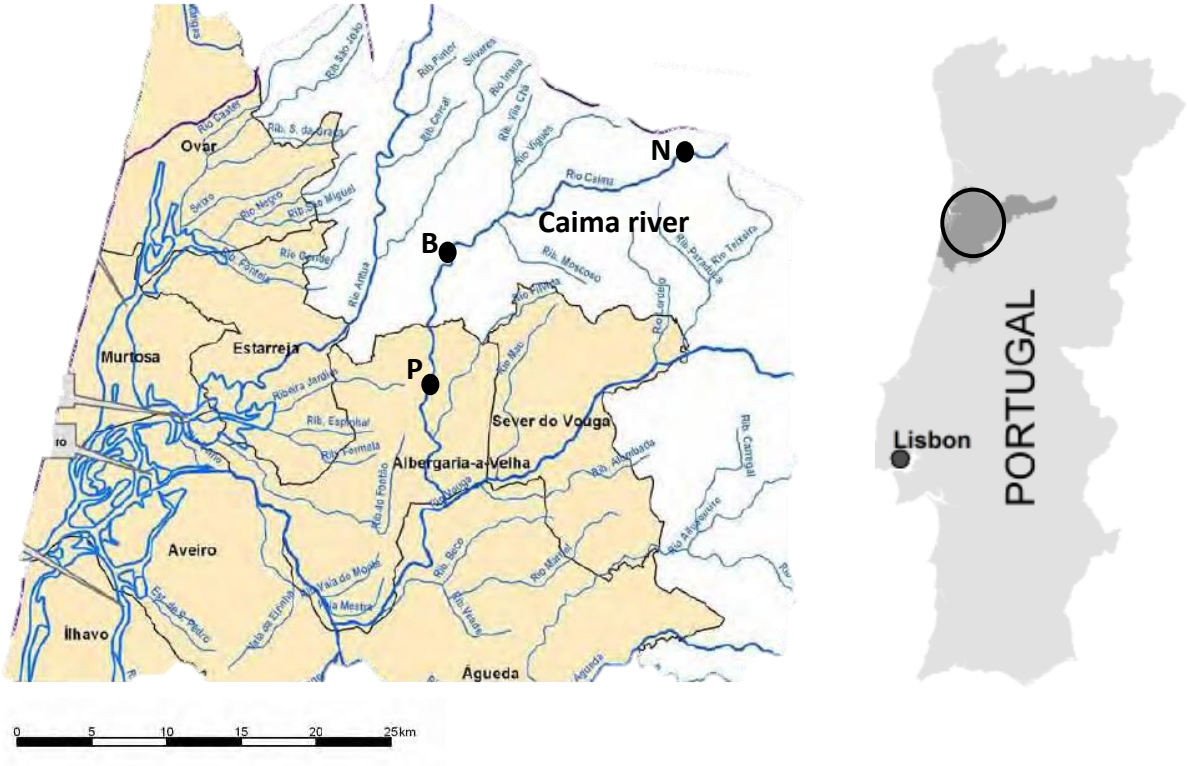

Figure 3- Location of sampling sites along the Caima River, Site 1- N (Nascente) river source; site 2- B (Bustelo) downstream WWTP and site 3- P (Palhal) Palhal mine.

Complementary hydromorphological parameters (Raven *et al*., 1998) were recorded: depth, channel and water width, flow velocity, presence of macrophytes and filamentous algae, continuity of riparian vegetation on both banks, shading cover of the channel.

2.2.2. Laboratory analysis

2.2.2.1. Sediment analysis and elutriate production

A small portion of the sediment was sorted to remove debris and oven-dried (70ºC for 24h) before determining organic content by loss-on-ignition (450ºC for 6h; Kirstensen & Anderson, 1987) (represented in the scheme of Fig 4).

The original sediment was readily used upon arrival to laboratory for preparation of elutriates. Sediment was mixed 1:4 (v/v) with ultrapure water and shaken in an orbital shaker at 200 rpm during 2 hours at 20ºC and left overnight. The overlying layer was centrifuged at 2,500 x g for 15 min at 4˚C and transferred to a clean Erlenmeyer ready to use. pH, conductivity and dissolved oxygen were recorded of the elutriates produced using the same multiparameter water quality probe Aquaprobe AP-2000 (Aquaread®).

2.2.2.2. Quantifications in water column and elutriate samples

Both water samples collected and elutriates were used for quantifying biochemical oxygen demand (BOD), dissolved organic carbon (DOC), turbidity, total phosphorus (TP), total nitrogen (TN), total suspended solids (TSS), ammonia parameters according to APHA (1995) following the scheme in Fig 4.

Both water samples and elutriates were vacuum filtered through VWR® glass microfiber filters (1.2 µm pore and 47 mm Ø). The residue was used to quantify total suspended solids (TSS) (APHA, 1995). The filtrate was used to quantify colored dissolved organic carbon (CDOC). Unfiltered water and elutriates samples were used for total phosphorus (APHA, 1989) and nitrogen content (Lind, 1979) (organic forms) after mineralization of samples with potassium persulphate (Ebina *et al*., 1983). Turbidity was indirectly measured according to the absorption coefficient at 450nm of unfiltered water samples and elutriates as well as the Ammonia (NH_4^+ and NH_3) and (Biological Oxygen Demand- BOD_5) following APHA (1995) procedures.

For metal analysis (Al, Mn, Fe, Cu, Zn, Cd, Ba, Pb, As and Cr) and total S, water and elutriate samples were acidified to pH < 2 with nitric acid PA 65% and analyze by Atomic Absorption Spectrometry (AAS).

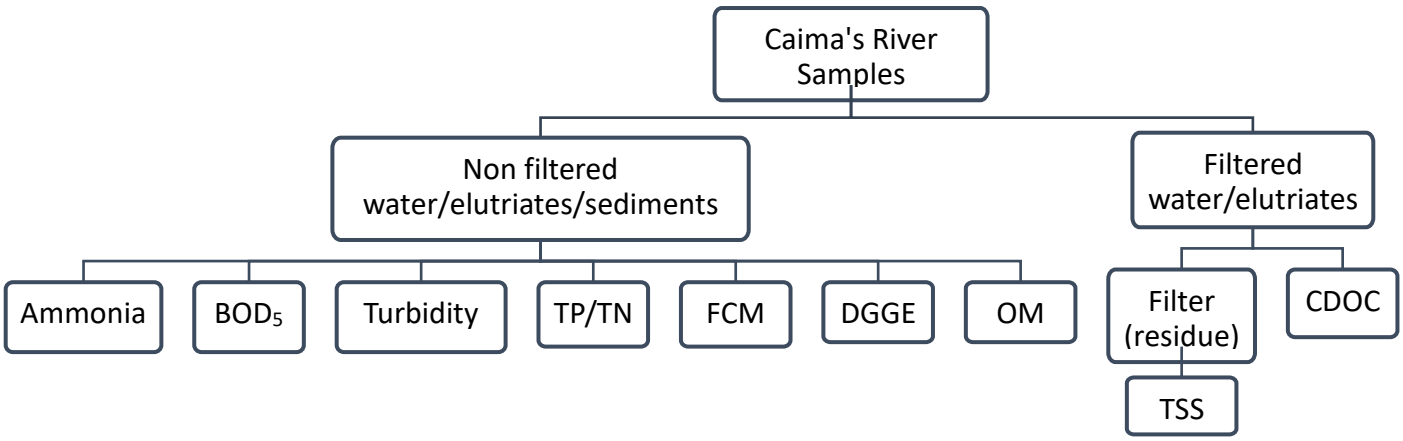

Figure 4- Simplified scheme of quantifications carried out for water, elutriates and sediment samples.

2.2.2.3. Biological communities sensu WFD approach

Macroinvertebrate community

Macroinvertebrates were kept preserved in alcohol 80-90% until sorting. Preserved samples were washed over 2mm and 0,5mm mesh-size sieves, respectively. These two sieves allow separating the major fraction of the smallest fraction, making it easier and more accurate the sorting process. Organisms were then counted and identified to the lowest practicable taxonomic level, generally family and stored in plastic vials with 70% ethanol (Edington & Hildrew, 2005; Elliott & Humpesch, 2010; Hynes, 1993; Pawley, Dobson, & Fletcher, 2011; Sundermann *et al*., 2007; Tachet, 2000; Wallace *et al*., 2003). The following macroinvertebrate community metrics were calculated based on family level identification: richness (S), diversity (Shanno's H'), and equitability (Pielou's J'). Three biotic indices were also calculated: EPT- the number of Ephemeroptera, Trichoptera and Plecoptera taxa; IBMWP- the sum of pre-defined tolerance (to pollution) scores for each taxon (Alba-Tercedor & Sánchez-Ortega, 1988; Jaímez-Cuéllar *et al*., 2002) and IASPT- the average score taxon, derived from IBMWP.

The ecological quality of each sample was determined as an Ecological Quality Ratio (EQR) according to the criteria designed to conform to the WFD. For this watershed, EQRs were derived from the multimetric index $IPtI_N$ (North Invertebrate Portuguese Index; INAG, 2009):

$IPtI_N = 0,25 \times S + EPT \times 0,15 + Evenness \times 0,1 + (IASPT - 2) \times 0,3 + Log\ (sel.\ ETD + 1) \times 0,2$, where S, EPT, Evenness, IASPT and Log (sel. ETD + 1), where sel. ETD is the sum of family abundances of Heptageniidae, Ephemeridae, Brachycentridae, Goeridae, Odontoceridae, Limnephilidae, Polycentropodidae, Athericidae, Dixidae, Dolichopodidae, Empididae and Stratiomyidae. The $IPtI_N$ index is calculated as the weighted sum of all metrics, each normalized as the ratio between the obtained value and the corresponding reference value. Reference values for all metrics were obtained from official guidance documents (INAG; 2009), for Northern rivers medium/large dimension due to the Caima river basin has over 100km^2 catchment. The $IPtI_N$ index itself was normalized for the river typology to obtain the EQR and the ecological status are categorized by the following intervals: High, if EQR > 0.87; Good, if 0.87 > EQR > 0.65; Moderate, if 0.65 > EQR > 0.44; Poor, if 0.44 > EQR > 0.22; and Bad, if EQR < 0.22 (INAG, 2009).

Periphyton community

In the laboratory, the preserved periphyton samples were oxidized with HCl (37%, Merck), several times until the organic material being totally removed. During the oxidization, the glass tubes were gentle warmed using alcohol lamp for 2-3 min helping to speed the process. A small amount of each sample was placed on the top of one coverslip and left to dry out at room temperature. The coverslip was used to prepare permanent slides using Naphrax (Brunel Microscopes Ltd, UK) gentle heated to evaporate the toluene. From each sample, approximately 400 valves were counted and identified to species or infra-specific level under a light microscope (Olympus CX 31) equipped with 100x immersion objective of 1.25 NA, mostly according to (Krammer and Lange-Bertalot, 1986, 1988; 1991a, 1991b; Levkov, 2009; Werum and Lange-Bertalot, 2004). The IPS diatom index (Cemagref, 1982) is based on Zelinka and Marvan's (1961) equation with differences in indicator and sensitivity values. Species are grouped in 5 classes from 1 (tolerant species)

to 5 (intolerant species). The CEE index is based on a two-way entry table, which includes 208 taxa. In this table, taxa are horizontally placed in eight groups arranged in descending order to sensitivity to pollution (group 1 most sensitive and group 8 most tolerant). Vertically, there are four subgroups of taxa (9 to 12) with restricted geographical distribution based on alkalinity and mineralization. For this catchment, the ecological status are categorized by the following intervals: High, if EQR > 0.98; Good, if 0.98 > EQR > 0.74; Moderate, if 0.74 > EQR > 0.49; Poor, if 0.49 > EQR > 0.25; and Bad, if EQR < 0.25 (INAG, 2009). The diatom indices were calculated with software OMNIDIA (v 6.0 - Lecointe *et al*., 1993).

Surface water Ecological status evaluation: integration of WFD data

All the data related to biological (macroinvertebrates and periphyton), physicochemical elements, including specific pollutants and hydromorphological elements, which are within the ecological status, and priority substances, referring to the chemical status, were collected and evaluated as required by INAG (2009). After the integration of all these elements, the worst classification overlaps and dictates the classification of the site in a given season (Table 5).

2.2.2.4. Bacteria community analysis by FCM and DNA extraction for DGGE analysis as rapid bioassessment tool to complement the WFD methodology

FCM analysis was performed on unfiltered water and elutriate samples using a commercial kit (Bacteria Counting Kit, Molecular Probes™, Invitrogen) for accurate enumeration of bacteria, following the manufacturer's protocol.

Briefly, 1 mL of both water and elutriate samples were transferred to Eppendorf tubes together with 1 µL of SYTO® BC and the mixture was then incubated at 37˚C for 5 min. Ten µL of the suspension of microsphere standard with 6 µm were added to the previous mixture after resuspension by sonication in a water bath for about 5 min. Samples were then briefly vortexed and analyzed in an Attune® Acoustic Focusing Cytometer (TermoFisher Scientific) equipped with a 488 nm laser.

The SYTO BC was excited at 488 nm and fluorescence measured with 530/30 bandpass filter (BL1). Light FSC and SSC were also recorded. For statistical significance, at least 10^5 cells were analyzed in each sample and bacteria populations were selected based on BL1 and FSC profiles using the FlowJo software (Tree Star Inc., Ashland, OR, USA). In BL1 vs SSC cytogram, a polygonal region was defined to include only bacteria populations and the concentration of cells in this region was recorded. A marker for separation of LNA and HNA bacterial was defined in the BL1 vs SSC cytogram and the concentrations were counted separately.

2.2.2.4.1. DNA extraction and PCR amplification of bacterial 16S rRNA fragments

For the bacteria DNA extraction of sediment samples, a commercial kit was applied (PowerSoil® DNA Isolation Kit, MO BIO Laboratories Inc.) following the manufacturer's protocol. In brief, 0.25g of sediment sample was added to a PowerBead tube and 60 µL of solution C1 (SDS and other disruption agents for cell lysis) was added, tubes were inverted several times and then they were positioned horizontally on a vortex at maximum speed for 20 min. Tubes were centrifuged at 10000 g for 30 s at room temperature. The supernatant was transferred to a clean tube and 250 µL of solution C2 (reagent to precipitate non-DNA organic and inorganic material) was added, followed by vortex for 5 s and incubation at 4˚C for 5 min. Next, the tubes were centrifuged at 10000 g for 1 min and 600 µL of supernatant was moved to a clean tube. 200 L of solution C3 (reagent to precipitate non-DNA organic and inorganic material) was added, briefly stirred and incubated under the above conditions. Centrifugation was performed as previously mentioned. 750 µL of supernatant was transferred to a clean tube and 1200 µL of solution C4 (high concentration salt solution for a stronger bond of DNA to silica) was added to the supernatant and briefly stirred. Six hundred seventy-five µl were moved to a spin filter (tube with a silica membrane) and then centrifuged under the same conditions. The flow-through was discarded and this step was repeated with the remaining supernatant. Next was added 500 µL of solution C5 (ethanol based wash solution) and centrifuged. The spin filter was placed in a clean tube and was added 100 µL of solution C6 (elution buffer). After another centrifugation, the spin filter was discarded and the DNA was stored at -20˚C until further applications.

Total DNA from environmental water samples was extracted by filtering 2 bottles of 1.5L of water samples through 0.22 µm polycarbonate sterile filters, 47 mm Ø (Whatman, Kent), each bottle works as 1 replicate. When it was not possible to filter 1.5L of water due to filter clogging the volume of water filtered were recorded for further calculations. The filters were frozen -20˚C in sterilized petri dish covered with parafilm M® until further extractions were done. The bacteria DNA extraction kit applied for water column samples were the same used for sediment extraction (PowerSoil® DNA Isolation Kit, MO BIO Laboratories Inc.) following the manufacturer's protocol (see above Bacteria community analysis by FCM and DNA extraction for DGGE analysis). The bacteria DNA extraction from the filter starts by cutting each filter into pieces with a sterile scalpel and transferring each sample it to one PowerBead tube in laminar flow chamber conditions.

The highly variable V3 region of the 16S rRNA gene fragments was PCR amplified using universal primers 338F-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-ACTCCTACGGGAGGCAGCAG-3'), and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer *et al*., 1993). For amplification, the 25 µL reaction mixture contained 14,7 µL of sterilized water, 2 µL of 25 mM $MgCl_2$, 5 µL of Flexi buffer 5× colorless, 0,5 µL of 10 nM dNTP mixture, 0,2 µL of dream Taq DNA polymerase and 0,3 µL of each primer (100 uM). The samples were amplified in an IQ™5 thermocycler PCR system (by a Touchdown PCR protocol: 5 min at 94˚C to denature, 1 min at 65˚C to anneal. The annealing temperature was decreased 1˚C every second cycle until touchdown at 55˚C, at which temperature five additional cycles were carried out. This procedure reduces nonspecific sequences amplification. A final extension was conducted at 72˚C for 3 min. A negative control reaction without any template DNA was performed simultaneously. The PCR amplicons were electrophoresed on a 1,5% agarose gel and compared with a molecular weight marker (GeneRuler™ 1 kb DNA ladder). Electrophoresis was performed at 95V for 40 min. The gel was then visualized by greensafe premium (Nzytech) staining on a UV transilluminator (Syngene G:BOX)

2.2.2.4.2. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed in a DCode™ universal mutation detection system (Bio-Rad Laboratories, Hercules, California, USA) using 0.5x TAE buffer containing 20 mM Tris, 10mM acetic acid and 0.5 mM EDTA (pH 8.0). Amplification products were separated in a 1 mm

vertical polyacrylamide gels (8% [wt/vol] acrylamide in 0.5x TAE buffer) using a 35%-60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized formamide). Electrophoresis was executed at 60˚C during 16h at 75V. The gel was then stained for 5 min in an ethidium bromide solution (5%), rinsed for 5 min with distilled water and scanned with a Molecular Imager FX™ system (Bio-Rad Laboratories, Hercules, California, USA).

2.2.2.5. Data analysis: multivariate approach

Each community of macroinvertebrate, periphyton and bacteria abundance data were analyzed individually by Detrended Correspondence Analysis (DCA, unconstrained ordination technique) on biotic data matrix, which analyzes gradients in community structure, including spatial and temporal patterns. DCA is an improved eigenvector ordination technique based on reciprocal (weighted) averaging and is commonly used in community ecology, as it assumes an underlying unimodal mathematical model (ter Braak, 1995, Gauch, 1982). In the case of macroinvertebrate and periphyton communities, manual downweighting of rare families was used, and species with less than 0.048% and 0.015%, respectively, of the total abundance in each sample, were discarded.

The distribution of each sample according to environmental parameters was assessed through a multivariate redundancy analysis (RDA) and canonical correspondence analysis (CCA) for periphyton community, after standardization of environmental data (by subtracting the mean from each observation and dividing to the corresponding standard deviation). RDA was the choice to explore seasonal and spatial gradients in the biotic data communities individually for macroinvertebrates and bacteria and CCA were performed for periphyton (ter Braak, 1995) according the theory on gradient analysis (ter Braak and Prentice 1988). In spite of species abundances tend to follow a unimodal response (CCA) to environmental gradients, RDA was best suited to deal with species abundance data when the length of gradient is small (usually below 3-4 s.d. units) thus approaching a linear response. CCA analysis were performed to the periphyton community due to the length of gradient be above 4, following a unimodal response pattern. Both RDA and CCA constrains the biotic matrix to the environmental gradients, which makes it direct gradient analysis technique (ter Braak 1995). The length of the arrow refers to the importance of the

explanatory variable in the ordination, and arrow direction indicates positive and negative correlations. The data were centered and standardized before redundancy analysis, and the Monte Carlo permutation test ($p<0.1$) was used to examine the significance of the RDA method, i.e., a selection procedure was performed a priori on the environmental data sets, including only significant explanatory variables in the model using symmetric scaling (Gabriel, 2013). All multivariate analysis were performed using CANOCO 4.5 software.

2.3. Results

2.3.1. Sampling sites water and sediment elutriate quantifications performed – abiotic framework

The variation of the physicochemical parameters in the Caima River is shown in Table 1. The temperature values varied between 6.3˚C in winter and 19.3˚C in summer, with minimum values registered at Nascente in all stations due to its altitude. The pH values in water did not demonstrate significant alterations in winter and spring, with values between 9.14 and 7.52. In summer all the stations decreased their pH to low than 7. However, the pH measured in sediment samples had higher values only in winter, being spring and summer very similar to each other.

Caima River was very heterogeneous among sampling seasons. Parameters such as ammonium (NH_4), ammonia (NH_3), total nitrogen (TN), phosphate (PO_4), total phosphorus (TP) and nitrate (NO_3) registered the higher values at Bustelo in the 3 seasons, in water samples (with the exception of four sediment samples: TP, PO_4 with higher values at Nascente in spring and NH4, NH3 with higher values at Nascente summer). Besides that, in these parameters, the water samples always had higher values than sediment samples. The minimum value was observed for NH_4 and NH_3 at Nascente for water samples and at Palhal for sediment samples, both in summer. The conductivity (cond) in water was significantly greater at Bustelo and Palhal in all the seasons, unlike sediment samples, that the minimum values corresponded to Bustelo in winter and spring. The dissolved oxygen (O_2) was relatively homogeneous among the sites and seasons, with a slight decrease at Bustelo sites. This parameter was always higher in water samples than sediment samples as well.

Dissolved organic carbon (CDOC) in water had its maximum value at Palhal in spring (24.38 mg/L) and its minimum value at Nascente in summer (1.61 mg/L), while in sediment the maximum value was registered at Nascente in winter (34.96 mg/L) and the minimum value was registered at Palhal in summer (0.92 mg/L). The values of biochemical oxygen demand (BOD_5) were similar among sampling sites, for the same season, but different between seasons, being strangely the maximum values in winter and the minimum values in spring and summer, for both water and sediment samples. These two last parameters had values of sediment samples higher than water samples in all seasons. Total suspended solids (TSS) in water had its maximum value (62.52 mg/L) at Palhal and its minimum value (0.133 mg/L) at Nascente, both in winter. Organic matter (OM) values, in sediments, were higher at Nascente and lower at Palhal in all seasons. No consistent pattern was found among all these parameters, although most of the nutrients had higher concentrations at Bustelo throughout the year, followed by Palhal and Nascente.

Low levels of zinc (Zn), cadmium (Cd) and barium (Ba) were found in water and sediment samples. It was observed that in all metals, the concentration was always higher in sediments than in water along the seasons (Table 2). The opposite was detected for the sulphur element, which had higher values in water. All the metal quantifications performed in water samples collected were below the thresholds values considered as safe in WFD legislation (INAG, 2009). Curiously, some elements such as arsenic (As), copper (Cu) and aluminum (Al) were found to be at higher concentrations in sediments at Nascente in winter (see Discussion). These metals were decreasing gradually throughout the seasons, reaching the lowest values in summer. Also, the maximum values of Al and Cu in water samples were found at river source in spring. Concentrations of manganese (Mn), lead (Pb) and total S were found to vary among seasons, although it seems that in spring the values are lower compared to the other seasons. The most worrying concentrations of iron (Fe) and chromium (Cr) in sediments were recorded at Nascente, summer and spring respectively, where the values are much higher than the rest of the sampling sites and the water measurement.

2.3.2. Biological communities sensu WFD approach

Macroinvertebrates

A total number of 13346 individuals belonging to 56 taxa were identified. Relatively to total abundance, the number of individuals increased over the seasons in all sites. At Bustelo, it was observed the greater increase, being the site with most abundance in all the seasons studied, followed by Palhal and Nascente. The total number of families were almost always below the reference value (26) for medium-large dimension rivers of northern Portugal. The only exception occurred at Nascente in summer, with a value of 33. The maximum values of richness (number of families) were registered at Nascente in every season, while the site Bustelo, downstream WWTP, recorded always the lowest values. In both sites Nascente and Palhal, the richness increased over the 3 seasons, with maximum values in summer, whereas in Bustelo the opposite occurred, having less richness in the summer.

The Ecological Status of Caima River throughout the 3 seasons of the year, according to macroinvertebrate community, is represented in Table 3. The taxa Ephemeroptera, Plecoptera and Trichoptera, which compose the EPT index, are known as intolerant to organic pollution (Chessmann & McEvoy, 1998; Lydy *et al*., 2000) and intolerant to toxic chemical products (Wallace *et al*., 1996) and were found in highest quantity at Nascente site, chosen as reference location. Although some families of Ephemeroptera and Trichoptera were found in Bustelo and Palhal, in general, the quantity and the sensitivity degree of these species are relatively low compared to Nascente.

The different metrics (IASPT, S, J', log (sel. ETD + 1) and EPT taxa) which composes the $IPTI_N$, were intercalibrated based on its reference values and the type of river that best suits Caima River, medium-large dimension rivers of northern Portugal. At first, the typology of the river raised some questions that were then clarified by the fact that the river has an extension of more than 100 km^2 (INAG, 2008b).

Nascente obtained high ecological status in winter and summer and good in spring. Bustelo was classified as moderate in winter and spring and its ecological status decreased in summer to poor. High temperatures and decrease in river flow worsened the impact of

WWTP on macroinvertebrate communities, making Bustelo the most impacted site. Palhal ecological status remained good along the 3 seasons sampled (Table 3).

Periphyton

A total of 44 species belonging to 26 genus were identified. The composition, abundance and ecological status of periphyton communities in the 3 seasons of the year are represented in Table 4. *Navicula* e *Gomphonema* were the genus more frequently represented, with 6 and 5 taxa, respectively. The lowest values of richness occurred at Nascente in every seasons, with minimum value in spring (5), while Bustelo registered the highest values. Nascente and Palhal share the same fluctuation, with decreasing values of taxa richness from winter to spring and an increase in summer, while Bustelo increased over the seasons. Some taxa were found exclusively in one station over the 3 seasons, for example, *Peronia fibula* (PERF), *Surirella angusta* (SANG) and *Anomoeoneis serians* (ANON) at Nascente, *Psammothidium subatomoides* (PSAT), *Navicula gregaria* (NGRE) and *Neidium dubium* (NEDU) at Bustelo, *Achnanthes minutissima* (AMIN), *Planothidium frequentissimum* (PLFR), *Gomphonema parvulum* (GPAR) and *Cocconeis placentula* (CPLA) at Bustelo and Palhal. Some species of the genera *Gomphonema*, *Nitzschia* and *Navicula* are known to be tolerant to organic and metal pollution (Kwandrans *et al*., 1998; Bere & Mangadze, 2014), being concordant with their distribution at Bustelo and Palhal sites. Moreover, in general, *Navicula* and *Gomphonema* genus occurred manly at pH <7, preferentially in meso-eutrophic environments and oxygen saturation about 70-85% (van Dam, 1994).

Ecological Status according to periphyton communities were calculated taking into account the typology of the river for the values of IPS and respective RQE (Table 4). In winter and spring the river water quality was good in all sites and in summer it was good at Nascente, high at Bustelo and moderate at Palhal.

Table 1- Environmental characteristics of the water and sediment samples: Nasc- river source; Bust- downstream WWTP; Palh- palhal mine in 3 seasons (winter, spring and summer) (maximum values are in bold)

	N_winter	B_winter	P_winter	N_spring	B_spring	P_spring	N_summer	B_summer	P_summer
Temp(ºC)_water	6.3	6.9	7.3	9.1	12.7	12.9	17.6	**19.3**	18.2
pH_water	**9.14**	7.52	7.8	8.5	7.71	7.93	6.93	6.5	6.86
pH_elutriate	**8.07**	7.5	7.71	5.39	5.6	5.53	5.88	6.14	5.88
cond (µS/cm)_w	14	82	80	14	61	67	10	**94**	91
cond (µS/cm)_e	43	11	**50**	43	11	**50**	19	21	17
O2 (%)_w	98.6	94	99.8	100.2	95	101.9	**103.9**	91.1	99.1
O2 (%)_e	92.7	89.5	**95.8**	75.8	69.5	92.5	79.6	64.3	84.7
O2 mg/L_w	11.07	11.4	**12.05**	10.44	10.05	10.79	8.89	8.29	9.26
O2 mg/L_e	8.88	8.79	9.15	**9.72**	9.26	9.82	8.6	7.69	8.89
CDOC (m^{-1})_w	6.9	2.99	2.53	23.92	23.46	**24.38**	1.61	4.14	3.22
CDOC (m^{-1})_e	**34.96**	13.11	9.66	29.44	26.68	30.13	23.92	5.29	0.92
BOD5 (mg/L)_w	5.1	5.1	**6.25**	0.68	0.62	0.65	0.31	1.1	0.5
BOD5 (mg/L)_e	**8.17**	7.39	6.72	1.09	2.72	1.35	1.59	2.94	1.19
Turbidity (m^{-1})_w	**6.44**	1.38	0	0	0	0	0.92	2.76	2.76
NH_4 (mg/L)_w	0.1161	**1.5996**	0.1419	0.129	0.8256	0.1419	0.0774	0.9288	0.129
NH_4 (mg/L)_e	0.1161	**0.8256**	0.1548	0.2322	0.3612	0.1419	0.5031	0.4644	0.0516
NH_3 (mg/L)_w	0.1098	**1.5128**	0.1342	0.122	0.7808	0.1342	0.0732	0.8784	0.122
NH_3 (mg/L)_e	0.1098	**0.7808**	0.1464	0.2196	0.3416	0.1342	0.4758	0.4392	0.0488

Table 1- Environmental characteristics of the water and sediment samples: Nasc- river source; Bust- downstream WWTP; Palh- palhal mine in 3 seasons (winter, spring and summer) (maximum values are in bold) (cont.)

	N_winter	**B_winter**	**P_winter**	**N_spring**	**B_spring**	**P_Spring**	**N_summer**	**B_summer**	**P_summer**
TP (mg/L)_w	0	0.159	0.0204	0	**0.2425**	0.0062	0	0.1696	0.0719
TP (mg/L)_e	0.3331	0.16254	0.05062	**0.389942**	0.219891	0.107467	0.11635	0.372176	0.04884
PO_4 (mg/L)_w	0	0.4865	0.06247	0	**0.742008**	0.018981	0	0.51912	0.220124
PO_4 (mg/L)_e	1.01926	0.49738	0.154888	**1.193221**	0.671336	0.32885	0.356031	1.138858	0.149452
TN (mg/L)_w	0	**1.518822**	0.872886	0	0	0	0	1.14017	0.895159
TN (mg/L)_e	1.296085	**1.296085**	0	0	0	0	0.29377	0.627875	0
NO_3 (mg/L)_w	0	**6.728381**	3.866883	0	0	0	0	5.050951	3.965556
NO_3 (mg/L)_e	5.741658	**5.741658**	0	0	0	0	1.301403	2.781488	0
TSS (mg/L)_w	0.13333	2.466667	**62.52**	0.653333	1.306667	0.206667	1.366667	1.46	1.926667
OM(%)	1.694246	0.418908	0.383996	3.589699	0.566382	0.278018	**14.93949**	0.598511	0.516087

Table 2- Metal concentrations (µg/L) found in both water and elutriate samples Nascente (N), Bustelo (B) and Palhal (P) in 3 seasons throughout the year (maximum values are in bold).

(µg/L)	N_winter	B_winter	P_winter	N_spring	B_spring	P_spring	N_summer	B_summer	P_summer
Al_w	45	29	19	**59**	58	25	39	39	54
Al_e	**3680**	410	350	400	63	430	86	18	49
Mn_w	13,2	**21,8**	5,4	9,9	8,5	4,2	4,7	15,6	2,3
Mn_e	10,4	30,3	30,5	10,9	16,7	23	17,8	**570**	17,2
Fe_w	<500	<500	<500	<500	<500	<500	<500	<500	<500
Fe_e	<500	<500	<500	<500	<500	<500	**2200**	720	<500
Cu_w	<2	3,5	7,6	**22**	<2	<2	<2	<2	2,3
Cu_e	**9,1**	4,7	8,6	7,9	2,3	7,6	2,1	3,4	3
Zn_w	<50	<50	<50	<50	<50	<50	<50	<50	<50
Zn_e	<50	<50	<50	<50	<50	<50	<50	<50	<50
Cd_w	<1	<1	<1	<1	<1	<1	<1	<1	<1
Cd_e	<1	<1	<1	<1	<1	<1	<1	<1	<1
Ba_w	<10	10	<10	<10	<10	<10	<10	<10	<10
Ba_e	<10	<10	<10	<10	<10	<10	<10	<10	<10
Pb_w	<3	<3	<3	<3	<3	<3	<3	<3	3,1
Pb _e	<3	<3	**9**	<3	<3	<3	4	5	5,7
Total S_w	276	1750	**2140**	289	1450	1660	172	2050	2030
Total S_e	266	728	949	317	382	1880	**2260**	728	1100
As_w	<3	<3	<3	<3	<3	<3	<3	<3	3
As_e	**33**	3,4	26	<3	<3	16	5	<3	12
Cr_w	<5	<5	<5	<5	<5	<5	<5	<5	<5
Cr_e	<5	<5	<5	**170**	<5	<5	<5	<5	<5

Table 3 - Composition of macroinvertebrate communites and ecological status of each sampling site (Nascente (Nasc), Bustelo (Bust) and-Palhal (Palh)) in the 3 seasons (winter, spring and summer), using $IPTI_N$ and respective RQE, according the Portuguese WFD.

	Dryo	Dytis	Elmid	Gyri	Hydra	Hydr p	Scirt	Athr	Cert	Chiro	Dixd	Dolc	Empd	Limn	Musc	Psyh	Rhai	Siml	Tipu	Baeti	Caen
Nasc_w	0	0	4	3	3	0	38	0	0	19	0	0	3	0	0	0	0	4	0	70	0
Bust_w	0	0	3	1	0	0	0	0	0	88	0	0	0	0	0	0	0	1	0	192	242
Palh_w	1	0	46	0	0	0	0	6	1	77	0	0	3	0	0	1	0	2	0	85	291
Nasc_sp	0	0	32	0	4	1	103	0	0	277	0	0	2	0	0	0	0	1	0	48	1
Bust_sp	0	0	5	0	0	0	0	1	1	819	0	0	6	0	0	0	0	8	0	228	399
Palh_sp	0	0	24	0	0	0	0	6	13	139	0	0	8	1	0	0	0	0	1	544	285
Nasc_s	0	0	51	0	2	0	4	0	0	270	3	1	1	1	0	0	1	18	0	207	0
Bust_s	0	0	3	0	0	0	0	0	0	3320	0	0	0	0	4	0	0	59	1	210	300
Palh_s	0	4	13	0	0	0	0	0	3	1421	0	0	3	0	0	0	0	0	0	104	705

	EPH	Hept	Lept	Ancy	Phys	Aphel	Corix	Gerr	Hydrom	Aesh	Calo	Cord	Gomph	Hydrac	Oligo	Duge	Plana	Tricl	Chlo	Leuct	Nemou
Nasc_w	0	29	20	0	0	0	0	0	0	2	0	0	2	2	0	0	1	0	1	4	15
Bust_w	0	0	0	3	18	0	0	0	0	1	0	0	3	1	84	3	0	0	0	0	0
Palh_W	0	0	0	9	0	0	0	0	0	1	1	0	2	28	38	1	0	0	0	0	0
Nasc_sp	5	1	19	0	0	0	0	0	0	0	1	0	2	35	0	0	0	0	0	21	46
Bust_sp	0	0	0	1	0	0	0	0	0	0	0	0	5	9	10	0	0	5	0	0	0
Palh_sp	0	0	0	6	0	2	0	0	0	1	0	0	6	40	1	1	0	0	0	0	0
Nasc_s	66	13	109	0	0	0	2	0	1	15	2	5	14	87	2	0	2	1	0	392	21
Bust_s	0	0	0	3	19	0	0	0	0	0	0	0	0	48	16	0	0	0	0	0	0
Palh_s	0	0	0	4	0	6	1	1	0	2	1	0	6	550	0	4	0	0	0	0	0

Table 3- Composition of macroinvertebrate communites and ecological status of each sampling site Nascente (Nasc), Bustelo (Bust) and Palhal (Palh) in the 3 seasons (winter, spring and summer), using $IPTI_N$ and respective RQE, according the Portuguese WFD (cont.).

	Perli	PLEC	Erpo	Ecnom	Gloss	Hydrop	Hydrot	Lept	Philo	Polyc	Psyc	Rhyac	Seri	Trich	IASPT	IBMWP	$IPTI_N$
Nasc_w	1	0	0	0	0	33	0	1	2	0	0	0	113	0	1.1679	146	0.982926
Bust_w	0	0	8	0	0	3	0	0	0	0	0	0	1	0	0.6927	76	0.498767
Palh_w	0	0	0	0	0	12	0	1	3	2	0	1	0	0	0.916	124	0.786632
Nasc_sp	0	8	2	0	0	5	7	0	0	0	0	13	214	0	0.9847	130	0.829836
Bust_sp	0	0	5	0	0	9	0	0	0	0	0	0	0	0	0.6657	65	0.556583
Palh_sp	0	0	0	0	0	10	1	1	3	0	5	0	0	0	0.9476	121	0.783999
Nasc_s	0	1	0	0	0	23	0	15	0	29	5	7	2	0	0.9832	183	1.064985
Bust_s	0	0	12	0	0	2	0	0	0	0	0	0	0	0	0.4844	51	0.3487
Palh_s	0	0	1	2	1	27	3	16	1	5	0	0	0	1	0.9131	135	0.809411

	RQE	RQE ref.	Ecological status
Nasc_w	0.9829	0.87-0.65	High
Bust_w	0.499	0.44-0.22	Moderate
Palh_w	0.787	0.87-0.65	Good
Nasc_sp	0.8298	0.87-0.65	Good
Bust_sp	0.557	0.65-0.44	Moderate
Palh_sp	0.784	0.87-0.65	Good
Nasc_s	1.065	> 0.87	High
Bust_s	0.349	0.44-0.22	Poor
Palh_s	0.809	0.87-0.65	Good

Table 4- Composition of periphyton communities and ecological status of each sampling site (Nascente (Nasc), Bustelo (Bust) and Palhal (Palh)) in the 3 seasons (winter, spring and summer), using IPS and respective RQE, according the Portuguese WFD.

	ACON	AMIN	ANON	CER	CPLA	CMEN	CTUM	DMES	ENMI	EBIL	EEXI	EMIN	FBCP	FCVA	FULN	FVUL	GACO	GGRA	GPAR	GPUM	MVAR	MCIR
Nasc_w	0	0	8	0	0	0	0	0	2	26	5	12	0	0	0	0	0	0	0	0	0	0
Bust_w	40	0	0	0	18	2	0	0	2	0	4	6	0	0	0	6	0	0	10	0	0	2
Palh_w	4	283	0	1	44	0	3	1	0	0	0	1	2	1	6	13	1	1	0	0	17	2
Nasc_sp	0	0	0	0	0	0	0	0	0	0	2	19	0	0	0	0	0	0	0	0	0	0
Bust_sp	15	130	0	8	2	3	0	7	0	0	0	17	0	0	13	3	0	0	53	6	0	0
Palh_sp	0	310	0	0	67	0	0	0	0	0	0	5	0	0	0	3	0	0	1	0	0	0
Nasc_s	2	0	2	0	1	0	0	0	0	0	8	79	0	0	0	0	0	0	0	0	0	0
Bust_s	0	8	0	0	4	3	0	2	7	0	0	1	0	2	1	1	1	20	9	2	1	0
Palh_s	3	100	0	0	126	168	4	1	6	0	0	0	0	0	2	0	0	0	4	0	14	0

NCRY	NCTE	NGRE	NLAN	NRHY	NEDU	NDIS	PERF	PGIB	PMIC	PSCA	PCLT	PLFR	PSAT	RSIN	SPUP	SPHO	SANG	SLIN	IPS	RQE	RQE ref.	Ecological Status
0	0	0	0	0	0	0	53	0	0	0	0	0	0	0	0	0	305	0	17.2	0.90052	0.98-0.74	Good
8	0	6	0	0	2	0	0	0	0	18	0	4	300	6	0	0	0	0	18.4	0.96335	0.98-0.74	Good
0	4	0	4	1	0	0	0	4	0	4	2	1	0	0	1	0	0	8	18.1	0.94764	0.98-0.74	Good
0	0	0	0	0	0	0	10	1	0	0	0	0	0	0	0	0	390	0	15.8	0.82723	0.98-0.74	Good
0	0	0	0	0	7	1	0	0	8	21	8	49	120	70	5	1	3	14	16.9	0.88482	0.98-0.74	Good
0	0	0	2	0	0	0	0	0	2	0	2	5	0	0	0	0	0	3	18.8	0.98429	> 0.98	Good
0	0	0	0	0	0	0	8	2	0	2	0	0	0	0	2	6	312	0	16.4	0.85864	0.98-0.74	Good
0	0	6	0	1	1	1	0	1	1	1	1	6	390	0	0	0	1	1	18.9	0.98953	> 0.98	High
0	0	0	0	0	0	0	0	0	0	0	1	84	0	0	0	0	0	1	12.6	0.65969	0.74-0.49	Moderate

Surface water Ecological evaluation: integration of WFD data

According to INAG (2009), some physicochemical parameters have limit reference values for the establishment of good ecological status in medium-large dimension rivers of northern Portugal. Parameters such as O_2 (≥5 mg/L), O_2% (60%-120%), BOD_5 (≤6 mg/L), pH (6-9), NH_4 (≤1 mg/L), NO_3 (≤25 mg/L) and TP (≤0.10 mg/L) have already defined their limit values. These values were taken into account in the final water quality assessment of Caima River (Table 5), existing only a border between good and moderate class, where a value above the limit requires the decreasing to good for moderate quality.

Bustelo obtained moderate along the three seasons, with high values of TP in all seasons (0.159, 0.2425 and 0.1696, respectively) and high levels of NH_4 (1.5996) in winter. Also in winter, Palhal and Nascente obtained moderate due to an elevated value of BOD_5 (6.25) and a high value of pH (9.14), respectively (Table 1).

Relatively to hydromorphological quality elements (Table 5), specific pollutants and chemical status, all the sampled sites were qualified with good ecological status. The specific pollutants evaluated in water of the sampling sites were never above threshold values considered as safe for the ecosystems, published in WFD guidance document and therefore were classified as good for all sampling site. The final ecological status was defined by the elements that present worse classification.

The application of WFD to the river sampling sites and comparing the discriminatory power given by the two biological communities, it was observed that they did not agreed with each other several times evidencing that macroinvertebrates were more sensitive to organic matter contamination after the WWTP than the periphyton, and periphyton communities were more sensitive to ashes runoff at Nascente or other conditions, never reaching high ecological status, according to those bioindicator communities. Meanwhile, the same community showed not to be sensitive to the WWTP effluent discharge in river at Bustelo and classifying the sampling site as high ecological status.

Table 5- Summarized data of the final water quality assessment of the different Caima River sites for winter, spring and summer.

		Biological Quality Elements		Hidromorphological Quality Elements	Specific Pollutants	Chemical and Physicochemical Quality Elements	Ecological Status	Chemical Status	Final Water Status
		Periphyton	Macroinvertebrate						
winter	Nascente	Good	High	Good or less	Good	Moderate	Moderate	Good	Moderate
	Bustelo	Good	Moderate	Good or less	Good	Moderate	Moderate	Good	Moderate
	Palhal	Good	Good	Good or less	Good	Moderate	Moderate	Good	Moderate
spring	Nascente	Good	Good	Good or less	Good	Good	Good	Good	Good
	Bustelo	Good	Moderate	Good or less	Good	Moderate	Moderate	Good	Moderate
	Palhal	Good	Good	Good or less	Good	Good	Good	Good	Good
summer	Nascente	Good	High	Good or less	Good	Good	Good	Good	Good
	Bustelo	High	Poor	Good or less	Good	Moderate	Poor	Good	Poor
	Palhal	Moderate	Good	Good or less	Good	Good	Moderate	Good	Moderate

2.3.3. Bacteria community analysis by FCM

The data obtained from the flow cytometry are shown in Figure 6 and 7, respectively from water samples and elutriates. As an example of the gatings that were applied in all the samples, Figure 5 corresponds to a cytogram and a histogram of the sediment sample at Nascente in spring. On the histogram, it's possible to see two peaks, corresponding to two different populations, with different nucleic acid content, one with low DNA content (LNA) and the other with high DNA content (HNA). All the water and sediment samples were subjected to the same gates for comparable results.

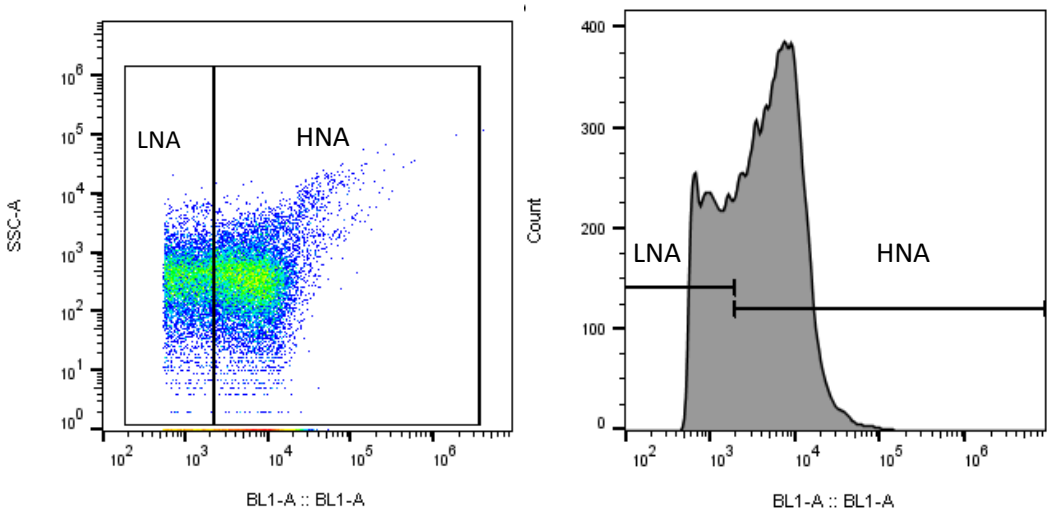

Figura 5- Example of HNA and LNA bacteria gatings applied at all the samples studied. On the left, the cytogram and its corresponding histogram (on the right), illustrates the differentiation of two populations based on the nucleic acid content.

As shown in the flow cytograms (Fig 6 and 7), LNA and HNA bacteria in water and sediment samples of the Caima River were discriminated by their DNA content and fluorescence intensity in all the 3 seasons for Nascente, Bustelo and Palhal. The increasing of bacteria abundance is proportional to the fluorescence intensity, being the greater abundance marked by the green color.

In general, the LNA bacteria tends to decrease throughout the seasons, with maximum values in winter and lower values in summer, suggesting a tendency to be higher

with lower temperatures and vice versa. The opposite occurred with HNA bacteria that had its maximum concentrations in summer and minimum in winter (except Bustelo in sediment samples). By analyzing the cytograms from Figure 6, referring to water samples, it´s clear that the greatest abundance happened at Bustelo in all seasons, with superior abundance in winter and summer, followed by Palhal and Nascente. For both Nascente and Palhal, there was a significant decrease of HNA bacteria from winter to spring and then an increase to summer, reaching higher concentrations in summer. The same occurred for LNA bacteria, but with higher values in winter. At Bustelo, there was a slight decrease of HNA from winter to spring and an increase to summer, reaching the highest value in summer.

Relatively to sediment samples, HNA bacteria concentration reached lower values at Nascente and Palhal in winter and then increased along the seasons. The opposite happened with LNA bacteria at Nascente and Bustelo, where the maximum value was registered in winter and decrease over the seasons reaching lower values in the warmer season. At Bustelo, the higher value of HNA bacteria occurred in winter, decreasing in spring and increasing again in summer (Figure 7). The same happened to Palhal but with high values of LNA bacteria.

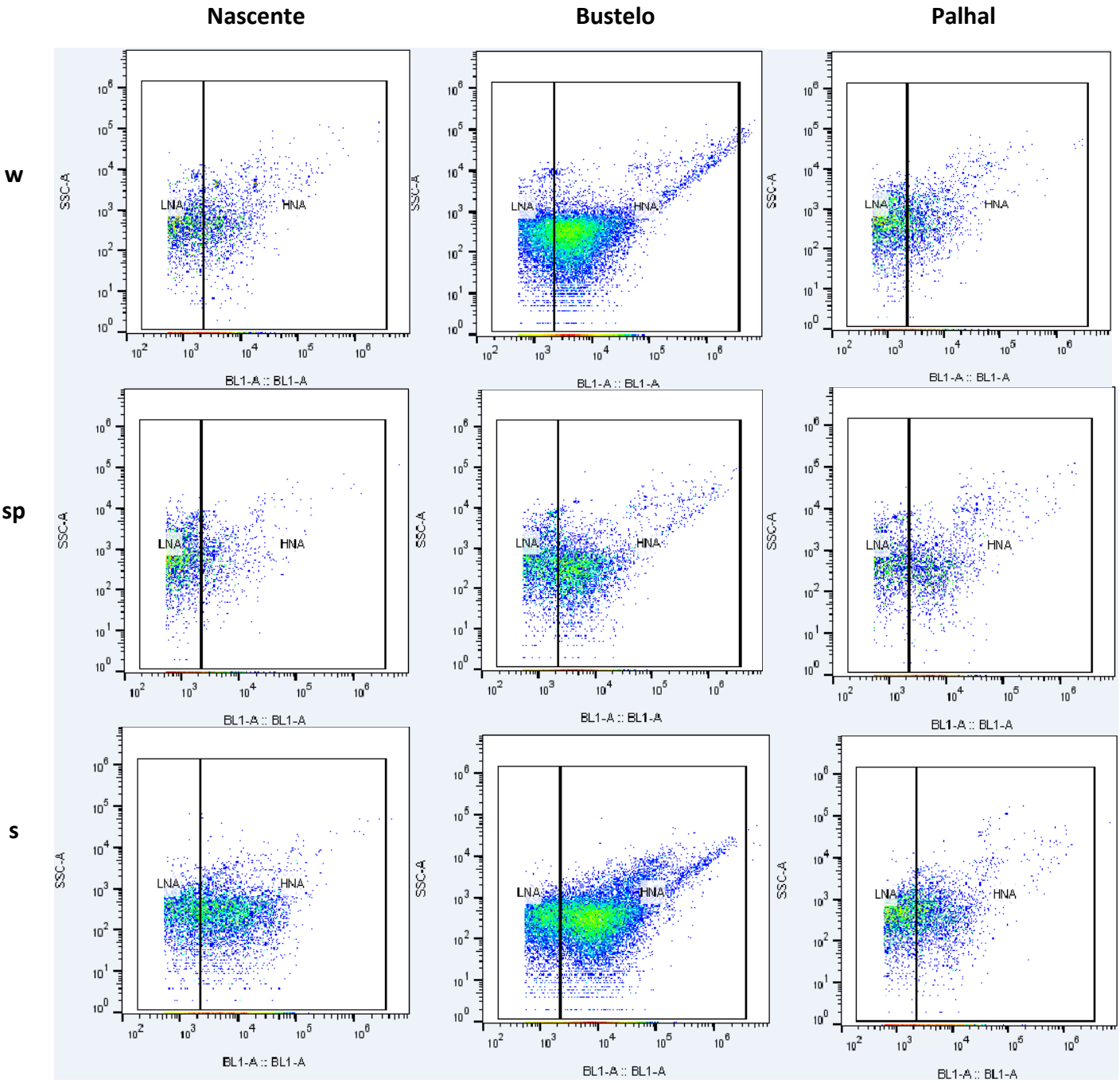

Figure 6- Flow cytogram are represented in dot-plots of total cell counts, and LNA (left side) and HNA (right side) bacteria water samples are indicated by solid lines. Samples were discriminated based of their side scatter (SSC) and fluorescence intensity (BL1). All the seasons are represented, being the first line the winter, spring and summer, respectively.

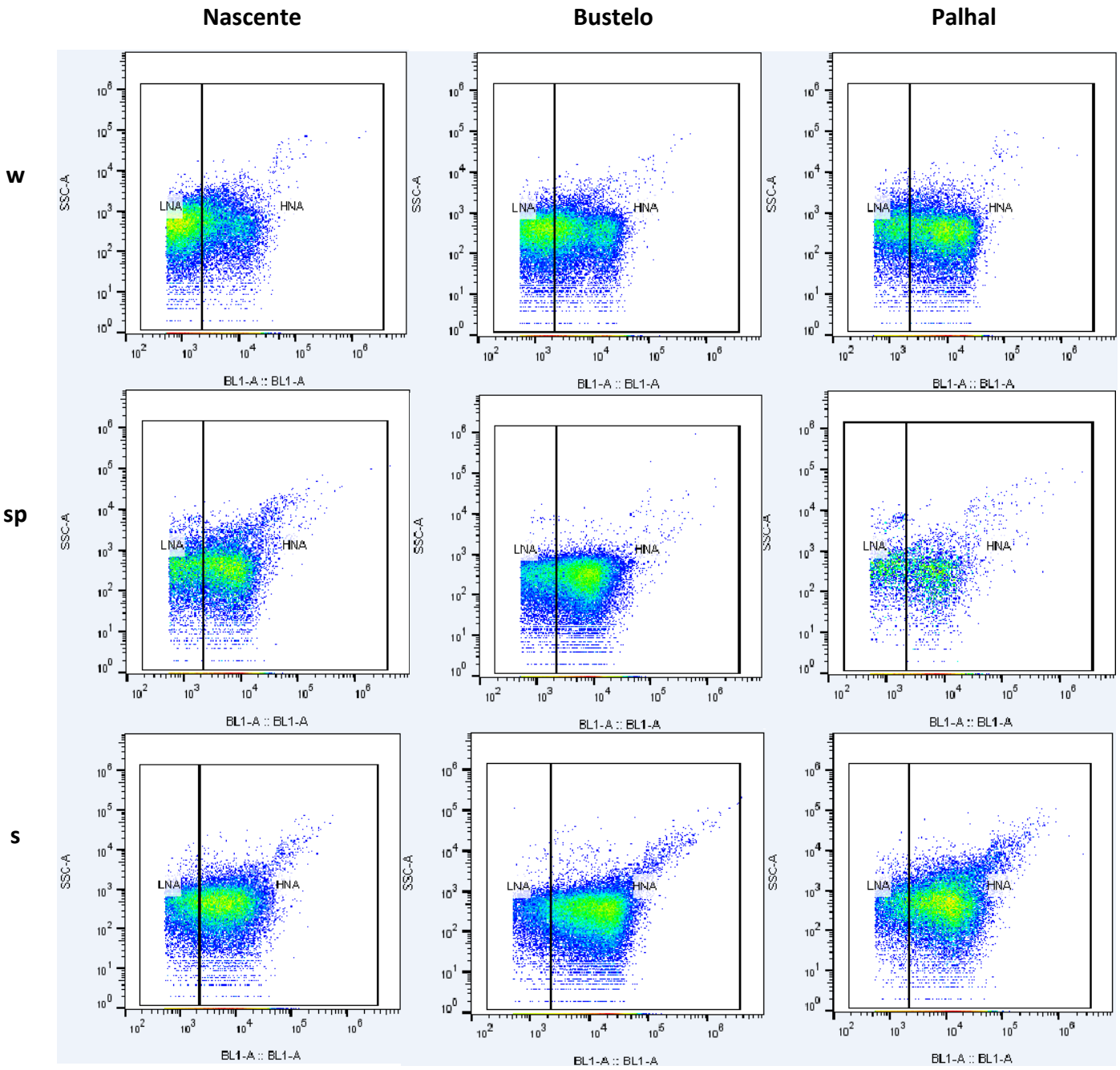

Figure 7- Flow cytogram are represented in dot-plots of total cell counts, and LNA (left side) and HNA (right side) bacteria sediment samples are indicated by solid lines. Samples were discriminated based of their side scatter (SSC) and fluorescence intensity (BL1). All the seasons are represented, being the first line the winter, spring and summer, respectively.

Bacteria communities densities from FCM analysis are shown in Table 6. Total cells, HNA and LNA bacteria were analyzed for each sampling site in all seasons, both for water and sediment samples. In general, there was always a higher concentration (total cells/mL) of bacteria in sediments than in water. Maximum values recorded for Bustelo sediment samples, in summer (1344998 cell/mL) and for Bustelo water samples, in winter (347480 cell/mL). Relatively to water samples, there was a similarity in bacterial density between Nascente and Palhal in all seasons. Bustelo was the sampling site with higher density in all seasons, wherein spring and summer had practically even and winter was the highest (see Fig A.1 in the Annex).

Table 6- Bacteria community densities obtained by Flow Cytometry using a commercial kit (Bacteria Counting Kit, Molecular Probes™, Invitrogen) for both water (w) and sediment samples (e) (maximum values of HNA and LNA for each site are in bold) of 3 sampling sites Nascente (Nasc), Bustelo (Bust) and Palhal (Palh) for winter, spring and summer.

		Total (cells/ml)	HNA (cells/ml)	LNA (cells/ml)
winter	Nasc_w	36389	13037	**24946**
	Bust_w	347480	**175547**	61173
	Palh_w	42235	13148	**22122**
spring	Nasc_w	43190	15743	**19517**
	Bust_w	100402	**47993**	27418
	Palh_w	41840	**17838**	15097
summer	Nasc_w	35244	**17834**	5878
	Bust_w	99940	**54498**	15715
	Palh_w	47965	**19767**	18812
winter	Nasc_e	1034357	337674	**479408**
	Bust_e	1030062	**456455**	285698
	Palh_e	679280	**352413**	143520
spring	Nasc_e	247183	**118538**	64467
	Bust_e	832612	**430897**	130576
	Palh_e	40006	**17893**	11135
summer	Nasc_e	1021044	**565647**	200015
	Bust_e	1344998	**814534**	113930
	Palh_e	537986	**354632**	66319

Bacterial density in sediment suffered more fluctuations over the seasons, with a very low bacterial concentration in spring in all sampling sites, comparatively to water column. Bacterial density in Nascente and Palhal share the same pattern of seasonal response for water and for sediment. In case of water samples (both Nascente and Palhal), it increases from winter to spring and decreases from spring to summer. Otherwise, for sediment samples (Nascente and Palhal), it decreases from winter to spring and rises again in summer to densities similar to winter. The exception, for bacteria density in water, was observed for Palhal from spring to summer which instead of decrease it increases but not significantly (see Fig A.1 in the Annex).

Regarding the HNA and LNA bacteria community composition, it can be observed that the quantity of HNA and LNA, for all the sites, was higher in sediments, with the exception of Palhal in spring that had similar values with the water column bacteria community composition, as shown in Figure 8. The HNA bacteria was mostly dominant throughout the seasons for all sampling sites, although with some exceptions (Nascente in winter and Palhal in spring) (Figure 8). This one presents elevated concentrations in sediments at Bustelo in all seasons, in relation to the other sites. Relatively to LNA bacteria in sediments, the density was greater in winter, decreasing over the seasons up to minimum values at Palhal in spring and summer and at Nascente in spring. In water samples, the concentration of HNA and LNA bacteria were very low and the values were very close to each other. However, it is possible to observe a peak at Bustelo in winter and summer, with maximum values in HNA bacteria. In water samples, it was found more sampling sites with a higher concentration of LNA bacteria, corresponding to the colder season. All these patterns can be observed more easily in the cytograms that are presented above.

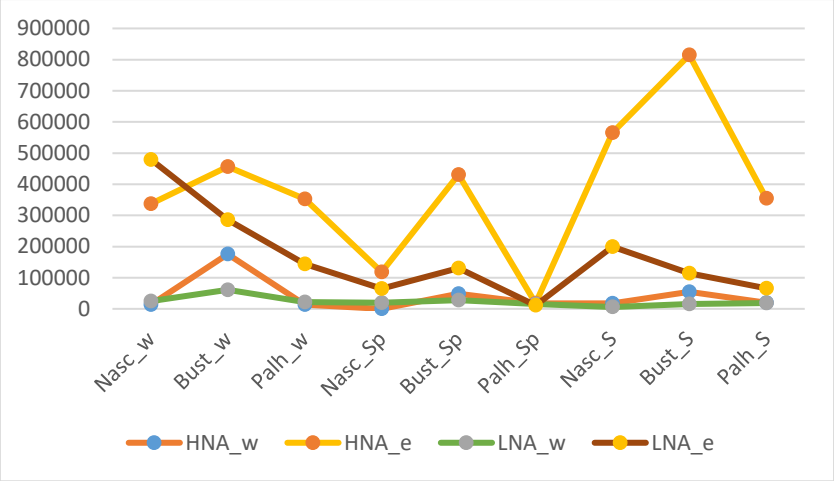

Figure 8- Variation of HNA and LNA bacteria concentrations along the 3 seasons (winter, spring and summer) for both water and sediment samples.

2.3.4. Data analysis – multivariate approach

Macroinvertebrates

The DCA performed on macroinvertebrate communities matrix obtained a low length of gradient of the first axis (2.022) (Figure 9). Different macroinvertebrate community composition and abundances are responsible for the separation of clusters.

Meanwhile, DCA provides some interesting discrimination based on the community composition only, between sampling sites, separating river source (Nascente sites) from the other sampling sites that are grouped together. Exception made for Palhal in spring season and Nascente in summer, which show some intermediate position between the two major groups indicating that community composition, in that seasons, were different from the other groups.

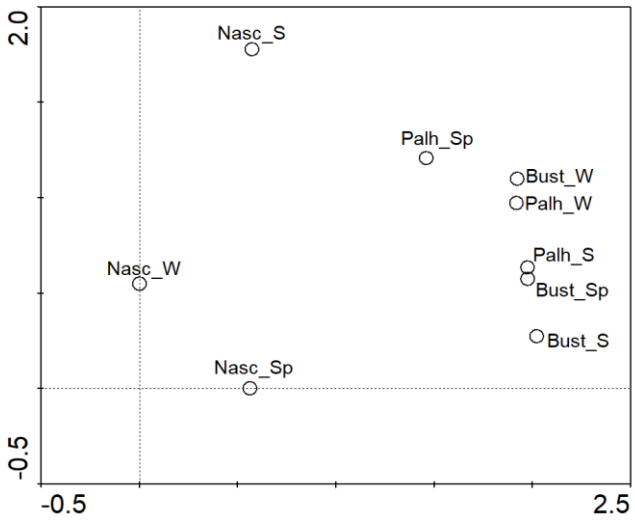

Figura 9- Sample scores of DCA on macroinvertebrate community abundances for 3 sampling sites Nascente (Nasc), Bustelo (Bust) and Palhal (Palh) in winter spring and summer. Eigenvalues are 0.520 and 0.307 for axes 1 and 2, respectively.

The RDA model selects the following parameters: O_2(%)_w, OM(%) and S_w as the most important explaining the macroinvertebrate variation (Figure 10 and 11). This RDA model has a percentage variance of 50.4% for species-environment relation. The diagram from Fig 11 suggests a separation of samples in three main groups: one of the groups is constituted by more tolerant taxa to contamination, with a lower sensitivity degree (solid line circle), consistent with the distribution of Palhal and Bustelo (Figure 10). The two remaining groups (dotted) are related to river source communities, with high taxa score (IBMWP) and high sensitivity to contamination, which were differentiated between them. In summer the increase in dissolved oxygen (%) organic matter and total sulfur, both in sediments, provide conditions for the development of highly diversified macroinvertebrate community with different taxa that were not observed on the other seasons, for the same sampling site, being characterized by a higher abundance of Leptophlebiidae, Leucridae, Leptoceridae, Elmidae, Calopterygidae, Cordulegastridae, Polycentropodidae, Ephemerellidae, Dixidae and Aeshnidae (dashed line circle –top right quadrant. The third group is characterized by Ephemeroptera (Heptageniidae), Plecoptera (Nemouridae), Trichoptera (Sericostomatidae, Hydroptilidae, Hydropsychidae and Rhyacophilidae) and

Coleoptera (Gyrinidae and Hydraenidae) taxa, corresponding to sensitive organisms, founded mostly at Nascente in winter and spring (Figure 10).

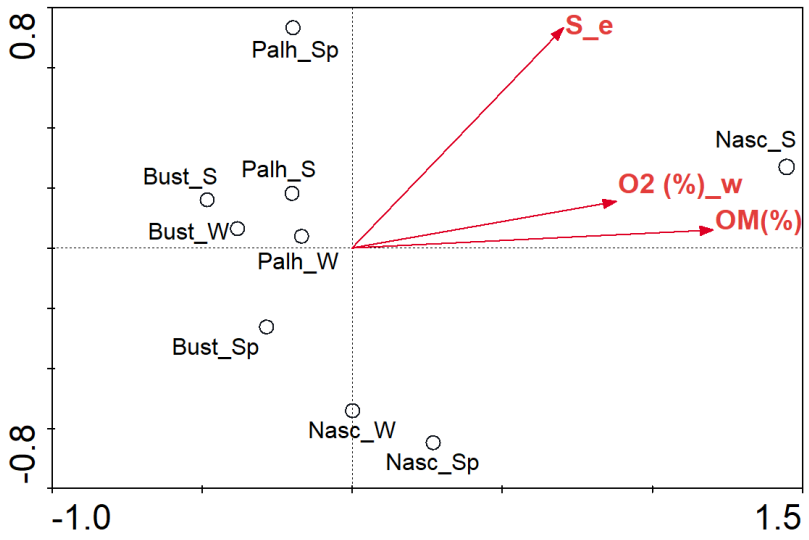

Figure 10- RDA biplot sample score and environmental gradients (represented by arrows) of macroinvertebrate data matrix. S_w stands for total sulfur in sediment, O2 (%)_w stands for percentage of dissolved oxygen in water and OM (%) stands for percentage of organic matter in sediment. Eigenvalues are 0.304 and 0.162 for axes 1 and 2, respectively.

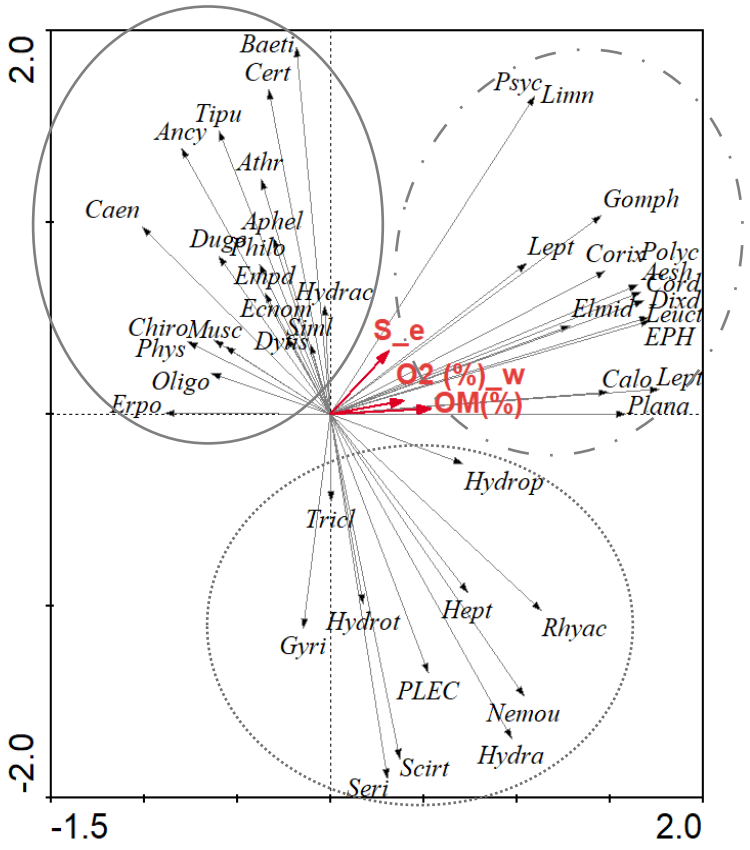

Figure 11- RDA biplot species scores (represented by grey arrows) and environmental gradients (represented by red arrows) of macroinvertebrate data matrix. S_w stands for total sulfur in sediment, O2 (%)_w stands for percentage of dissolved oxygen in water and OM (%) stands for percentage of organic matter in sediment. Eigenvalues are 0.304 and 0.162 for axes 1 and 2, respectively.

Periphyton

The DCA performed on periphyton communities matrix obtained a length of gradient of the first axis of 6.742. Bustelo and Palhal have very similar periphyton communities and therefore, both sampling sites were positioned together for all seasons. On the other side of the DCA, it can be observed Nascente sampling sites all together evidencing their similar biotic community composition and very different from the other sampling sites (Figure 12).

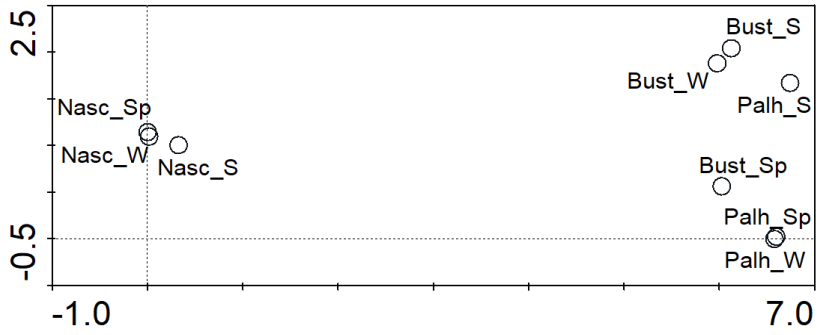

Figura 12- Sample scores of DCA on periphyton community abundances for 3 sampling sites Nascente (Nasc), Bustelo (Bust) and Palhal (Palh) in winter spring and summer. Eigenvalues are 0.954 and 0.407 for axes 1 and 2, respectively.

The CCA diagram integrated the periphyton data with selected significant physicochemical and metal concentrations: NH_3_w, NH_4_w, S_w and Pb_w, which explained 48.6% of species-environment relation (Figure 13 and 14). The resulting diagram proposes a separation of samples in three groups: one of the groups is constituted by species that are more intolerant to pollution (dashed line circle), with no nutrients or metals associated; the two other groups corresponded to species tolerant to nutrients, thus more abundant in impacted environments. Even so, these two groups were very differentiated by the type of pollutant sources and its abundance. Black solid circle associated all the Bustelo sites (Figure 14), which were influenced by the presence of NH_3 and NH_4 in water column, characterized by high abundances of *Gomphonema parvulum, Gomphonema pumilum, Neidium dubium, Nitzschia dissipata* and *Psammothidium subatomoides*. Grey solid line group is associated to higher values of sulfur (S_w) and lead (Pb_w) in water and incorporated all the Palhal sites that were characterized by the diatom community of *Cyclotella meneghiniana, Achnanthes minutissima, Cocconeis placentula, Melosira varians, Cymbella tumida, Navicula cryptotenella, Navicula lanceolata, Navicula rhynchocephala, Gomphonema acuminatum, Planothidium frequentissimum, Fragilaria biceps, Encyonema minutum, Pinnularia gibba* and *Surirella linearis*. The dashed line circle, in the opposite direction were negatively related with nutrient input and metals and were observed at Nascente in all seasons sampled (Figure 13), characterized *by Eunotia exigua, Eunotia minor, Eunotia bilunaris, Anomoeoneis serians, Peronia fibula, Stauroneis*

phoenicenteron and *Surirella angusta*. These results confirm the choice of Nascente as reference location due to the negative relation to nutrient input and metals.

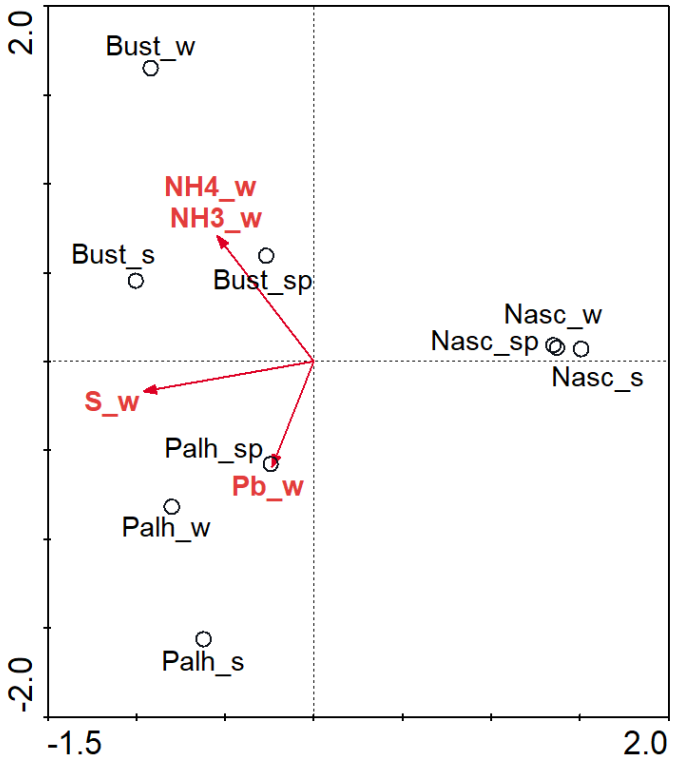

Figura 13- CCA biplot os sample score and environmental gradients (represented by red arrows) of macroinvertebrate data matrix. Pb_w, NH_3_w, NH_4_w and S_w stands for lead, ammonia, ammnium and sulfur in water, respectively. Eigenvalues are 0.914 and 0.607 for axes 1 and 2, respectively.

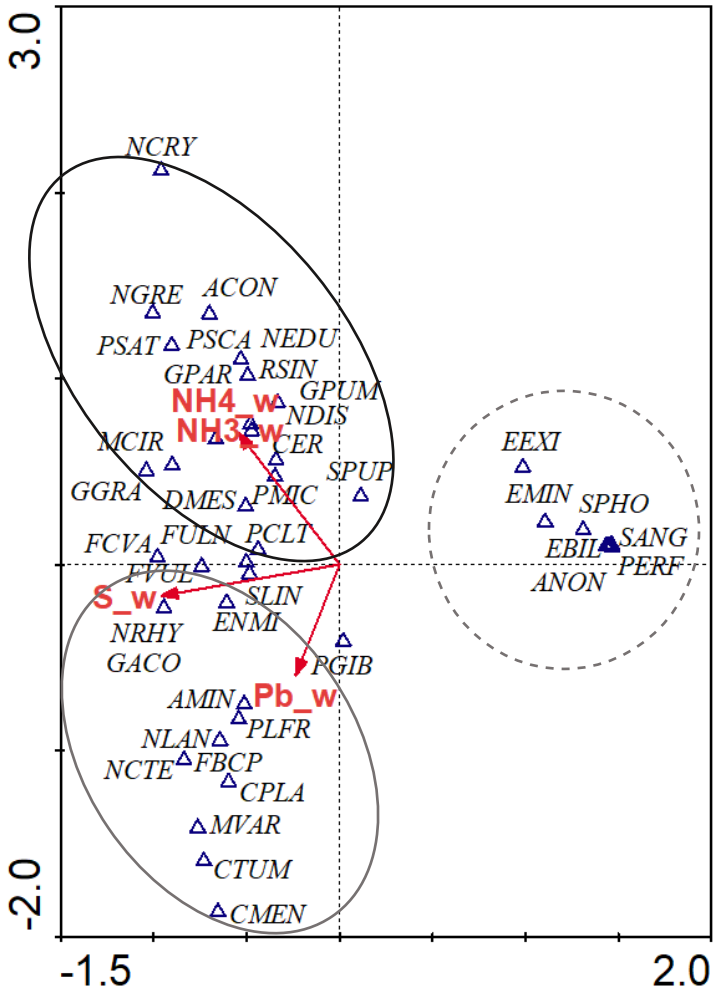

Figura 14- CCA biplot of species (represented by triangles) and environmental gradients (represented by red arrows) of periphyton data matrix. Pb_w, NH_3_w, NH_4_w and S_w stands for lead, ammonia, ammonium and sulfur in water, respectively. Eigenvalues are 0.914 and 0.607 for axes 1 and 2, respectively.

Bacteria

The DCA for bacteria community was made with total density and LNA/HNA bacteria abundances, both in water and sediment, and it was obtained a length of gradient for the first axis of 1.029. Apparently, Palhal in spring and Nascente in winter were the two sites that were less related to the other sites, being more distanced in the graphic (Figure 15). It was not possible to see a clear pattern in the sampling sites related to bacteria community.

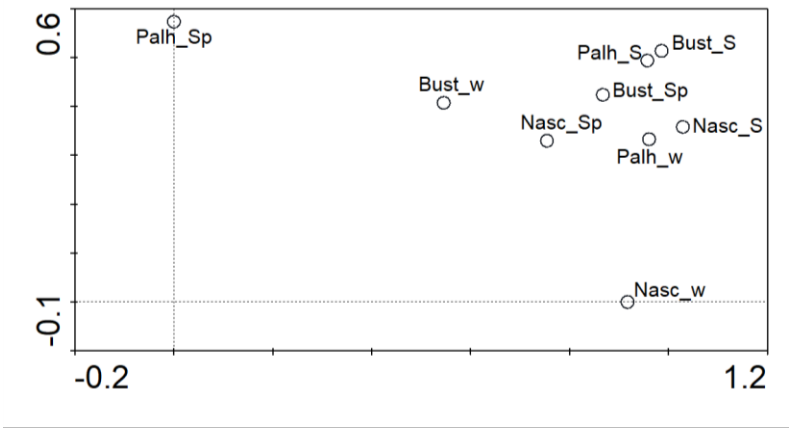

Figura 15- Sample scores of DCA on bacteria community abundances for 3 sampling sites Nascente (Nasc), Bustelo (Bust) and Palhal (Palh) in winter spring and summer. Eigenvalues are 0.081 and 0.038 for axes 1 and 2, respectively.

The RDA model included the bacteria data with extracted gradients from significant physical and chemical and metal concentrations parameters: BOD5_w, TP_w, PO4_w, Mn_e, cond_w and CDOC_w, which explained 90.7% of the data variability (Figure 16 and 17). As shown in Figure 17, the graphic was separated in two groups: one group is constituted by HNA bacteria in sediment, influenced by high levels of total phosphorus, phosphates and conductivity in water and manganese in sediment (solid line circle), which were related to polluted sites, Bustelo in summer and spring; another group were composed by HNA, LNA bacteria, total density in water and LNA bacteria in sediment, being the last one highly influenced by BOD_5_w (dashed line circle). While LNA, HNA and density in water samples were very similar to each other, sediments were discriminated apart from the water on the graphic. Total density in sediment was associated with low values of CDOC in water.

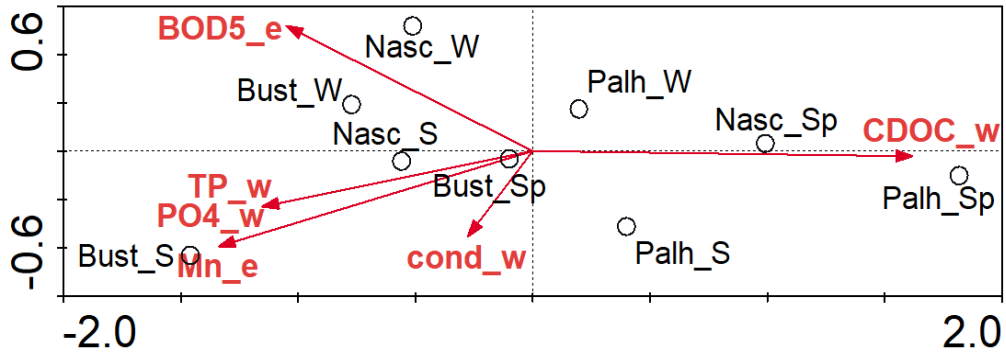

Figura 16- RDA biplot of samples score and environmental gradients (represented by red arrows) of bacteria data matrix. BOD_5_e and Mn_e stands for biochemical oxygen demand and manganese in sediment and TP_w, PO_4_w, cond_w and CDOC_w stands for total phosphorus, phosphate, conductivity and dissolved organic carbon in water, respectively. Eigenvalues are 0.866 and 0.070 for axes 1 and 2, respectively.

Bustelo and Palhal in summer were associated with high levels of conductivity in water. Furthermore, Bustelo in summer and spring were also related to elevated values of phosphates, total phosphorus in water, and manganese in sediment (Figure 16). This sampling site corresponds to high concentration of HNA bacteria in sediment, where the ecological impact is greater than in the other sites. Nascente in winter were mostly influenced by high values of BOD_5 and associate also with high levels of LNA in sediment. BOD5 also correlates with Bustelo in winter and CDOC in water was found in high concentration in spring at Nascente and Palhal. High levels of LNA in sediment and LNA_w, HNA_w and dens_w were associated to Nascente in summer and winter and Bustelo in winter.

The DGGE methodology was not successful, due to difficulties in the DNA extraction and in the optimization of protocols (e.g. temperatures cycle), making it impossible to present any result in this work.

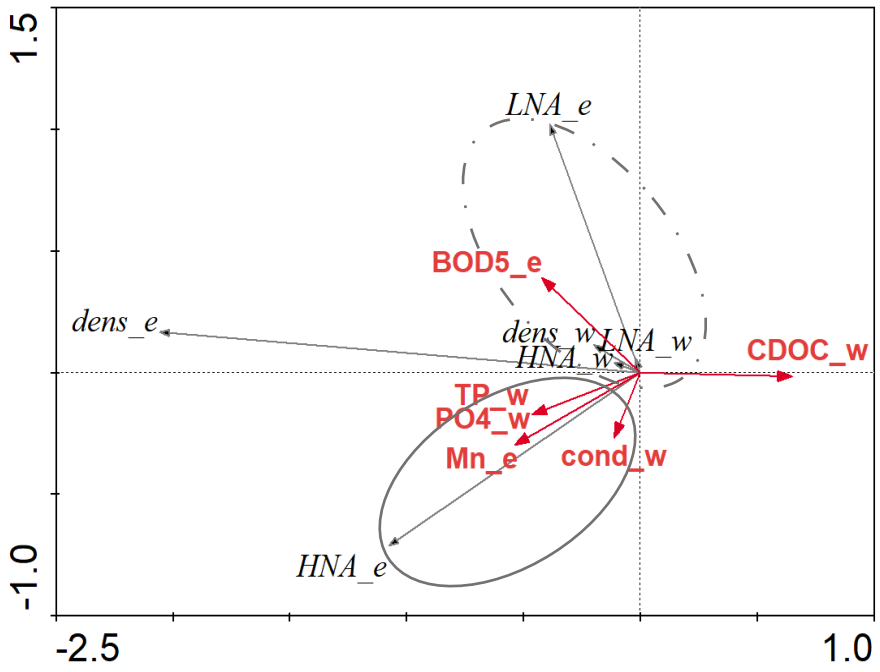

Figura 17- RDA biplot of species (represented by grey arrows) and environmental gradients (represented by red arrows) of bacteria data matrix. BOD_5_e and Mn_e stands for biochemical oxygen demand and manganese in sediment and TP_w, PO_4_w, cond_w and CDOC_w stands for total phosphorus, phosphate, conductivity and dissolved organic carbon in water, respectively. Eigenvalues are 0.866 and 0.070 for axes 1 and 2, respectively.

2.4. Discussion

Caima River was chosen for this study as a model for the appliance of the Portuguese WFD (using macroinvertebrates and periphyton communities) and in addition, to evaluate the bacterial communities present in river water and sediment, as biological indicator using a rapid screening method of FCM for different effects of multiple stressors along its course (abandoned mine drainage, domestic and industrial waste), comparatively to a possible unpolluted site, the river source (Nascente). Although Nascente proved to be a good reference point, the summer wildfires followed by winter rainfall events promote the ashes runoff into the river course diffusing the contamination by chemical elements in the aquatic ecosystem (Pereira *et al.*, 2013). Some metals such as S, Al, Cu, As, Cr and Fe

(Campos *et al*., 2012; Silva *et al*., 2014) and nutrients, especially phosphate, nitrate and ammonium (Spencer *et al*., 2003; Earl & Blinn, 2003; Hosseini *et al*., 2017), were found to be attached to ashes and subsequently transported to different environmental ecosystems. At Nascente, the higher concentrations of these metals and nutrients occurred mainly in sediment samples, while in water samples, metals like Cu and As were found at minimum concentrations and the phosphates and nitrates weren't even detected (Table 1 and 2). CODC and BOD_5 were also found very high in winter, probably due to the recent transport of organic matter into the river, by the rainfall. In general, all these concentrations were significant in the first season of sampling (winter) and decreased over the year. Hoissini *et al.,* (2017) found the highest value of N and P exports in runoff and sediment loss in the second year after the fire but several nutrient peaks were found in autumn and winter after the fire has occurred and first raining events happened. The same authors pointed the high concentration of suspended solids and nutrients on runoff capable of affecting the water quality of downstream aquatic systems and the same authors also stress about the repeated fire frequency on Mediterranean ecosystems and land management possibly returning to prefire conditions. High values of pH recorded in winter at Nascente can be also related to the ashes runoff into the river course as documented by the authors (Earl & Blinn, 2003). It's worth to mention the high amount of organic matter (%) in sediment, dissolved organic carbon and BOD_5 obtained in Nascente elutriates can also being overestimated by the effect of composed sample which, in case of Nascente, the sediment collection takes also into consideration a downstream dam, that due to the retention effect, presented higher amounts of organic matter. Likewise, in all sampling sites, a composed sample of sediments collected reflect the river microhabitats.

Regarding the application of WFD to the river sampling sites, macroinvertebrate and periphyton communities didn't show to be affected by wildfires since these communities classified the Nascente as high in winter just after the runoff of ashes from the summer fires in spite off in contrary, Rugenski *et al* (2014) observed a community structure change in macroinvertebrate assemblage increasing taxa disturbance adapted like chironomid midges and *Baetis* mayflies. We hypothesize that ashes runoff occurred soon before the sampling collection and the community changes have not been felt, yet or the

characteristics and duration of the ash flow produces minimal reductions in density of macroinvertebrate community (Earl and Blinn 2003). The periphyton community was classified in Nascente as good, in all seasons, opening the possibility for some degree of sensitivity to that particular event (ashes runoff). Earl & Blinn (2003), found small changes in periphyton community mostly change in community assemblage to smaller more adnate taxa not considering the total periphyton biomass being significantly affected by wildfires.

Previous studies on WWTP (Waiser *et al*., 2010; Drury *et al*., 2013; Perujo *et al*., 2016) reports an increase in nutrients concentration (PO_4, NH_4, NH_3, TP and TN) downstream the effluent, as observed in this study at Bustelo sites, where the highest values occurred for all samples seasons, both in water and sediment samples. Curiously, all these parameters were found to be in higher quantity in water samples, than on sediments, probably due to the high flow of the discharge that does not allow to settle down in sediments. Contrary to what was expected, there were no significant differences in BOD_5 and CDOC concentrations relatively to the other 2 sites, showing that the WWTP was working properly and not dumping dangerous amounts of organic matter and dissolved carbon into the river. The literature related to European WFD (STAR project) monitoring of running water has been using phytobenthos for decades and benefit from existing information on the sensitivity of indicator taxa to various impairments. They are incorporated in the methodological approaches, standard and practice of water management due to their known sensitivity to eutrophication/organic pollution, acidity, salinity and current velocity (Brabec and Sczoszkiewick, 2006; Besse-Lototskaya *et al*., 2006). Several studies performed to different river typologies, in Europe, have compared the discriminant power of different biological communities to detect change and conclude that benthic diatoms and macroinvertebrate assemblages were reliable indicators of changes in nutrient status, eutrophication and acidification (Johnson *et al*., 2016a). Furthermore Johnson *et al*., (2016b) regarding the organism-response relations to environmental gradients, advanced that diatoms would respond strongly when the sites became more impacted with nutrient enriched and that diatoms would be an early warning indicator in detection of early changes in nutrients levels. Having all these in consideration, in our study, the periphyton community did not show any early warning capacity in

discriminating the presence of nutrient enrichment even in small amounts (our case) and still classified Bustelo as good in winter and spring and high in summer.

Palhal was expected to be impacted by runoff rich in metals from the deactivated mine as already been confirmed by Vidal *et al*., (2012) and Nunes *et al*., (2003). Comparatively to previous studies (Vidal *et al*., 2012; Nunes *et al*., 2003) Cd and Zn were below detection level, but Pb, S, Cu and Al were quantified in higher quantities than in previous studies. Metal were consistently observed at higher concentrations in sediment than in water samples, supporting the idea that some are strongly bound to the sediments (Morillo *et al*., 2004) and can persist for centuries (historic contamination) after the mine closure (Byrne *et al*., 2013) and, still, the actual contamination resulting from deactivated mine without a recovery plan to mitigate the effects on river communities (Vidal *et al*., 2012).

From the ecological status evaluation point of view, in Palhal in summer, an inversion of response pattern towards Bustelo can be observed, being the macroinvertebrate community less discriminatory than benthic diatoms. Several studies have shown that some of the metrics used in WFD approach (richness and EPT taxa) are sensitive to metal contamination (Hickey and Clements, 1998; Mebane, 2001; Beasley and Kneale, 2003), particularly in mining areas (Hoiland *et al*., 1994; Maret *et al*., 2003; Iwasaki *et al*., 2009). However natural factors such elevation (Clements and Kiffney, 1995) or fine sediment accumulation (Mebane, 2001), also change the response of such metrics. Therefore the use of generic metrics will always produce confounding factors, and may either over-or underestimate environmental quality, especially in multiple stress scenarios. The solution to this problem could be the inclusion of stress-specific biotic indices as suggested by Extence *et al*., (1999; 2013) or use fine-resolution community tools like multivariate analysis.

In contrast to WFD approach, community structure analysis (multivariate analysis) enables the extraction of gradients that explain much better the proximity or separation among groups of samples and their relationship with abiotic parameters, one of the most criticism presented to the WFD approach that does not establish a cause-effect relationship. In general, when comparing both approaches (WFD and multivariate analysis)

for macroinvertebrates and periphyton can be concluded easily that multivariate analysis were able to separate clearly the sampling sites, except for Nascente in winter where the ecological status was superimposed by the physico-chemical parameters (Table 5) and not by the biological communities itself due to WFD rule, one out - all out, and both methodologies agree with the final results obtained. Seasonality were important for WFD approach (since the ecological status varies according with the season sampled) but not in multivariate analysis, being the most important the sampling site and the communities associated with it (Figs. 10,11,12,13) disregarding the sampling season. Multivariate analysis discriminate unanimously Bustelo and Palhal as impacted sites independent from the biological community used (macroinvertebrate and periphyton). Looking further into the macroinvertebrate community assemblage composition, some pollution-sensitive taxa associated with Nascente sites were identified. The EPT richness was very high in the river source along the seasons, reached the maximum value in summer. EPT species richness are significantly higher in undisturbed sites (Compin & Céréghino, 2003), being the Plecoptera order one of the most sensitive to water pollution (Lazaridou-Dimitriadou, 2002), supporting the Nascente site as a reference zone with little human impact. At Bustelo and Palhal the values were stable but very low, as expected since the community is influenced by stressed and low oxygenated sites (Pastuchová, 2006). In Bustelo and Palhal were found a variety of organisms tolerants to pollution such us Chironomidae, among others Diptera, Oligochaeta and Gastropoda. Species of Oligochaeta are frequently associated with organically polluted sites as well as Chironomidae that are in environments with low concentrations of dissolved oxygen (high organic matter) (Rosa *et al*., 2014) (Oliveira *et al*., 2010). Concerning the same approach to periphyton, different taxa founded in polluted sites, as *Gomphonema*, *Nitzschia* and *Navicula,* are known to be tolerant to organic and metal pollution (Almeida, 2001; Kwandrans *et al*., 1998; Bere & Mangadze, 2014) preferring eutrophic environments and relatively low oxygen saturation (Van Dam *at al*., 1994; Dalu *et al*., 2017). Palhal in spring and summer was associated with CPLA, CMEN, FBCP and MVAR that, according to Van Dam (1994), are classified as facultatively nitrogen-heterotrophic taxa, needing periodically elevated concentrations of bound nitrogen and normally founded in eutrophic places. The periphyton community at Nascente was mostly

composed by species known to be indicators of oligotrophic and mesotrophic waters (Van Dam, 1994) and intolerants to nutrient contamination.

Analyzing the multivariate community structure analysis among biological communities (macroinvertebrates, periphyton and Bacteria) studied using this methodology and its comparison in order to stress about the bacterial community response as biological indicator using fast screening methodology like FCM. Both the macroinvertebrates and periphyton community individualized the river source (Nascente), in all sampling seasons, as clean and on the other hand associated, also for all seasons, Palhal and Bustelo with metals and anthropogenic variables measured in water, lead (Pb) plus total sulfur and ammonia and ammonium, respectively. Bacteria also associate Nascente as clean sampling site in all seasons but include also Bustelo and Palhal, in winter as free of impacts. The multivariate periphyton community analysis considers both Palhal and Bustelo, in winter, as contaminated as Palhal and Bustelo in any other season. Meanwhile, macroinvertebrates place Palhal_w and Bustelo_w very near the reference position (Fig. 10) showing that its relationship with contamination is weak, but present (WFD classified Bust_w as moderate signaling the contamination presence). Multivariate analysis on Bacteria (Fig. 16) allows to clear identify Bustelo and Palhal as contaminated in spring and summer associated HNA bacteria (high nucleic acid content bacteria), in this case, measured on sediment elutriate. Zhao *et al*., (2010) and Ke *et al*., (2015) already suggest that microorganisms in water column or in sediments in aquatic environments can functioning as an instantaneous indicator of water quality respond positively and strongly to total phosphorus, total nitrogen and ammonium-nitrogen contents and can be also strongly associated with metals (Cao *et al*., 2006). Futhermore, Córdova-Kreylos *et al*., (2006) found that sediment microbial total biomass is positively correlated with organic carbon or total nitrogen in sediments. Sediment microorganisms can work also as efficient indicators of long-term impact of impact in the overlying water column (US EPA 2002, Goodrich *et al*., 2005). The data found in this work fully confirms the literature and found positive correlation between the HNA, in sediments, and total phosphorous, phosphates, increase in conductivity, all in water, and the manganese, in elutriates. HNA bacteria as representative of the most active part and contribute to most of the total microbial

production (Gasol *et al*., 1999; Lebaron *et al*., 2001). From the point of view of using FCM for fast screening of bacterial communities changes in water quality evaluation, LNA bacteria was previously considered as not being active or even dead, but thereafter were shown to be viable and active in low nutrient environment by several authors (Prest et al., 2013; Servais *et al*., 2003; Longnecker *et al*., 2005; Bouvier *et al*., 2007 and Wang *et al*., 2009) and is confirmed by data obtained, being LNA both in water and sediment associated with Nascente where the contamination was shown to be minimum in comparison with other sampling sites. On the other hand, HNA is considered to be more dynamic and sensitive to changes than LNA, what was completely corroborated by the results presented and as shown, highly related with increased of nutrients especially phosphates, conductivity and manganese (see Fig.17).

Therefore the discriminant power of the Bacteria community analyzed by FCM was not perfect but provides responses good enough to continue the search and invest more time and energy in refinement of this fast screening methodology as a tool to complement and prioritize sampling sites requiring further intervention and WFD appliance. It would be quite interesting also to compare the results obtained and the results from DGGE analysis and more efforts would also be put on in order to refine the methodology and solve the problems raised so far. A trial approach of performing multivariate analysis merging all the biological communities surveyed (macroinvertebrates, periphyton and bacteria) does not allow to understand the relationships between the communities and environmental variables selected by forward selection in canonical analysis as clearly as performing them individually.

In rivers, the contamination of water column is very variable depending on the river flow, turbulence and dilution factor and hence assessment of ecological status should consider the sediment matrix. Sediment can contain several amounts of organic and inorganic material bounded to particles, but when disturbed by stormnwater runoff, they can turn bioavailable as an important pollution source for both benthic and planktonic organisms communities (Burton, 2002) Elutriate sediment toxicity test are widespread useful tools to address toxicity of complex environmental samples (USEPA 2001; Vidal *et*

al., 2012). Therefore this study comprises sediment elutriate quantifications and analysis in addition to the analysis on running water requested by WFD.

2.5. References

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Chapter 3 - Final remarks

Since the rise of industrialization has occurred that freshwater ecosystems have been threatened. The overexploitation of this resource for all types of human activities had devastating consequences for the biodiversity that lives in it (Maksimovic *et al*., 1996; Allan & Castillo, 2007). For that reason, the European Union reached an agreement and implemented the Water Framework Directive (WFD) in 2000, to stimulate water quality improvement and contribute to the management of all surface waters and groundwaters (van Puilenbroek *et al*., 2015). As an aim of WFD to reach good ecological status by 2015 or 2027 at the latest (Voulvoukis *et al*., 2017), the Member States have dedicated a lot of work in the optimization and harmonization of techniques and methodologies. However, WFD is very complex, methodologies are time-consuming and costly, and barely provide a clear view of cause-effects relationships. Thus, it is important to find new complementary methodologies to simplify the technical complexity of WFD methodologies.

In order to develop a rapid and cost-effective methodology, we proposed the study of bacteria community by flow cytometry, as a possible bioindicator for water quality assessment. By choosing sites with different environmental impacts, and studying them throughout the seasons, we were able to better understand how the bacterial abundance differ from place to place. One of the aims of this work was to study the bacterial abundance also by DGGE, and compare the two techniques. This part was not possible to present, due to difficulties in protocols appliance (DNA extraction protocol and temperature cycles) and reagent contaminations.

The use of multivariate analysis in the addition to the methodology WFD application to Caima river allowed to conclude that the river has good ecological status in river source, and not so good in impacted sites (Bustelo and Palhal), as expected due to the different sources of pollutants along the river WWTP, agricultural runoffs and mine drainage, respectively. On the other hand, community structure analysis was more discriminatory, allowing the study of spatial and temporal patterns with the factors that best explain the species-environmental relation and identifying clearly the pollution sources in each sampling site. Bacteria community analysis was also able to distinguish the majority of impacted sites from clean sites, being clear the separation of LNA and HNA bacteria

community in sediment according to the different environmental stress. These results showed that bacteria in sediment has more reliable information about the possible effects that may arise, being a good indicator of long-term environmental impacts. Despite that, further studies needed to recognize the applicability of this method, possibly complemented with DGGE methodology.

Annex

Table A.1- Abbreviation (Abbr) list of macroinvertebrate taxa collected in Caima River for 3 sampling sites during the study period.

Abbr.	Class/Order	Family	Nasc_w	Bust_w	Palh_w	Nas_sp	Bust_sp	Palh_sp	Nasc_s	Bust_s	Palh_s
Dytis	Coleoptera	Dytiscidae									4
Elmid	Coleoptera	Elmidae	4	3	46	32	5	24	51	3	13
Gyri	Coleoptera	Gyrinidae	3	1							
Hydra	Coleoptera	Hydraenidae	3			4			2		
Scirt	Coleoptera	Scirtidae	38			103			4		
Athr	Diptera	Athericidae			6		1	6			
Cert	Diptera	Ceratopogonidae			1		1	13			3
Chiro	Diptera	Chironomidae	19	88	77	277	819	139	270	3320	1421
Dixd	Diptera	Dixidae							3		
Empd	Diptera	Empididae	3		3	2	6	8	1		3
Limn	Diptera	Limoniidae						1	1		
Musc	Diptera	Anthomyiidae								4	
Siml	Diptera	Simuliidae	4	1	2	1	8		18	59	
Tipu	Diptera	Tipulidae						1		1	
Baeti	Ephemeroptera	Baetidae	70	192	85	48	228	544	207	210	104
Caen	Ephemeroptera	Caenidae		242	291	1	399	285		300	705
EPH	Ephemeroptera	Ephemerellidae				5			66		
Hept	Ephemeroptera	Heptageniidae	29			1			13		
Lept	Ephemeroptera	Leptophlebiidae	20			19			109		
Ancy	Gastropoda	Planorbidae		3	9		1	6		3	4
Phys	Gastropoda	Physidae		18						19	
Aphel	Heteroptera	Aphelocheridae						2			6
Corix	Heteroptera	Corixidae							2		1
Aesh	Odonata	Aeshnidae	2	1	1			1	15		2
Calo	Odonata	Calopterygidae			1	1			2		1
Cord	Odonata	Cordulegastridae							5		
Gomph	Odonata	Gomphidae	2	3	2	2	5	6	14		6
Hydrac	Acari	Hydracarina	2	1	28	35	9	40	87	48	550
Oligo	Oligochaeta	Oligochaeta		84	38		10	1	2	16	
Duge	Turbellaria	Dugesiidae		3	1			1			4
Plana	Turbellaria	Planariidae	1						2		
Tricl	Turbellaria	Tricladida n.i.					5		1		
Leuct	Plecoptera	Leuctridae	4			21			392		

Table A.1- Abbreviation (Abbr) list of macroinvertebrate taxa collected in Caima River for 3 sampling sites during the study period (cont).

Abbr.	Class/Order	Family	Nasc_w	Bust_w	Palh_w	Nas_sp	Bust_sp	Palh_sp	Nasc_s	Bust_s	Palh_s
Nemou	Plecoptera	Nemouridae	15			46			21		
PLEC	Plecoptera	Plecoptera n.i.				8			1		
Erpo	Hirudinea	Erpobdellidae		8		2	5			12	1
Ecnom	Trichoptera	Ecnomidae									2
Hydrop	Trichoptera	Hydropsychidae	33	3	12	5	9	10	23	2	27
Hydrot	Trichoptera	Hydroptilidae				7		1			3
Lept	Trichoptera	Leptoceridae	1		1			1	15		16
Philo	Trichoptera	Philopotamidae	2		3			3			1
Polyc	Trichoptera	Polycentropodidae			2				29		5
Psyc	Trichoptera	Psychomyiidae						5	5		
Rhyac	Trichoptera	Rhyacophilidae			1	13			7		
Seri	Trichoptera	Sericostomatidae	113	1		214			2		

Table A.2- Abbreviation (Abbr) list of periphyton taxa collected in Caima River for 3 sampling sites during the study period (continues next page)

Species	Abbr.	Nasc_w	Bustelo_w	Palhal_w	Nas_Sp	Bustelo_Sp	Palhal_Sp	Nascente_S	Bustelo_S	Palhal_S
Achnanthes conspícua A.Mayer	ACON		40	4		15		2		3
Achnanthes minutissima Kützing var. *minutíssima*	AMIN			283		130	310		8	100
Anomoeoneis serians (de Breb.) Cleve var. *brachysira* (de Breb.) Kützing	ANON	8						2		
Ceratoneis arcus Kützing	CER			1		8				
Cocconeis placentula Ehrenberg var. *placentula*	CPLA		18	44		2	67	1	4	126
Cyclotella meneghiniana Kützing	CMEN		2			3			3	168
Cymbella tumida (Brebisson) Van Heurck	CTUM			3						4
Diatoma mesodon (Ehr.) Kützing	DMES			1		7			2	1
Encyonema minutum (Hilse in Rabh.) D. G. Mann	ENMI	2	2						7	6
Eunotia bilunaris ((Ehr.) Mills	EBIL	26								
Eunotia exigua (Breb.) Rabenhorst	EEXI	5	4		2			8		
Eunotia minor (Kützing) Grunow in Van Heurck	EMIN	12	6	1	19	17	5	79	1	
Fragilaria biceps (Kützing) Lange- Bertalot	FBCP			2						
Fragilaria capucina Desmazieres var. *vaucheriae* (Kützing) Lange-Bertalot	FCVA			1					2	
Fragilaria ulna (Nitzsch.) Lange-Bertalot var. *ulna*	FULN			6		13			1	2
Frustulia vulgaris (Thwaites) De Toni	FVUL		6	13		3	3		1	
Gomphonema acuminatum Ehr. var. *coronata* (Ehr.) W.Smith	GACO			1					1	
Gomphonema gracile Ehr.	GGRA			1					20	

Species	Abbr.	Nasc_w	Bustelo_w	Palhal_w	Nas_Sp	Bustelo_Sp	Palhal_Sp	Nascente_S	Bustelo_S	Palhal_S
Gomphonema parvulum (Kützing) Kützing var. *parvulum* f. *parvulum*	GPAR		10			53	1		9	4
Gomphonema pumilum (Grunow) Reichardt & Lange-Bertalot	GPUM					6			2	
Melosira varians Agardh	MVAR			17					1	14
Meridion circulare (Greville) C.A.Agardh var. circulare	MCIR		2	2						
Navicula cryptocephala Kützing	NCRY		8							
Navicula cryptotenella Lange-Bertalot	NCTE			4						
Navicula gregaria Donkin	NGRE		6						6	
Navicula lanceolata (Agardh) Ehr.	NLAN			4			2			
Navicula rhynchocephala Kützing	NRHY			1					1	
Neidium dubium (Ehr.) Cleve	NEDU		2			7			1	
Nitzschia dissipata (Kützing) Grunow	NDIS					1			1	
Peronia fibula (de Brebisson et Arnott) Ross.	PERF	53			10			8		
Pinnularia gibba Ehr.	PGIB			4	1			2	1	
Pinnularia microstauron (Ehr.) Cleve	PMIC					8	2		1	
Pinnularia subcapitata Gregory	PSCA		18	4		21		2	1	

Species	Abbr.	Nasc_w	Bustelo_w	Palhal_w	Nas_Sp	Bustelo_Sp	Palhal_Sp	Nascente_S	Bustelo_S	Palhal_S
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot	PLFR		4	1		49	5		6	84
Psammothidium subatomoides (Hustedt) Bukht. Et Round	PSAT		300			120			390	
Reimeria sinuata (Gregory) Kociolek & Stoermer	RSIN		6			70				
Sellaphora pupula (Kützing) Mereschkowsky	SPUP			1		5		2		
Stauroneis phoenicenteron (Nitzsch.) Ehr.	SPHO					1		6		
Surirella angusta Kützing	SANG	305			390	3		312	1	
Surirella linearis W.M.Smith	SLIN			8		14	3		1	1

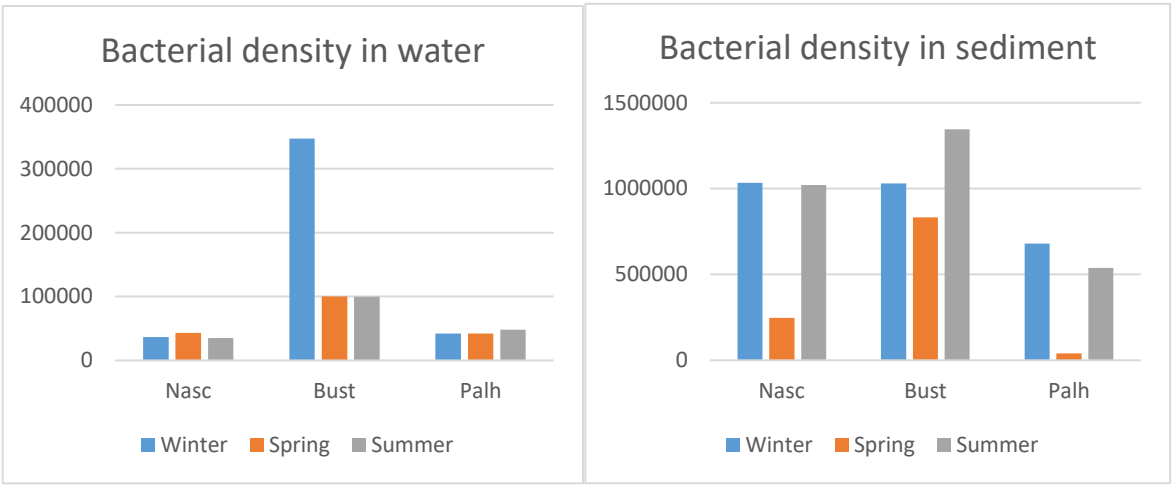

Figure A.1- Total bacterial density in both water (left side) and sediment (right side) samples, throughout the seasons, for Nascente, Bustelo and Palhal sites.