Avaliação ecológica e ecotoxicológica das Lagoas de Gens

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Dedicated to Vó

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Publications of the work developed during this dissertation:

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ii. Resumo

As atividades mineiras polimetálicas portuguesas foram importantes para o desenvolvimento da economia. No entanto, essas atividades deixam quantidades de rejeitos suscetíveis à erosão e ao intemperismo químico, representando um risco potencial para o meio ambiente. As lagoas Gens surgiram devido à mineração a céu aberto numa mina de ouro-antimónio em Gondomar inserida no Parque das Serras do Porto (PSeP). Assim, este trabalho tem como objetivo caracterizar o ecossistema de três lagoas artificiais, no que diz respeito aos parâmetros físico-químicos, e elementos biológicos em diferentes períodos de amostragem, durante o ano 2022/2023. E avaliar o efeito ecotoxicológico de amostras naturais de água destas lagoas em *Lemnna minor* (inibição do crescimento) e *Daphnia magna* (imobilização aguda, sobrevivência e ensaios de inibição da taxa de alimentação). Os bioensaios com as amostras de água de cada lagoa foram realizados em dois tratamentos diferentes: i) amostras de água natural e ii) amostras de água natural com ajuste de pH. De acordo com a análise do FQ, as lagoas são classificadas como de potencial ecológico razoável, devido aos valores de pH ácido e altas concentrações de nutrientes. Em relação aos elementos biológicos, a concentração de clorofila registada obedece aos valores dos padrões. A comunidade de macroinvertebrados é diversificada e é constituída por espécies resistentes (*Oligochaeta*), tolerantes (*Haliplidae*) e sensíveis (*Perlodidae*). Por outro lado, a comunidade fitoplanctônica também é diversificada, sendo influenciada pelos parâmetros físicos e químicos, os resultados obtidos a partir da análise CCA. Os ensaios ecotoxicológicos mostraram-se sensíveis à água das lagoas em todos os períodos de amostragem. O ajuste do pH no caso de *L. minor* provocou um aumento significativo, mas insuficiente para superar os valores de controlo. A existência de metais pesados e aspeto ácido das lagoas Gens, causou toxicidade. No caso de *D. magna*, a mortalidade foi registada nas amostras de água natural tanto no ensaio de sobrevivência quanto nas maiores diluições e amostras diretas no ensaio agudo, enquanto com as amostras de água natural com o ajuste de pH nenhuma mortalidade foi registada em ambos os ensaios. Em relação à taxa de alimentação, as características ácidas e a presença de metais pesados afetam a taxa de alimentação em *D. magna.* As três lagoas apresentaram um potencial ecológico moderado. A biota de Gens mostrou diversidade em ambas as comunidades. O conjunto de bioensaios realizados neste estudo, demonstram toxicidade.

Keywords: Ecotoxicologia; DQA; *Daphnia magna; Lemna minor*

iii. Abstract

Portuguese polymetallic mining activities were important for local development of economy. However, these activities leave amounts of tailings susceptible to erosion and chemical weathering, representing a potential risk to the environment. Gens ponds arose due to open pit mining in a gold-antimony mine in Gondomar (north of Portugal) inserted in the Parque das Serras do Porto (PSeP). Thus, this work aims to characterize the ecosystem of three artificial ponds, regarding the physical-chemical parameters, and biological elements in different sampling periods, during the year 2022/2023. And evaluate the ecotoxicological effect of natural water samples from these ponds in *Lemnna minor* (growth inhibition) and *Daphnia magna* (acute immobilization, survival and feeding rate inhibition assays). For conduct the bioassays with the water samples from each pond two different treatments were carried out: i) natural water samples and ii) natural water samples with pH adjustment. According to the analysis of PC, the ponds are classified as reasonable ecological potential, due to acidic pH values and high nutrient concentrations. Regarding the biological elements, the chlorophyll concentrations recorded comply with the environmental quality standards values. The macroinvertebrate community is diverse and is constituted by resistant species (*Oligochaeta*), tolerant species (*Haliplidae* and *Mesoveliidae*) and sensitive species (*Perlodidae*). On the other hand, the phytoplankton community is also diverse, and is influenced by the physical and chemical parameters, the results obtained from the CCA analysis. The ecotoxicological assays, showed to be sensitive to the water of the ponds in all of the sampling periods. With the pH adjustment in the case of *L. minor* a significantly increased was observed but insufficient to surpass the control values. The existing of heavy metals and acidic aspect of Gens ponds caused toxicity. In the case of *D. magna*, mortality was registered in the natural water samples both in the survival assay and at highest dilutions and direct samples (without dilutions) in the acute assay, meanwhile with the natural water samples with the pH adjustment no mortality was registered at both assays. Regarding the feeding rate, the acidic characteristics, and the presence of heavy metals, affect the feeding rate in *Daphnia magna.* The water quality of Gens ponds, according to WFD, showed that the three ponds presented an ecological potential of moderate. The biota of Gens showed some diversity in both communities. The cluster of bioassays performed in this study, reveal that the ponds of Gens displayed toxicity.

Keywords: Ecotoxicology; WFD; *Daphnia magna; Lemna minor*

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vi. List of Abbreviations

APA	Agência portuguesa do ambiente
ASTM	American society for testing and materials
AWB	Artificial water bodies
BOD	Biological oxygen demand
BW	Buffer water
EQR	Ecological quality ratio
EP	Ecological potential
HMWB	Heavily modified water bodies
HRMP	Hydrographic regions management plans
IBMWP	Iberian biological monitoring working party
IPMA	Instituto português do mar e da atmosfera
NW	Natural water
PSEP	Parque das serras do porto
P1	Pond 1
P2	Pond 2
P3	Pond 3
RV	Recommend value
SPS	Suspended sediments
TSS	Total suspended solids
VSS	Volatile suspended solids
WFD	Water framework directive

1. Introduction

Portuguese polymetallic mining activities were essential for the economy and were actively developed until the early 1970s, which turned out to be one strong pillar in the essence of the economy's structure at that time (Antunes et al. 2013). Over several decades, metal production tends to decline, due to the depletion of the mineral content or the fact that the exploitation of the mine ends up becoming unnecessary due to the new needs of the population, contributing to the high number of closed/abandoned mines (Ericsson et al. 2019). And the high number of closed/abandoned mines represents a sensitive concern in today's environmentally conscious society. Normally the main reasons that leave mine closures are: (a) resource depletion or exhaustion, (b) economic-sharp decline in mineral prices, (c) geologic accessibility, (d) government interventions and (e) society pressure, among others (Pokhrel et al. 2013). However, the abandonment of this activity let large surfaces susceptible to erosion and chemical weathering, representing a potential risk to the environment (Antunes et al. 2013). Moreover, the surrounding ecosystems (adjacent soils, groundwater, etc) are also exposed to a high concentration of heavy metals and acid drainage (Pereira et al. 2004), bearing high concentrations of heavy metals. Indeed, several studies already reported significant effects on aquatic ecosystems, namely (a) a decrease in biota richness and diversity (the low pH can make an environment incapable to support many aquatic life forms) due to the mobilization of heavy metals from the sediment to the water column (Martinez-Haro et al. 2022), (b) communities are restricted to tolerant organisms which can survive in these conditions and (c) alteration in nutrient cycles and abiotic changes may also occur (Oberholster et al. 2013).

Despite abandoned mining activities, other problems cause alterations in the ecological quality of freshwater ecosystems, like demographic pressure, urban and agricultural land use, climate changes, inappropriate waste management, and others (Akhtar et al. 2021). This has been a constant fight since it is urgent to implement measure to protect aquatic resources and ensure water quality in these ecosystems. The Water Framework Diretive (WFD) is the central piece of European water quality legislation (União Europeia 2000). The WFD through the implementation of Hydrographic Regions Management Plans (HRMP), aims to achieve a good ecological status for all surface waters (rivers, lakes, coastal and transitional waters) and groundwater, as well as a good ecological potential for heavily modified and artificial water bodies (e.g., reservoirs) (Diogo et al. 2022). The WFD pursues the sustainable management of water resources, whilst considering environmental, economic, and social dimensions. The “Ecosystem Approach” inherent within the WFD reflects Europe’s increasing efforts to preserve the ecological integrity of the aquatic ecosystems, which is also in line with the aims of other European Directives, described in article a from Directive/60/EC, regarding the concern of deterioration and the protection and enhancement of aquatic ecosystems and it’s sustainable use for diverse activities (União Europeia 2000; Martinez-Haro et al. 2022). As part of the implementation of the WFD in Portugal, the Instituto da Água carried out a series of works to develop tools for assessing the ecological status of the different Portuguese water bodies. In addition, Agência Portuguesa do Ambiente (the national water authority) aims to define management policies and instruments that ensure the application of the following objectives: (a) guarantee the availability of water with quality and quantity for the future generations and (b) water access for everyone, including its protection and efficient use as environmental good and scarce resource (APA 2005). To achieve this objective, it is necessary to know and assess the status of water bodies, whether surface waters (rivers, reservoirs, coastal waters, or transitional waters) or groundwater. The results obtain are based on specific physical, chemical, biological and hydromorphological parameters for each type of water body describe. Based on this information it is possible to classify the water body in Ecological Quality Ratio (EQR) in a scale with five-classes: bad, poor, moderate, good, or excellent. To fulfil the objectives of WFD mentioned above, were defined plans/programs to establish guidelines and criteria for the environmental quality and territory management associated with regional development (APA 2010). In a structured and pragmatic way, these plans (HRMP) are instruments aimed at the management, protection, and environmental, social, and economic enhancement of water at the level of the hydrographic region, making their uses compatible with their availability (APA 2010). Furthermore, these plans are drawn up in planning cycles, and are reviewed and updated every six years. As result, there are currently three planning cycles where a temporal scale of classification is recorded: 1st planning cycle (2009-2015); 2nd planning cycle (2016-2021), and 3rd planning cycle (2022-2027). Over the three cycles, it is possible to observe an effort made by APA to integrate all types of water bodies in WFD, providing more information on the parameters used to assess the different cases and have established reference values for the parameters, in a way it provides a more detailed evaluation. A clear example of the effort made by APA, can be seen on the group of Artificial Water Bodies (AWB) throughout the three cycles.

In the 1st planning cycle, two main groups were defined: superficial water bodies, that was subdivided in rivers, transition water, Heavily Modified Water Bodies (HMWB) and artificial water bodies. Initially HMWB were associated with reservoirs and for AWB, this type did not properly fit into any category, so its evaluation was carried out according to the characteristics which the AWB most resembles with, in this case they are associated with reservoirs. From the 1st to the 3rd cycle, the HRMP went under some modifications in the 3rd cycle the AWB were conceded their own assessment criteria, this type of water body received their own physical and chemical parameters, biological parameters and hydromorphological parameters. A clear example of the evolution from the 1st cycle to the 3rd cycle can be applied to the artificial ponds, for example due to anthropogenic activities (mining).**Table 9**, **Table 10** and **Table 11** in the sector **6. Attachments** describes a simple example of how an artificial pond would be assessed according to the rules described in the 1st cycle HRMP, i.e., an artificial pond, considered an artificial body of water, and in the other hand compare it to the principles of the 2nd and 3rd cycle. On the other hand, a lack of information for the assessment of lentic water bodies was recorded, over the three planning cycles. Moreover, and according to the WFD, the assessment of lentic ecosystems is limited regarding the biological parameters, with phytoplankton being the only parameter used for the ecological potential evaluation. The concern for lack of biological parameters has been established in the scientific literature for lentic ecosystems (García-Chicote et al. 2018a; Trottier et al. 2019a; Pinto et al. 2021a). For example, several authors suggest the study of other communities to be inserted into the biological parameters (e.g, bacterial, benthic macroinvertebrates, and zooplankton) as potential indicators of water quality (Llirós et al. 2014; García-Chicote et al. 2018b; Trottier et al. 2019b; Pinto et al. 2021a).

Although WFD allows the assessment of water quality across physical, chemical, and biological parameters, there are some limitations, since this actual evaluation does not consider the cause-effect relationships, ecosystem functioning, and individual organism responses (Pinto et al. 2021b; Martinez-Haro et al. 2022). Thus, in recent years, several studies proved that using ecotoxicological tools (to understand the various biological responses) provides a more integrative and realistic approach to assessing water bodies quality (Allan et al. 2006; Diogo et al. 2022). Based on this, standardized ecotoxicological assays with aquatic organisms of different trophic levels (such as microalgae, macrophytes, and microcrustaceans) have demonstrated sensitivity and be good indicators of water pollution (Diogo et al. 2022). Due to the ecotoxicological assays, sublethal effects can be determined, that are biologically linked with key ecological processes such as feeding and disorders in specific biochemical and metabolic pathways (Rodrigues et al. 2021).

For this study two main objectives were defined to evaluate the water quality and characterize the biota of the three ponds of Gens using different approaches. At a first step was conducted a complete evaluation of physic and chemical parameters and an overview of the biological communities that constitute the unknown biota of the artificial ponds. To achieve the second objective, regarding the water quality, an ecotoxicological evaluation was applied with a several bioassays conducted with natural waters, namely a growth inhibition assays using *Lemna minor* and a survivor, acute toxicity, and feeding rate inhibition with *Daphnia magna*.

2. Material and Methods

2.1 Study Area

This study was conducted in three artificial ponds (P1, P2 and P3), designed as Gens Ponds, located in the municipality of Gondomar (**Figure 1**). These ponds arose due to mining activities of gold-antimony in the surrounding area, P1 (A = 1002.64 m^2), P2 (A = 2942.45 m^2) and P3 (A = 1604.89 m^2), that occur during the last century. However, other mineral riches (charcoal, silver, lead, tin, tungsten, and zinc) are also present and exploited in the surrounding area (Vieira De Sousa et al. 2017). The mines are located at the confluence of the localities of Gens, Foz do Rio Sousa, and Covelo parishes. Despite the lack of information about this mining activity, it is known that extraction was carried out in layers, with the richest ones being close to 20 meters thick. The exploration started in 1872 and ceased in 1940. The high period of exploitation is documented between 1916 and 1920, with an average of 8046 tons being extracted during this period (Vieira De Sousa et al. 2017). The current artificial ponds, resulting from this activity, are inserted in an area classified as Regional Protected Landscape (from 2017), the Parque das Serras do Porto (PSeP), in the municipality of Gondomar.

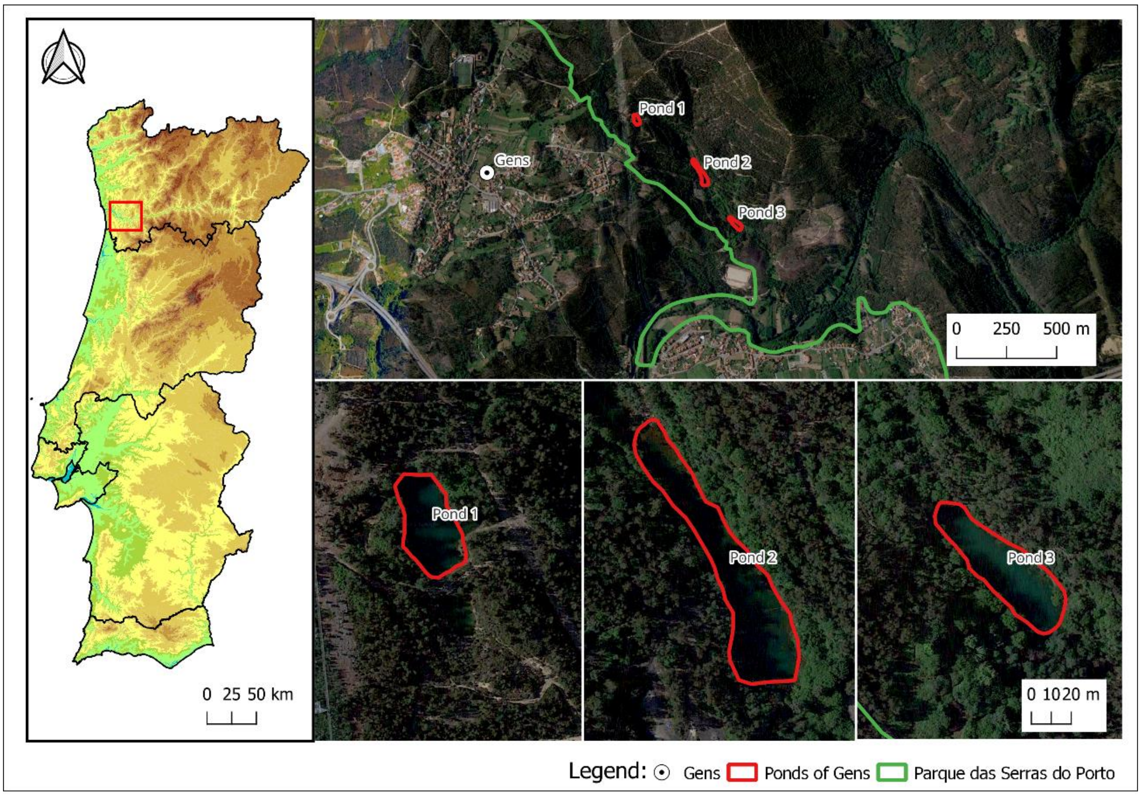

Figure 1 Map of the location of sampling sites in Gens. P1 – 41.1163939, -8.4727097; P2 – 41.1144045, -8.4691684; P3 – 41.1117144, -8.4665426

PSeP is an area that aggregates a series of mountains (Santa Justa, Pias, Caniçal, Flores, Santa Iria, Banias - **Figure 2**) running northwest/southwest, which makes up the Valongo anticline, bringing together some of the oldest geological formations in Portugal, dating back over 540 million years (Parque Das Serras Do Porto 2018). PSeP area presents a diverse and rich natural capital of geology and geomorphology aspects which contributed significantly to the settlement of the region. The geology that characterizes this territory continues to be a hallmark in the construction of houses and walls and the mineral riches (gold, silver, lead, antimony, tin, tungsten, and zinc) have aroused interest at least since Roman times until today. PSeP also presents a landscape with extreme significance for the Porto metropolitan area, due to the extensive and diverse set of natural and cultural values. The PSeP project was created to integrate management that seeks to leverage new and innovative ways of promoting research, strengthening protection measures, defining, and applying policies for the conservation of nature and geo biodiversity, and promoting sustainable management of the territory.

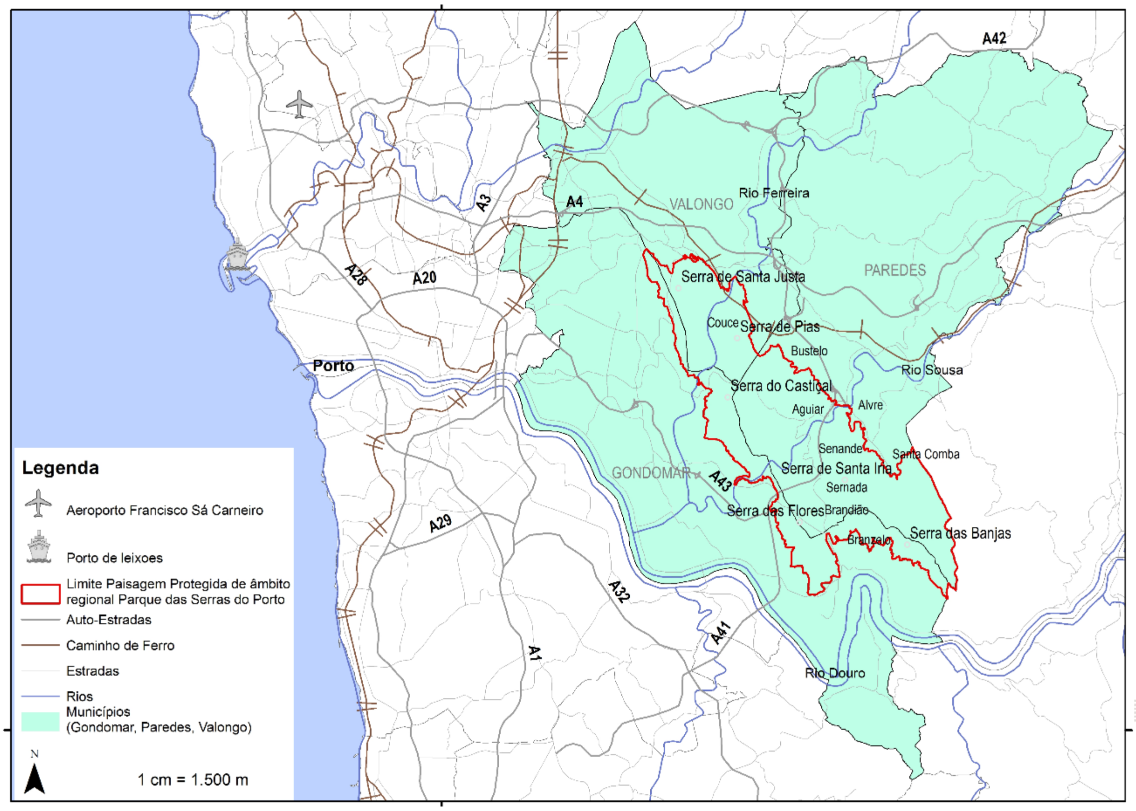

Figure 2 Representation of PSeP area (Total area: 6000 ha) and the complex of Mountains (Santa Justa, Pias, Caniçal, Flores, Santa Iria, Banias). Font: https://serrasdoporto.pt/enquadramento/

The PSeP has the status of a protected landscape at regional level. The values in terms of biodiversity, related to the uniqueness of the area in terms of climate, geological and cultural heritage, become factors that fully justify its classification as a Protected Area. The area's natural vegetation clearly reflects the influence of the transition between the Mediterranean and Atlantic climates that characterized the Douro River valley. This influence is visible in the **Figure 3**, natural forest vegetation characteristic of the territory, dominated by the oak (*Quercus robur*) and the cork oak (*Quercus suber*) in areas with drier soils, but also in the predominance of eucalyptus (*Eucalyptus globulus*) and maritime pine (*Pinus pinaster*) in forest plantations. In addition, two floristic species of high interest for conservation stand out in the territory: Film Fern (*Trichomanes speciosum)* namely the species *Lycopodiella cernua*, thus being the only known place in all continental Europe where it exists, and the hairy fern (*Culcita macrocarpa*), the only one detected in the entire continent (Lima et al. 2018).Despite the high pressures suffered by the main biotopes, several species listed in the Annexes of the Habitats Directive (União Europeia et al. 1992) and classified as threatened in the Red Book of Vertebrates of Portugal still occur in the territory (Cabral et al. 2005). Due to its conservationist importance and the relevance of the population of the species present in the territory of the Serras do Porto, the Lusitanian salamander (*Chioglossa lusitanica*) is probably the best example of the importance of this territory (**Figure 3**).

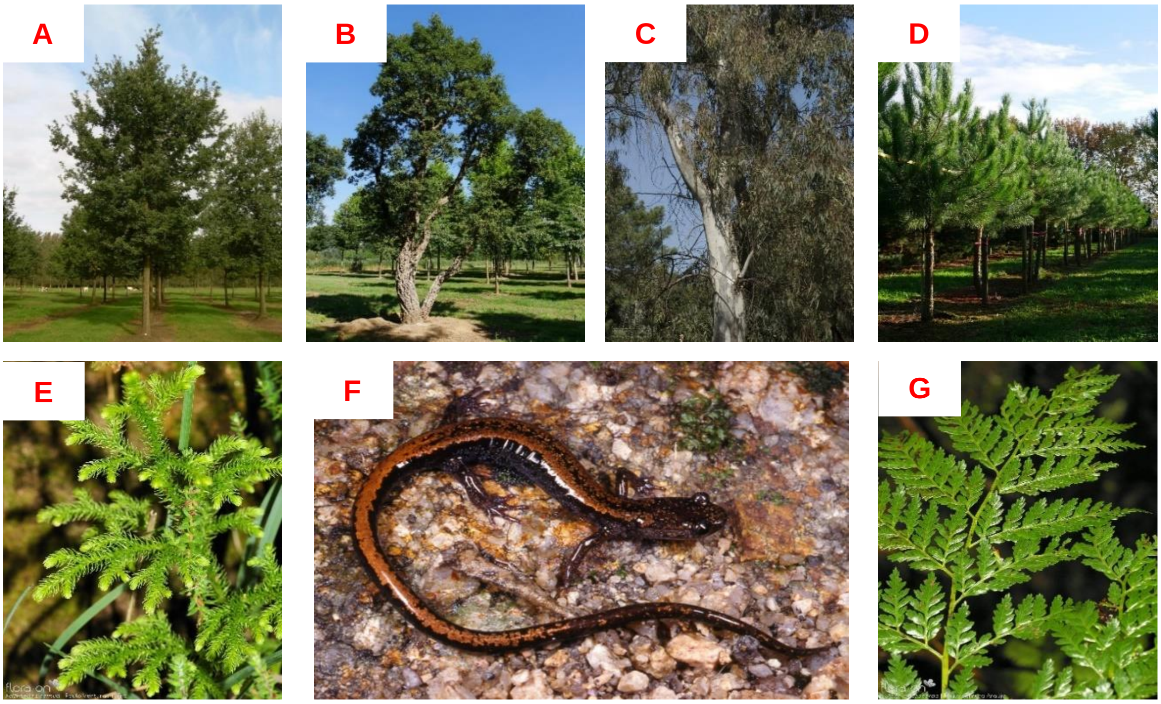

Figure 3 Examples of biodiversity of PSeP (A – *Quercus robur*, B – *Quercus suber*, C – *Eucalyptus globulus*, D – *Pinus pinaster*, E – *Lycopodiella cernua*; F – *Chioglossa* lusitanica; G – *Culcita macrocarpa*

2.2 Sampling Procedure

The present study was performed in 6 sampling periods, along the year 2022/2023: July (Jul22), August (Aug22), September (Set22), November (Nov22), February (Feb23), and May (May23), regarding the guidelines from Water Framework Directive to assess water quality of lentic ecosystems (APA 2022a). In each sampling period, the 3 ponds (P1, P2, and P3 – **Figure 4**) were sampled regarding several physical and chemical parameters measured *in situ* [dissolved oxygen (mg/L) and % saturation), pH, conductivity (µS/cm), salinity, and temperature (ºC)] using a multiparameter probe (Multi 3630 IDS SET F). Additionally, water samples from each pond were collected in plastic bottles (10 L) and transported in the dark at 4 ºC to a chemical industry to perform a more advance chemical analyses (HPLC analysis) during July-August-September and February, for the following groups: Priority substances, Specific pollutants, and other pollutants. And more samples were also collected for the study of phytoplankton, macroinvertebrate communities and for the performance of bioassays within a maximum period of 24 h after the water collection.

Figure 4 Ponds of Gens; Left) pond P1 (41.1163939 N -8.4727087 W); Middle pond P2 (41.1144045 N -8.4691684 W); Right) pond P3 (41.1117144 N -8.4665426 W).

The phytoplankton analysis was conducted in an aliquot of 500 mL of water sample that was stored with 5 mL of Lugol (1%, v/v), according to the (INAG 2009) protocol. The macroinvertebrates community was collected using a hand net (0.5 mm of mesh; 0.5 m of length) and a composite sample was collected comprising all the habitats recorded in each pond (e.g substrate and vegetation), according to (INAG, 2008). The macroinvertebrates samples were preserved in formaldehyde (4 %) until analysis.

2.3 Laboratory Procedure

2.3.1 Physical and chemical parameters

In the laboratory, water samples were analysed regarding the parameters proposed by the WFD for surface artificial waterbodies (APA 2022a). Priority substances, specific pollutants and other pollutants were analysed during two seasons, summer (July, August and September) and winter (February) establishing two extremes of meteorological conditions. Standard guidelines are conducted to determination of biochemical oxygen demand (BOD_5) (APHA, 1999) nitrites (NO_2^-) (NF EN ISO 10304-1), nitrates (NO_3^-) (NF EN ISO 10304-1), total nitrogen (N_{total}) (NF EN 25663), ammoniacal nitrogen (NH_4^+), phosphates (PO_4), and total phosphorus (P_{total}) (NF EN ISO 17294-2). (APHA, 1999). Total suspended solids, and volatile suspended solids were determined in triplicate per pond where the filtrate is dried to constant weight representing the suspended solids retained on the filter. After the remaining solids are subjected to a constant temperature of 430ºC, and the weight lost on ignition is the volatile solids (Ali Khan 1989; APHA 1999).

2.3.2 Biological parameters

2.3.2.1 Phytoplankton

Phytoplankton communities' quantification followed the INAG's protocol (INAG 2009) and the samples went through sedimentation for one week. After this period, samples were decanted until obtained a pellet with a final volume of 5 mL. Subsequently, the phytoplankton quantification and identification from each water sample were conducted in an optical microscope (Leica DMLB), using a Neubauer camera, counting at least 800 cells in 3 replicates. The Identification of phytoplankton was performed using guides and specific identification keys (Lund et al. 1958; Carlson et al. 1977). For the quantification of Chlorophyll *a* concentration, an aliquot of water samples from each pond was filtered through a Whatman GF/C filter (1.2 µm porosity, 47 mm diameter). Succeeding this procedure, the filters were immersed in 90 % acetone and stored in the dark at 4 ºC for 24 h, to accomplish the full pigment extraction (Strickland et al. 1972; Lind et al. 1979). To achieve the chlorophyll *a* concentration a set of monochromatic equations were used according to Lorenzo et al, (1967). The trophic state index was also obtained for each pond using the chlorophyll a concentration as endpoint with the following formula (Carlson et al. 1977):

$$TSI(CHL) = 9.81 \times \ln(CHL) + 30.6$$

Providing information about the biological productivity of the water body. The concentration of nitrogen, phosphorus, and other biologically useful nutrients are the primary determinants of a water body's trophic state index. Excess concentration of nitrogen and phosphorus tend to result in increased plant growth and increases subsequent trophic level.

2.3.2.2 Macroinvertebrates

The macroinvertebrates samples were washed with a 0.5 mm mesh sieve, to remove all the fine sediment and formaldehyde. After that sample was placed in trays and sorted the macroinvertebrates from the remaining detritus. Afterward, the harvested organisms were preserved in 70 % alcohol and stored in small bottles properly labelled for later identification. The macroinvertebrates were identified with a binocular magnifying glass to the family level in almost all taxa, and to the class level for Oligochaetes, using a specific dichotomous identification key (Tachet et al. 2000).

2.3.3 Bioassays

2.3.3.1 Test organisms and maintenance conditions

The model organisms selected to perform this study belongs to several biological trophic levels, that play different key functions in the aquatic food web. The test organisms used were the macrophyte *Lemna minor* and the Cladocera *Daphnia magna* key species in aquatic toxicology (Shaw et al. 2008; Klaus et al. 2013).

Lemna minor is an aquatic vascular plant, commonly used in ecotoxicological studies as a model organism (OECD et al. 2006; Lee et al. 2021). The use of this species has been shown several advantages including a rapid growth rate, easy of cultivation and handling, and high sensitivity to a wide range of pollutants e.g., Klaus et al. (2013). Phytotoxicity tests using the duckweed as a model organism, usually assess growth parameters such as the number of fronds, leaf area, and biomass, as well as the content of photosynthetic pigments (OECD 2006; Paczkowska et al. 2007). *L. minor* was cultivated in Steinberg medium, and the culture is maintained at a controlled temperature of 23 ± 2 ºC and continuous light, according to the standard guideline OECD 221 (OECD 2006). The genus *Daphnia* belongs to the class Crustacea and is widely distributed in aquatic ecosystems worldwide (Shaw et al. 2008). *Daphnia* is an important link between primary producers and consumers of higher trophic levels such as fish. *Daphnia* is internationally recognized as a standard species in aquatic ecotoxicological research because of its rapid reproduction, short life cycle, and sensitivity to pollutants (Ebert et al. (2022). Cultures of *Daphnia magna* were continuously kept in laboratory conditions for successive generations. Cultures were renewed on alternate days using a synthetic hard-water medium “ASTM hard water” (ASTM 1989), which was supplemented with a standard organic additive, *Ascophyllum nodosum* extract (Allen et al. 1995). The cultures were maintained in a culture chamber Incubator (TC 445 S, Lovibond Water Testing) under controlled conditions of photoperiod (16 h^L:8 h^D) and temperature (20 ± 2 ºC). Daphniids were fed with *Raphidocelis subcapitata* at a rate of 3.0×10^5 cells/mL/day.

2.3.3.2 Natural water treatments

For conduct the bioassays with the water samples from each pond two different treatments were carried out: i) natural water samples from each pond (P1, P2 and P3); and ii) natural water samples from each pond with pH adjustment (BP1, BP2 and BP3) for the optimal pH value regarding the culture medium. For *L. minor* the pH adjustment was 5.3 < pH < 5.7 and for *D. magna* was 7.0 < pH < 7.5. The use of two treatments, aims to demonstrate that in fact the acidic pH of the artificial ponds is one of the main problems. Due to its capacity to control the concentration of metals and its availability in the aquatic environments, pH is an important factor to determine the chemical and biological properties of water (Saalidong et al. 2022).

2.3.3.3 *Lemna minor* inhibition assays

Lemna minor growth inhibition assays were conducted following the standard guideline (OECD 2006). Six-well micro-plates were used to perform each assay, each well was full of 12.5 mL of the different samples (P1, P2, P3 or BP1, BP2 and BP3), and 5 fronds of *L. minor* were added. A control group was performed with fronds exposed to Steinberg medium. The microplates were incubated in a climatic chamber, in conditions of continuous light (~7000 lux), and temperature (23 ± 2 ºC) for 7 days.

2.3.3.4 *Daphnia magna* assays

For the survival assay, 24-well microplates were used, and the *D. magna* survivor was evaluated along a defined period (methodology adopted from Lopes et al. (1999) after exposure to 2.5 mL of each water sample (P1, P2, P3, BP1, BP2 and BP3). In each well, 2.5 mL of the samples were added, and the bioassay started with adding one *D. magna* (with < 24 h of age and born between the 3rd and 5th broods). The survival of the exposure organisms was monitored for 6 h and the regular observations were conducted at 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 240, 300, 380 and 1140 min. *D. magna* acute immobilization test was conducted based on the standard guideline 202 (OECD, 2004). A control group (ASTM) and a range of water samples dilutions [15%, 30%, 50%, 70%, 90%, 100% (direct sample – without dilution)] were prepared. For each water sample, four replicates were carried out in glass vessels with 25 mL of sample dilution or ASTM (control). In each replicate, 5 organisms, with less than 24 h old and born between the

3^{rd} and 5^{th} broods, were added to the vessels. The assay was conducted under controlled conditions of temperature (20 ± 2 ºC) and photoperiod (16 h^L:8 h^D). Daphniids were observed after 24 h and 48 h and immobilized or dead organisms were accounted.

Daphnia magna feeding inhibition rate assays were performed according to (McWilliam et al. 2002) with few adaptions described in (Queirós et al. 2019). The assay was conducted in 6 well-microplates, and in each well was added 12.5 mL of the water samples with pH adjustment (BP1, BP2 and BP3) and a *R. subcapitata* volume of 3.0×10^5 cells/mL/day. Five replicates per sample were used and 6 neonates (with 4 or 5 days old and born between the 3^{rd} and 5^{th} broods) were added to each well. To account for the potential algae growth during the test period, a blank with samples and microalgae, without organisms, was made. Before adding the organisms, the absorbance value was measured at ʎ = 440 nm (Abs Fl_0) in a spectrophotometer (Genesys TM 10Series Thermo Spectronic). After adding the organisms, the microplates were incubated in a climatic chamber with a controlled temperature (20 ± 2 ºC), and in total darkness, to avoid algal growth. After 24 h exposure, the organisms were collected and removed, and the absorbance values of each well were measured (Abs Fl_{24}). Feeding rate was calculated according to the following equation given by (Allen et al. 1995):

$$\mathrm{F} = \left[V * \left(\mathrm{AbsFl}_0 - \mathrm{AbsFl}_{24}\right)\right)/t]/n$$

Where *V* corresponds to the assay volume used (in this case 12.5 mL), *t* stands for the assay period (24 h) and *n* the number of organisms per well/replicate (n=6).

2.4 Statistical analysis

The Ecological Quality Ratio (EQR) is an indicator of the current deviation from the reference conditions of a water body. For the characterization of the phytoplankton community, the EQR calculation was performed following the equations presented in (APA 2022b). With the combination of the EQRs for chlorophyll *a* concentration, the total biovolume, the Cyanobacteria biovolume % and Algae Group Index (AGI), reflects the ecological potential of that body of water, which may be: Excellent or Good, Moderate, Mediocre or Bad. For the bioassays results, a one-way analysis of variance (ANOVA), followed by Dunnett test, was performed to determine differences between the treatment and the control group. The level of significance was 0.05. Additionally for the *L. minor* growth inhibition assay, a Pairwise t test was applied to discriminate significant differences between samples. The statistical analyses were performed using the SPSS Statistics v29.

3. Results and Discussion

3.1 Physical and chemical parameters

The physical and chemical parameters were measured in the three ponds (P1-P3) along the year 2022/2023: July (Jul22), August (Aug22), September (Set22), November (Nov22), February (Feb23), and May (May23). **Table 1** presents the range of values for general physical and chemical parameters and nutrients, proposed, and quantified in Water Framework Directive (WFD), for artificial water bodies in the third cycle. In the **Table 13**, are described additional parameters, which allow perceiving a better water characterization, however without threshold values to evaluate this water bodies. For the first values, the ecological potential (EP) for the three ponds was calculated and regarding the general physical and chemical parameters all the ponds were classified with an EP of moderate. The temperature fluctuated considerably throughout the sampling periods, ranging from 12.4 ºC at P1 in February to 27.6 ºC also at P1 in July. The three ponds showed acidic pH values throughout the whole sampling campaign, with pH values below the range 6-9 for the classification of Good Ecological Potential. Although in November the three ponds obtained the highest pH value registered (pH ≈ 5), due to the heavy rain faced in this month, the values of precipitation regarding November were around 138.7 mm, which correspond to 127% of the normal values. November of 2022 was considered a “raining month” by the report from Instituto Português do Mar e da Atmosfera (IPMA 2022). The concentration of TDS also exceeds the recommended value (640 mg/L) for this type of water body, namely in pond 2 in February (TDS = 669 mg/L) and May (TDS = 796 mg/L). The results of the nutrient concentrations were below the recommended values throughout most of the sampling campaign (**Table 1**), except total nitrogen values that exceeded the maximum recommended (8 mg/L) in July (P2 and P3), September (P1 and P3), and February (P1). The rising utilization of fertilizers and manure in regional agriculture, in conjugation other factors may serve as primary factors contributing to the accumulation of nutrients, including phosphorus and nitrogen. Warmer temperatures which are evident in July and September, tend to promote higher biological activity, including the activities of bacteria and algae. The microorganisms play a crucial role in nitrogen cycle, leading to the release of nitrogen compounds into the water. And high concentrations of nitrogen are often associated with algal blooms. The other physical and chemical parameters do not exceed the recommend values in all the sampling campaign (**Table 1**).

Table 2 showed the concentration of specific pollutants, and other pollutants quantified in each water pond collected in July, August, September, and February. The intuition of the selection of the months, comprehended the idea of having two extremes of meteorological conditions, summer and winter. There are cases for specific pollutants, (e.g. Arsenic, Copper, Chromium and Zinc) where the obtained value represents the analytical limits, which are above the respective reference value. It is a concerning factor because the real value of the respective specific pollutant may be above or below the RF. Meanwhile manganese concentration exceeded the RV throughout the entire campaign. Mining operations generate a significant amount of waste material known as tailings. These tailings may contain elevated levels of metals such as manganese. Over time, weathering of these tailing can lead to the release of manganese into nearby water bodies, and the pH and redox conditions in the pond can influence the solubility and mobility of manganese. Certain conditions may favour the release and accumulation of manganese in water. For example, manganese tends to be more soluble and mobile in acidic conditions.

Table 3 shows the concentration values of priority substances quantified in each water pond. In this case, the same factor occurs, that being the analytical limits of various priority substances (e.g. Cadmium, Nickel, and Lead) are above the reference values, meaning that the real value may me above or below the respective RF. Several authors already demonstrated that mine areas showed an increase of metals in water, sediment, and soils, for example Antunes et al. (2007) discuss the toxicity in the water column and sediment from an abandoned uranium mine. Another example, Lopes et al. (1999) the process of remediation of acid mine drainage, in both studies, several metals were quantified like the present study of the ponds of Gens. With the presence of these metals, they can affect the surrounding biota of these ecosystems and cause toxicity.

Zhang et al. (2014), analysed the spatial distribution of a set of metals (As, Cr, Pb, Cd, Cu, and Zn) in the water and sediment impacted by a gold mine. The authors describes that the elevated concentrations of As, Pb, Cd, and Zn can be associated to existence of the gold mine. On the other hand, the authors discuss that the sediment can act as contaminant sink through chemical reactions as precipitation and adsorption and under favourable conditions these metals can be released to the water column by desorption, particle resuspension and mineral dissolution, the high concentration of Zn, Cd, Pb, Ni, Cu and As are associated with acidic conditions (low pH) (Zhang et al. 2014). As the pond of Gens do in fact present these acidic conditions, the concentration of the metals is above the RV.

Table 1 Results of the general physical and chemical parameters (Sampling sites: P1, P2 and P3; Sampling periods: July, September, August, November, February, and May). Reference values (RV) according to (APA, 2022a); Bold values represent the values outside the established RV.

	Site	**Temp (ºC)**	**pH**	**BOD_5 (mg/L)**	**Cond (µS/cm)**	**TDS (mg/L)**	**TSS (mg/L)**	**NO_2^- (mg/L)**	**NO_3^- (mg/L)**	**PO_4^+ (mg/L)**	**PO4-P (mg/L)**	**Total nitrogen (mg/L)**	**Ammoniacal nitrogen (mg/L)**	**Ecological Potential**
Reference Values		10 to 27	6-9	7.0	1100	640	45	0.7	20	1	0.6	8	2.5	
July	**P1**	27.6	**3.69**	1.05	272	302	10.34	<0.05	<4.4	<0.18	<0.08	<5	<0.14	**Moderate**
	P2	26.9	**3.53**	1.84	511	510	26.5	<0.05	<4.4	<0.18	<0.08	**163**	<0.14	
	P3	25.9	**4.55**	0.65	447	446	9.83	<0.05	<4.4	<0.18	<0.08	**11**	<0.14	
August	**P1**	24.5	**3.82**	1.07	293	327	10.1	<0.05	<4.4	<0.18	<0.08	<5	<0.14	**Moderate**
	P2	24.1	**3.67**	0.17	495	552	27.7	<0.05	<4.4	<0.18	<0.08	<5	<0.14	
	P3	23.4	**4.44**	0.25	472	472	26.1	<0.05	<4.4	<0.18	<0.08	<5	<0.14	
September	**P1**	20.4	**4.04**	0.96	261	292	10.7	<0.02	<5	<0.12	<0.08	**600**	<0.03	**Moderate**
	P2	20	**3.77**	0.26	501	559	14.3	<0.02	<5	<0.12	<0.08	<5	<0.03	
	P3	20.6	**4.77**	0.5	416	464	9.9	<0.02	<5	<0.12	<0.08	**700**	<0.03	
November	**P1**	12.8	**5.17**	0.04	127	143	24.5	0.125	4.3	<0.03	<0.01	<0.02	<0.03	**Moderate**
	P2	12.6	**5.03**	1.06	286	319	18.1	0.093	<2.2	0.15	0.05	<0.02	<0.03	
	P3	13.3	**5.07**	0.51	255	284	27.9	0.103	1.2	<0.03	<0.01	<0.02	<0.03	
February	**P1**	12.4	**3.62**	1	312	313	14.0	<0.05	<4.4	<0.18	<0.08	**17**	<0.14	**Moderate**
	P2	17	**3.34**	0.13	595	**669**	37.0	<0.05	<4.4	<0.18	<0.08	<5	<0.14	
	P3	14.8	**3.78**	0.75	496	554	21.1	<0.05	<4.4	<0.18	<0.08	<5	<0.14	
May	**P1**	20.1	**3.26**	1.52	359	399	11.7	0.046	2.2	0.22	0.07	<0.02	<0.03	**Moderate**
	P2	21	**2.91**	1.18	713	**796**	23.7	0.128	2.9	0.05	0.02	<0.02	<0.03	
	P3	21.5	**3.65**	1.5	485	541	14	0.056	< 2.2	0.03	0.01	<0.02	<0.03	

Table 2 Results of specific pollutants, and other pollutants (Sampling sites: P1, P2 and P3; Sampling periods: July, September, August, November, February, and May). Reference values (RV) according to APA (APA, 2022a); Bold values represent the values outside the established RV. *stands for not quantified in the sampling period.

		Specific Pollutants				Other Pollutants				
	Site	**Arsenic (mg/L)**	**Copper (mg(L)**	**Chromium (mg/L)**	**Zinc (mg/L)**	**Lithium (mg/L)**	**Manganese (mg/L)**	**Iron (mg/L)**	**Aluminum (mg/L)**	**Ecological Potencial**
	Reference Values	0.05	0.0078	0.0047	0.0078	2.5	0.2	5.0	5.0	
July	**P1**	<0.0002	0.0029	<0.0002	**0.128**	0.0119	**0.76**	0.541	0.430	**Moderate**
	P2	<0.0002	0.0035	<0.0002	**0.147**	0.0116	**0.79**	0.501	0.480	
	P3	<0.0002	0.0032	<0.0002	**0.079**	0.0278	**1.02**	0.157	2.96	
August	**P1**	<0.0002	0.0018	<0.0002	**0.118**	0.0122	**0.86**	0.178	0.460	**Moderate**
	P2	**<3.0**	**0.1**	<0.001	**0.600**	0.0154	**0.85**	0.691	0.419	
	P3	**<3.0**	**0.1**	<0.001	**0.060**	0.0257	**1.02**	<0.050	0.271	
September	**P1**	<0.003	**<0.05**	**<0.01**	**0.080**	0.0071	**26.0**	0.470	1.61	**Moderate**
	P2	<0.003	**<0.05**	**<0.01**	**0.050**	0.0268	**23.0**	0.850	1.06	
	P3	<0.003	**<0.05**	**<0.01**	**0.050**	0.025	**23.0**	0.450	4.93	
November	**P1**	*	*	*	*	*	*	*	*	
	P2	*	*	*	*	*	*	*	*	
	P3	*	*	*	*	*	*	*	*	
February	**P1**	<0.003	**<0.05**	**0.01**	**0.080**	0.0192	**1.60**	0.289	1.37	**Moderate**
	P2	<0.003	**<0.05**	**0.05**	**0.090**	0.0417	**2.30**	2.80	0.698	
	P3	<0.003	**<0.05**	**0.01**	**0.150**	0,0414	**2.80**	0.980	1.17	
May	**P1**	*	*	*	*	*	*	*	*	
	P2	*	*	*	*	*	*	*	*	
	P3	*	*	*	*	*	*	*	*	

Table 3 Results of priority substances (Sampling sites: P1, P2 and P3; Sampling periods: July, September, August, November, February, and May). Reference values (RV) according to APA (APA, 2022a); a Moderate Ecological Potential in all of three ponds were presented; Bold values represent the values outside the established RV. *stands for not quantified in the sampling period.

		Priority substances			
	Site	**Cadmiun (mg/L)**	**Nickel (mg(L)**	**Lead (mg/L)**	**Ecological Potencial**
	Reference Values	0.00025	0.0012	0.004	
July	**P1**	**0.55**	**0.048**	0.001	**Moderate**
	P2	**0.603**	**0.05**	0.0014	
	P3	**0.33**	**0.06**	0.0003	
August	**P1**	**0.33**	**0.04**	0.00087	**Moderate**
	P2	**0.446**	**<0.060**	**177**	
	P3	**<0.01**	**<0.060**	**135**	
September	**P1**	**<0.01**	**<0.060**	**<3.0**	**Moderate**
	P2	**<0.01**	**<0.060**	**<3.0**	
	P3	**<0.01**	**<0.060**	**<3.0**	
November	**P1**	*	*	*	
	P2	*	*	*	
	P3	*	*	*	
February	**P1**	**<0.02**	<0.001	**0.31**	**Moderate**
	P2	**<0.02**	<0.001	**0.31**	
	P3	**<0.02**	<0.001	**0.32**	
May	**P1**	*	*	*	
	P2	*	*	*	
	P3	*	*	*	

3.2 Biological parameters

3.2.1 Phytoplankton community

The analysis of the phytoplankton community comprehended several parameters to evaluate the ecological potential of lentic ecosystems. The selection of the parameters was based on the quality standards described in the HRMP in the 3º cycle for artificial water bodies and for heavily modified water bodies (APA 2022b). For artificial water bodies, the concentration of chlorophyll *a* is the only factor used for evaluation. However, for HMWB, the phytoplankton community has been a biological element to evaluate this type of water body (for example reservoirs; Pinto et al. 2021a). The study of this community is crucial to evaluate the ecological potential of water masses, phytoplankton, which consists of microscopic algae and other photosynthetic organism, play a key role in aquatic ecosystems. And overall, this community should be included in the biological parameters for the quantification of AWB. In this case, the parameters evaluated are the concentration of chlorophyll *a*, the percentage of biovolume of cyanobacteria's, total biovolume and Algae Group Index (AGI) and the trophic state index. (APA 2022b).

Regarding the TSI, the results are described in the **Table 4**. In July, every pond obtained a classification of oligotrophic, and on the following months namely in August, Pond 2 and Pond 3 were classified as mesotrophic, meaning that biological activity increased during this period. This can be associated with the high concentration of total nitrogen in this period (see **Table 1**). And after November which was described as "raining month" as described before, the three ponds were classified as oligotrophic again.

For this study, the analysis of the phytoplankton community consolidated the criteria described above. However, the results obtained might have some discrepancy associated with it, due to the evaluation was made according to the characteristics of a reservoir and the reference values established for this type of water body and not for ponds. Considering the analysis of the phytoplankton community (**Table 5**), all the ponds were classified with a Good Ecological Potential, except P3 in August, and in P2 and P3 in September, where only a Moderate EP was obtained. Regarding the concentration of chlorophyll *a* the highest values were recorded on August and September (**Table 5**). This can be attributed to the heightened temperatures and increased sunlight typical of summer, which foster the growth of phytoplankton. These conditions support photosynthesis, resulting in an upsurge in chlorophyll-a concentration. Additionally, increased levels of nutrients, especially nitrogen and phosphorus, contribute to the flourishing of algae. The chemical analysis conducted during these months revealed elevated total nitrogen concentrations. And associated with these concentrations' values, in the same months, the values of total cyanobacteria biovolume were also high (P3 in August and P2 and P3 in September). Cyanobacteria, particularly certain species, thrive in conditions of eutrophication where is an abundance of nutrients for example *Microcystis* which was quantified in our samples. The AGI is a metric used to assess the composition and health of algal communities in water bodies. It is often calculated based on the relative abundance of different algal groups. In the case of Gens ponds, the values of AGI were low indicating poor water quality or degraded ecological conditions in the water body, additionally a decrease in AGI may be linked to the dominance of specific algal groups that indicative of poor water quality.(Catalan et al. 2003). However, Edward et al. (2015), described if the following conditions: high nutrient availability, temperature increase and light conditions, which occur in spring and summer, it favours the growth and diversity of the phytoplankton.

The multivariate analysis conducted with the physical and chemical, and phytoplankton community matrices showed and seasonality in the ponds. The first axis of CCA (**Figure 5**) explains 47.5% of the total variation in the data, while the second axis accounts for 21%. The spring sampling campaign (May) showed high similarity with high values of BOD_5, and high abundance of Baccilariophyceae (Bacc_2, Bacc_3, Pin, Fra, Aul and Eun) and Cynophyceae (Cya) are associated with this event. The high temperatures registered in July, September and November can be associated with the low pH also registered, additionally the high temperatures can also cause a higher evaporation rate which can lead to an increase in the concentration of chlorophyll *a*, favouring the appearance of Cynaphyceae. In P3_Jul, P1_Jul, P2_Sept and P1_Nov displayed a tendency in the CCA distribution comprehending the pH values, temperature and the concentration of Chl *a*, and due to that, there is an increase of Cyanophyceae (Rha_sp and Rha_cur).

Several authors suggest that the increase of nutrients (e.g., nitrogen and phosphorus), the variation of temperature, and the light conditions may affect the growth and diversity of the phytoplankton community (e.g. Edward et al. (2015)). In our case of study, the diversity of the pond of Gens phytoplankton community suffers alteration throughout the entire sampling campaign. In the first three months (July, August and September), there is an appearance of Cyanobacteria (Rha_sp and Rha_cur) which as previously described are associated with high temperatures and high concentration of Chl *a*, but in the other half (November, February and May) of the campaign, these two examples are not found in the sample due to the alteration of the physical and chemical proprieties, for example, a decrease in the temperature which followed by a decreased in the Chl *a* concentration. On the other hand, towards the months of February and May, there is an appearance of Baccilariophyceae. These diatoms are generally considered to be better adapted to lower temperatures than for example Cyanobacteria (Zhang et al. 2018). In fact, the values of temperature for these months varied between 12.4-21.5. Meanwhile the example of Microcystis and Peridinium occurred constantly throughout all sampling periods.

On the other hand, heavy metals can also affect the planktonic algae, reducing phytoplankton and the mean size of individuals as described by Cattaneo et al. (1998) in Lake D'Orta in the northern of Italy (acid lake polluted with Cu, Zn, Ni and Cr for more than 50 years). The acidity, it is also associated with low primary productivity, and with low levels of taxa of phytoplankton, causing a decrease of phytoplankton biomass (chlorophyll *a*) (Edward et al. 2015). Water transparency is also an important variable in the phytoplankton growth. Kwiatkowski et al. (1976), describes that an increase in water transparency associated with a low pH promotes a decreased in chlorophyll *a* concentration, causing alteration on the species composition and appearance of Cyanophyta. Similar results were observed on Gens ponds where acidic values were recorded and an increase of *Microcystis* sp. (Cyanophyta) was also observed affecting the productivity of the phytoplankton community.

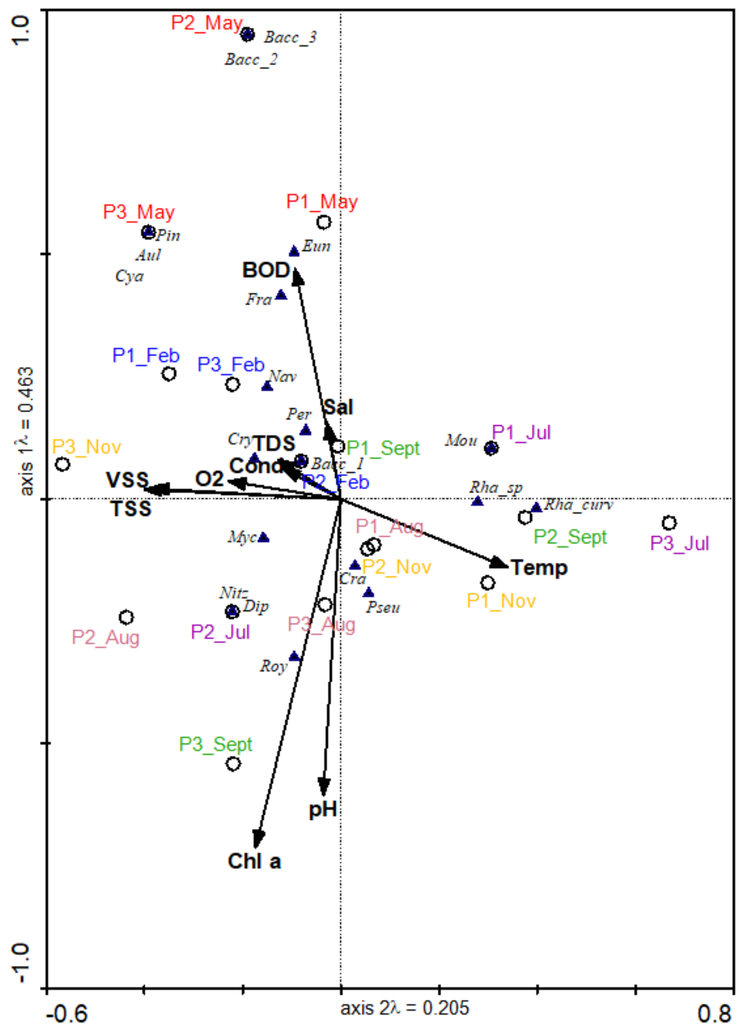

Figure 5 Canonical Correspondence Analysis (CCA) of the distribution of aquatic phytoplankton (see taxa abbreviation in Table 4). Sampling sites (P1, P2 and P3); Sample periods (Jul – July, Aug – August, Sept – September, Nov – November, Feb – February, and May); Physical and chemical parameters: temperature (Temp), conductivity (Cond), salinity (Sal), dissolved oxygen (O2), biochemical oxygen demand (BOD), total dissolved solids (TDS), total suspended solids (TSS), volatile suspended solids (VSS), pH, and chlorophyll a concentration.

Table 4 Results of the trophic state index, regarding the concentration of chlorophyll *a*. The classification is divided into four classes: Oligotrophic (TSI = <30; Chl = <0.95); Mesotrophic (TSI = 30-40; Chl = 0.95-2.6); Eutrophic (TSI = 50-60; Chl 7.3-20); Hypereutrophic (TSI = 70-80; Chl 56-155). *Is classified as Oligotrophy and the hypolimnion may become anoxic. **Negative result due to very low values of chlorophyll a concentration.

	Pond	TSI	Classification
July	P1	22.15	Oligotrophic
	P2	38.64	*Oligotrophic
	P3	18.45	Oligotrophic
August	P1	38.72	*Oligotrophic
	P2	42.51	Mesotrophic
	P3	41.47	Mesotrophic
September	P1	36.36	*Oligotrophic
	P2	36.58	*Oligotrophic
	P3	41.41	Mesotrophic
November	P1	25.97	Oligotrophic
	P2	**-3.79	Oligotrophic
	P3	13.77	Oligotrophic
February	P1	17.38	Oligotrophic
	P2	**-3.79	Oligotrophic
	P3	11.98	Oligotrophic
May	P1	12.37	Oligotrophic
	P2	9.03	Oligotrophic
	P3	19.84	Oligotrophic

Table 5 Phytoplankton abundances (number of cells per sampling effort) recorded in each pond and sampling campaign. Results obtained for the EQR of phytoplankton community is also presented. Reference values for EP calculation and EQR normalized (in parentheses below) for each parameter for High, Good, Moderate, Poor and Bad EP according to WFD (APA 2022a). High/Good – 0.80; Good/Moderate – 0.60; Moderate/Poor – 0.40; Poor/Bad – 0.20.

		July			**August**			**September**			**November**			**February**			**May**		
	Abbr.	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
Rhapidiopsis curvata	Rha_cur	551	64	655	79	15	-	28	1634	-	-	-	-	-	-	-	-	-	-
Rhapidiopsis sp.	Rha_sp	3429	615	997	-	-	-	1756	4416	56	1160	463	-	-	-	-	-	-	-
Perodinium sp.	Per	967	456	319	1436	824	282	310	199	283	78	171	331	586	494	308	2086	73	787
Naviculas sp.	Nav	19	10	1	109	9	53	46	6	40	-	-	134	27	349	173	143	176	16
Craticula sp.	Cra	6	3	-	-	-	15	-	-	-	-	-	-	-	-	-	-	-	-
Cryptomonas sp.	Cry	164	-	-	51	29	34	708	-	138	117	197	539	531	226	500	-	-	-
Mougeotia sp.	Mou	58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fragilaria sp.	Fra	20	16	3	23	10	42	8	43	2	-	-	27	-	-	192	185	183	35
Roya obtusa sp.	Roy	156	358	9	291	40	97	76	127	1233	38	49	-	-	-	-	-	-	-
Pseudonabaena sp.	Pseu	-	226	-	-	-	-	-	24	-	227	-	-	-	-	-	-	-	-
Nitzchia sp.	Nitz	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Diploneis sp.	Dip	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mycrocytis sp.	Myc	-	-	-	3067	1954	1996	1161	-	3531	245	573	1181	915	2317	617	351	527	413
Baccilariophyceae n.i.	Bacc_1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Eunotiaceae sp.	Eun	1	-	-	-	-	-	4	6	-	-	-	-	-	-	107	231	44	-
Baccilariophyceae n.i.	Bacc_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35	-
Baccilariophyceae n.i.	Bacc_3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-
Cyanophyceae n.i.	Cya	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
Pinnularia sp.	Pin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Aulacoseira sp.	Aus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10
	Ref Values																		
Chl a (mg/m³)	1.70	0.423 (1)	2.27 (0.87)	0.29 (1)	2.29 (0.868)	3.37 (0.748)	3.03 (0.776)	1.80 (0.971)	1.84 (0.961)	3.01 (0.778)	0.624 (1.88)	0.03 (158)	0.18 (26.6)	0.26 (1)	0.03 (1)	0.15 (1)	0.156 (1)	0.111 (1)	0.334 (1)
Biovolume Total (mm³/L)	1.20	43.1 (0.04)	45.5 (0.04)	22.9 (0.07)	33.6 (0.05)	28.6 (0.05)	21.8 (0.07)	25.9 (0.064)	18.5 (0.09)	27.1 (0.06)	25.2 (0.07)	23.6 (0.07)	29.6 (0.056)	15.7 (0.11)	36.3 (0.046)	21.9 (0.07)	58,01 (0.03)	4.99 (0.33)	15.9 (0.11)
Biovolume Cyanobacteria (mm³/L)	0.02	0.38 (0.61)	0.24 (0.62)	0.51 (0.61)	0.76 (0.6)	0.58 (0.60)	1.05 (0.46)	0.73 (0.6)	1.54 (0.31)	1.77 (0.27)	0.51 (0.61)	0.40 (0.61)	0.56 (0.61)	0.34 (0.61)	0.89 (0.53)	0.26 (0.62)	0.11 (0.66)	0.55 (0.6)	0.15 (0.64)
AGI	2.00	0.023 (1)	0.01 (1)	0.016 (1)	0.02 (1)	0.02 (1)	0.04 (1)	0.04 (1)	0.04 (1)	0.05 (1)	0.02 (1)	0.023 (1)	0.04 (1)	0.021 (1)	0.03 (1)	0.03 (1)	0.011 (1)	0.037 (1)	0.017 (1)
EQR		0.66	0.63	0.67	0.62	0.6	0.57	0.65	0.59	0.53	0.66	0.67	0.66	0.68	0.64	0.67	0.67	0.73	0.68
Ecological Potential		**Good**	**Good**	**Good**	**Good**	**Good**	**Moderate**	**Good**	**Moderate**	**Moderate**	**Good**	**Good**	**Good**	**Good**	**Good**	**Good**	**Good**	**Good**	**Good**

3.2.2 Macroinvertebrate community

Results of macroinvertebrate community for each site and sampling period are shown in **Table 7**. In general, summer presented the highest abundance values, with the maximum observed in the pond 3 in July (n=244). A major contribution for this high value is due to the 66.6% being namely part of the group *Oligochaeta*. The lowest value was observed in P2 in February (18). Once again, a major part of the organism is *Oligochaeta* (72.2%). Taxonomic richness varied between 4 and 12, with a higher variation of species registered in July, August and September, higher temperatures registered and a decline in the remaining sampling periods, (November, February, and May) there the opposite was registered this being, lower temperatures. Throughout the sampling periods there a variation on the diversity of the community, for example *Aeshnidae* and *Platycnemididae* are organisms that are present in the samples of July, August and September which are associated with high temperatures (**Table 1**), but in the other months, November, February and May there is a significant decrease in the presence of this organism. In another instance *Chironomidae* tends to only appear in July but has slight appearances in the following months. Margalef index measure the diversity of a sample by counting the number of species in the sample. Values inferior to 2.0 represent low diversity while values up to 5.0 stands for high diversity. Overall, the results of Margalef index were comprehend between the range (2.0-5.0) meaning that in general a moderate diversity was registered in the sampling ponds, (**Table 7**). According with the results, it is possible to see higher values of Margalef index in the first three months than in the other three months. The values don't vary much between ponds, although P1 has slightly higher values than the other two. Expect for November where the value was d=0.6, which can be caused by the low taxonomic richness (4) and a major dominance by one taxon, namely *Platycnemididae* (66.2%). Low taxonomic richness can affect the value of Margalef index. Regarding the Shannon-Wiener diversity was generally low in all sites (**Table 7**), the highest values observed were in P1 in July (H'= 2.1) and P2 in July and P1 in August both with the same value (H'=1.9) and the lowest value was registered in P2 in May (H'=0.2). There are differences between ponds meaning that each pond acts different to the macroinvertebrate community. Regarding evenness values that allows the measure of the relative abundance of the different species making up the richness of an area. Low values represent a low evenness in the community, meaning that in a sample, a major part of the organism may be from one species, therefore considered less diverse. Overall, low values of evenness were recorded with the lowest in P3 July and P1 February and the highest observed in P3 February (**Table 7**).

In ecology, alpha diversity refers to the diversity within a specific habitat or community, it is a measure of species richness and is concerned with understanding the variety and abundance of species within a localized and well-defined area. With that, P1 and P3 have an alpha diversity of 18 (see **Table 6**), indicating that they share the same species richness within their respective communities, and both can share similar environmental conditions. P3 has an alpha diversity of 17 it may have a distinct community composition or be missing some of the species found in P1 and P2. Regarding the beta diversity, provides a measure of the dissimilarity between pairs of communities: P1-P2$_{\beta}$ (6); P1-P3$_{\beta}$ (5); P2-P3$_{\beta}$ (7). For example, P1-P3$_{\beta}$ has the lowest beta diversity, indicating that these two ponds are some similar in species composition compared to the other pairs. Also, P1 and P3 may share similar environmental conditions, that allow and contribute to a more like species composition. Meanwhile a higher beta diversity value of 7 between P2 and P3 indicates a greater dissimilarity in species composition. At last, the gamma diversity is the overall species richness considering all the ponds together and for this case, the value was 13, suggests a more diverse and species-rich overall ecosystem.

The biota is constituted namely by tolerant and resistant species (e.g. *Haliplidae* and *Oligochaeta*), but there is evidence of the presence of sensitive species (e.g. *Perlodidae*).

Table 6 Alpha, Beta and Gamma diversity of Gens ponds.

Alpha diversity	**P1**	**P2**	**P3**
	18	18	17
Beta diversity	**P1-P2**	**P1-P3**	**P2-P3**
	6	5	7
Gamma diversity		13	

Table 7 Results of macroinvertebrate community recorded in the sampling sites along the sampling period. *No community of macroinvertebrates were found.

		July			**August**			**September**			**November**			**February**			**May**		
	Abbr.	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
Chironomidae	Chi	39	24	11	5	-	-	-	1	4	-	-	5	3	-	-	18	-	-
Ceratopogonidae	Cer	20	13	17	2	-	-	-	3	7	-	14	1	60	-	7	17	2	23
Dytiscidae	Dyt	14	6	4	2	14	15	-	-	-	-	-	-	2	1	-	2	104	8
Platycnemididae	Pla	14	24	41	26	2	1	151	39	15	-	-	1	-	-	-	-	-	-
Aeshnidae	Aes	8	9	3	10	-	3	7	2	-	-	-	-	4	2	-	-	1	1
Simuliidae	Sim	6	-	1	1	-	-	-	-	1	-	3	-	-	-	-	-	-	-
Coenagrionidae	Coe	6	10	5	6	-	-	46	14	1	-	-	2	-	2	8	4	2	22
Veliidae	Vel	5	-	-	4	7	5	-	-	-	-	-	-	-	-	-	-	-	-
Hydrochidae	Hydrochi	3	-	-	4	1	2	-	-	-	-	-	-	-	-	-	-	-	-
Thaumaleidae	Tha	2	-	-	-	-	-	-	-	2	-	-	-	13	-	3	-	-	-
Scathophagidae	Sca	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gomphidae	Gom	2	-	1	-	7	1	-	-	-	-	-	-	-	-	-	-	-	-
Oligochaeta	Oli	-	13	160	-	-	-	-	-	-	-	6	54	-	13	5	3	3	-
Mesoveliidae	Mes	-	2	-	1	1	1	-	1	-	-	-	-	-	-	-	-	-	-
Culicidae	Cul	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Hydropsychidae	Hydropsy	-	-	1	-	5	9	-	-	-	-	-	-	-	-	-	-	-	-
Notonectidae	Not	-	-	-	3	1	1	24	-	-	-	-	-	2	-	2	8	-	-
Haliplidae	Hal	-	-	-	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydracarina	Hydra	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Perlidae	Perl	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Perlodidae	Per	-	-	-	-	-	-	-	-	-	-	-		1	-	1	-	-	-
Noteridae	Not													3			2	2	
Abundance (N)		**121**	**102**	**244**	**65**	**41**	**38**	**228**	**61**	**30**	*****	**24**	**64**	**88**	**18**	**26**	**54**	**109**	**51**
Taxonomic richness		**12**	**9**	**10**	**12**	**9**	**9**	**4**	**7**	**6**	*****	**4**	**6**	**8**	**4**	**6**	**7**	**4**	**4**
Margalef (d)		**2.3**	**1.7**	**1.6**	**2.6**	**2.2**	**2.2**	**0.6**	**1.5**	**1.5**	*****	**0.9**	**1.2**	**1.1**	**1.0**	**1.5**	**1.5**	**0.6**	**0.8**
Shannon.Wiener (H')		**2.1**	**1.9**	**1.2**	**1.9**	**1.8**	**1.7**	**0.9**	**1.1**	**1.4**	*****	**1.1**	**0.7**	**1.1**	**0.9**	**1.6**	**1.6**	**0.2**	**1.1**
Simpson evenness (E)		**0.5**	**0.7**	**0.2**	**0.4**	**0.6**	**0.5**	**0.5**	**0.3**	**0.5**	*****	**0.6**	**0.3**	**0.2**	**0.5**	**0.8**	**0.6**	**0.3**	**0.7**

Figure 6 shows the spatial distribution resulting from the CCA applied to the physical and chemical parameters and macroinvertebrates observed at each sampling site and period. The first axis of Gens pond's CCA (**Figure 6**) explains 28.6% of the total variation in the data, while the second axis accounts for 24.3%. Spring and summer samples associate with high temperatures and high chlorophyll concentrations were characterized by Coleoptera (Not and Hal), Hemiptera (Mes), and Heteroptera (Vel) genera. In contrast, autumn and winter samples appear associated with high pH values and macroinvertebrates belonging to Annelidia (Oli), Hydracarina, and Plecoptera (Per).

The following organisms described above, have critical ecosystem functions, for example *Oligochaeta* can live in aquatic ecosystem and can break the dead or decaying plant and animal matter transforming that into to nutrients that helps with the soil replenishment. According to the Biological Monitoring Working Party (IBMWP) which is used for lotic water bodies was a helpful tool to correlate the existence of certain *taxa* found in the here present-study and the meaning according in the table of IBMWP (see **Figure 10**), for instance *Oligochaeta* has a score of 1 indicating that the presence of these type is generally considered to be an indication of poor ecological status of freshwater and they tend to prevalent in these conditions. Considered to be resistant organisms to different types of pollutions regarding the IBMWP which is designed for organic pollution or heavy metals pollution. In the opposite, Plecoptera are often indicators of cool, well oxygenated waters, in fact, these taxa are often used as indicators of high-water quality, meaning that they are sensitive to changes in water quality and have a classification of 10 according to the IBMWP table, in Gens ponds, these organism was found in P1 and P3 in February where according to **Table 13** the values of %O_2 and concentration of O_2 were high. And they perceived themselves as mediators of energy flow and nutrient cycling. Meanwhile, Coleoptera (Not and Hal), Hemiptera (Mes) and Heteroptera (Vel) have a classification of 5 in the IBMWP table, meaning that water quality can be reasonable, they are considered sensible but prevail in such conditions.

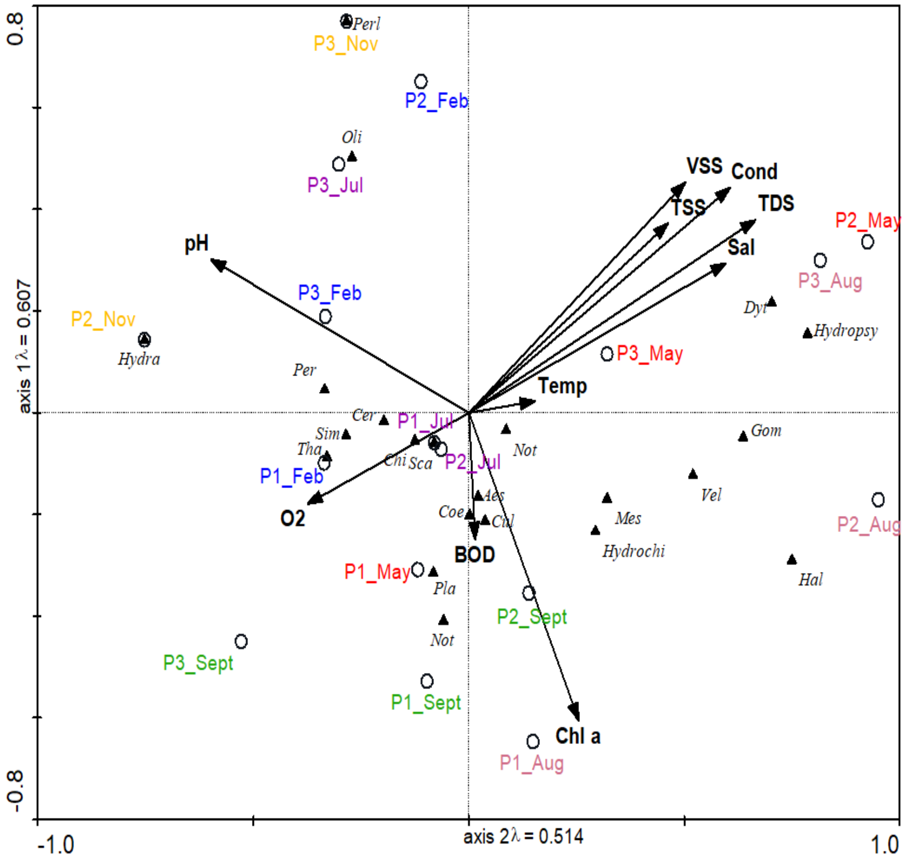

Figure 6 Canonical Correspondence Analysis (CCA) of the distribution of macroinvertebrates (*see* taxa abbreviation in **Table 7**); Sampling sites (P1, P2, P3); Sample periods (Jul - July, Aug - August, Sept - September, Nov - November, Feb - February and May); Physical and chemical parameters: temperature (Temp), conductivity (Cond), salinity (Sal), dissolved oxygen (O_2), biochemical oxygen demand (BOD_5), Total dissolved solids (TDS), Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), pH, and chlorophyll *a* concentration.

3.2.3 *Lemna minor* growth inhibition assays

The results obtained in the growth inhibition assays performed with *Lemna minor* are shown in **Figure 7**. The number of fronds and the dry weight of *L. minor* showed a significant decrease, when compared to the control group, after exposure to all the natural samples, with and without adjustment of pH, in all the sampling campaigns. These results showed that even with the pH adjustment (samples BP1, BP2 and BP3), there are other factors that may be affecting the growth of *L. minor* as for example the presence of heavy metals in water (e.g. high heavy metals concentration, see **Table 2** and **Table 3**). Although the concentration of these metals is not necessarily constant, but they can fluctuate over time, causing a variation between intense exposure and low exposure, either way this time of exposure can cause toxic injuries to the organism (Drost et al. 2007). Among the metals evidence in the table, Cu is classified as extremely toxic, and Zn is classified as moderately toxic to *Lemna* (Diritgen and Nel 1994). Cvjetko et al. (2010) refers the effect of Copper and Cadmiun on the toxicity in the duckweed. With that, the study shows that both metals do accumulate with time, and affect the organism meaning that they are toxic, but when in combination they can reduce each other's uptake, provoking a more complex response, affecting sub-individual parameters. Nickel and Zinc described to also cause damage in *Lemna minor*, Ni causes a decrease in growth rate and Zn was more tolerated by the duckweed according to (Khellaf et al. 2008a).

A significant increase in the number of *L. minor* fronds were observed after exposure to the water samples with pH adjustment (BP1, BP2 and BP3) when compared to the natural water samples (P1, P2, and P3) (**Figure 7**). Comparing the results obtained between treatments, in all samples P1 and P2 and after pH adjustment (BP1 and BP2, respectively), its verified that only in November no significant differences were observed in the number of fronds of *L. minor*. As mentioned previously (**section 3.1** Physical and chemical parameters) the meteorological conditions faced in November caused some alterations in the composition/physical and chemical characteristics of the ponds (e.g., pH and nutrients concentrations), which can explain the results obtained.

In the contrast, in the remaining months the number of *L. minor* fronds increased significantly after exposure to sample BP1 and BP2. Regarding the samples from pond 3, only in February the BP3 sample induce a significant increase in the number of fronds when compared to P3. Indeed, P3 showed a different response from P1 and P2 and reveals a similar behaviour to the water samples with pH adjustment (BP1, BP2 and BP3). Overall, the here-obtained results showed that the pH adjustment promotes an increase of number of fronds, however, the values are still lower to the control group. Indeed, this fact revealed that other factors may be responsible for causing *Lemna minor* growth. In fact the chemical characterization of water samples revealed high concentrations of metals (Zn, Mn, As, Cu, Cr, Ni, Cd, and Pb) in all of the three ponds, and these appearances occurred in the entire sampling period and concentration of nutrients (Total nitrogen) (see **Table 1**,**Table 2** and **Table 3**).

Dyck et al. (2023) analysed the effect of Co, Cs, Mn, Ni and Zn and how each effect on physiological and biochemical functions of *Lemna minor*. The results showed that high Zn concentrations induces phytotoxic effects such as reduce of growth and, biomass, chlorosis and a decreased of photosynthetic pigments content. Indeed, a similar result was observed in the present study where an inhibition of *L.* minor growth was observed after exposure of waters with high concentrations of zinc. The results obtained showed that pH value and the chemical composition of natural waters were two factors that induces phytotoxicity.

The results of *L.* minor dry weight after exposure of natural waters were also shown in **Figure 7**. The dry weight of *L. minor* decreases significantly, when compared to the control group, after exposure to all samples (P1, P2, P3, BP1, BP2 and BP3) from all sampling periods. Once again, the pH adjustment is not sufficient to prevent the growth inhibition after natural water exposure. Regarding the differences between the natural and pH adjustment samples, it was verified that BP1 and BP2 caused a significant increase in dry weight in all the samplings (except in July and November) when compared with P1 and P2 samples, respectively. BP3 also caused a significant increase in dry weight in February and May, while the opposite (significant decrease) was observed in August and September. In November, no significant differences were observed between natural (P1, P2, and P3) and pH adjustment (BP2, BP2, and BP3) samples.

Several aquatic plants are heavy metal pollution indicators (e.g., *Eichhornia crassispes*, *Hydrilla verticallata*, *Cabomba fuscata*, *Salvinia natans*, *Nelumbo nucifera* and *Pistia stratiotes*) and are successfully used as a method for monitoring environmental pollution (Ekperusi et al. 2019). The phytotoxicity of metals generates several visible symptoms in *L. minor*, like stunted growth, chlorosis, and necrosis (Radić et al. 2010). According to Khellaf and Zerdaoui (2009), the metals Cu, Ni, Cd and Zn can cause also visible damage to *L. minor.* A first effect recorded was chlorosis (a progression of green to yellow colour on the fronds) followed by necrosis when the presence of Cu, Ni and Cd. Severi et al. (1997) showed that Al can cause a severe physiological stress reaction, which increases peroxidase activity, leading to a decreased growth rate (Peters et al. 1989), interfering with the metabolism of cell wall polysaccharides (Huck 1972; Foy et al. 1978). On the other hand, Khellaf et al., 2008 states that Cu and Ni can stimulate the growth when exposure to low concentrations of Cu and Ni (0 and 0.2 mg/L, and 0 and 0.5 mg/L for Ni, respectively). Moreover, Gens ponds are located in a mining deactivated area and an high concentrations of metals occurs in the natural waters (see **Table 2** and **Table 3**), namely Zn, Mn, Cd and Pb. Despite the effects in *L. minor* growth and performance, a decrease in effects was observed after a pH adjustment in natural waters.

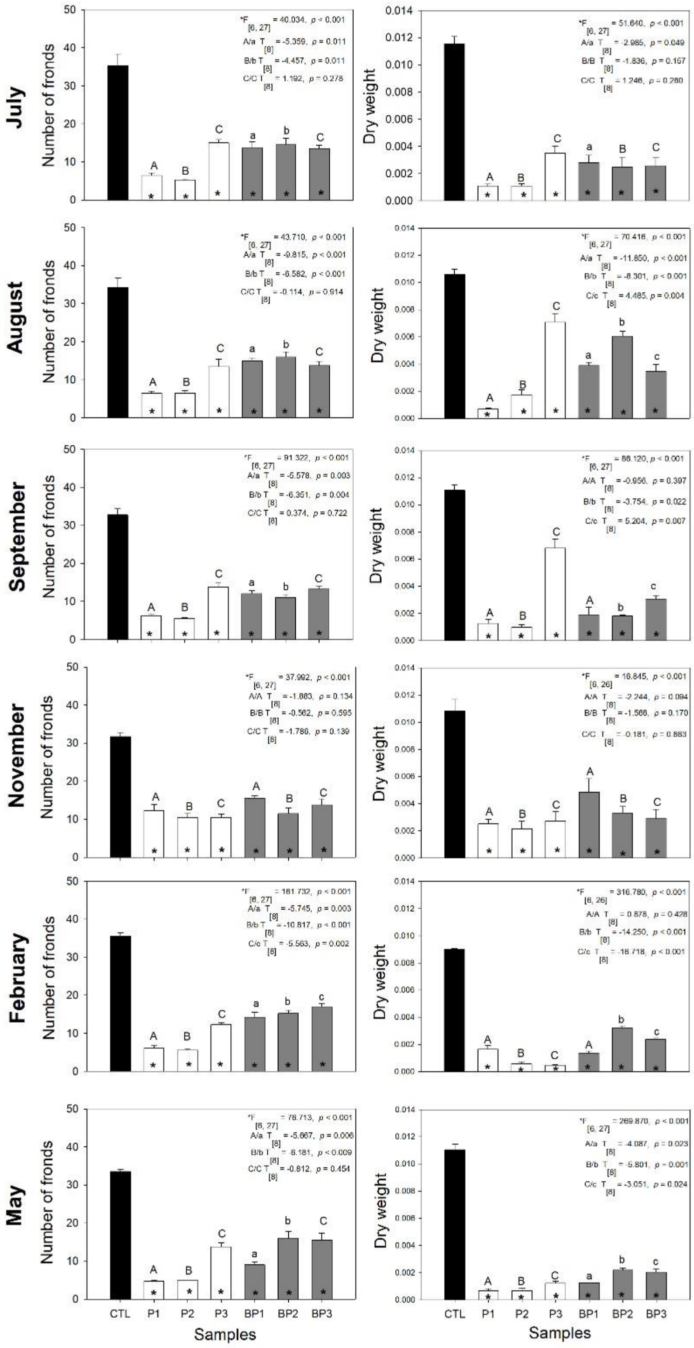

Figure 7 Results of growth inhibition assay (number of fronds and dry weight) of *Lemna minor* after exposure to the natural samples (P1, P2 and P3 – white bars) and with pH adjustment (BP1, BP2, and BP3 – grey bars). *Stand for significant differences between treatments and control group (Dunnett-test, $p < 0.05$). Different letters stand for differences between water treatment in each pond; same letters (AA, BB and CC) stands for no significant difference; different letters (Aa; Bb and Cc) stands for significant differences (T-test).

3.2.4 *Daphnia magna* survival assays´

The results of the *D. magna* survival assays after the exposure to the natural samples (P1, P2 and P3) are presented in **Figure 8**. The water samples with pH adjustment (BP1, BP2 and BP3) did not affect the survivability of *D. magna* (no mortality was registered). In contrast, all the natural water samples (P1, P2, and P3) affect the survivability of *D. magna* in all the sample periods (except the sample P3 in November - **Figure 8**). Overall, P3 tends to have a later effect on the mortality of *D. magna* when compared to P1 and P2, that cause mortality after less exposure time. In fact, between P1 and P2, P2 causes mortality at a faster rate (e.g., a high mortality was registered only after 20 minutes of exposure in July and May). Thus, P2 reveals more toxic relative to the other ponds. El-Deeb Ghazy et al. (2011) reported that pH can affect the survival, growth, and reproduction of Cladocera *Daphnia magna*. It also describes that the survival rate at the end of experiments was 47 % at pH 4.44 this percentage gets higher with higher values of pH, for example at pH of 4.61 the survivability is around 80 %. Gens ponds as described above, present acidic values of pH throughout the whole campaign (see **Table 1**). For instance, in July, August and September, P1 and P2 had inferior values when compared to P3 (**Table 1**), and according to **Figure 8** both P1 and P2 affect the survival of *D. magna* at an earlier stage than P3, suggesting that the slight difference of pH can help buffer the toxicity of the heavy metals present. In November as described above due to the weather conditions, in general, pH values of the three ponds raised (**Table 1**) to values similar to those described in the study above, despite the change, it was insufficient to denied the toxicity registered by the metals although at a slower rate. In February and May, the presented similar values of pH (**Table 1**) and P3 this time didn't differentiate itself from the other two.

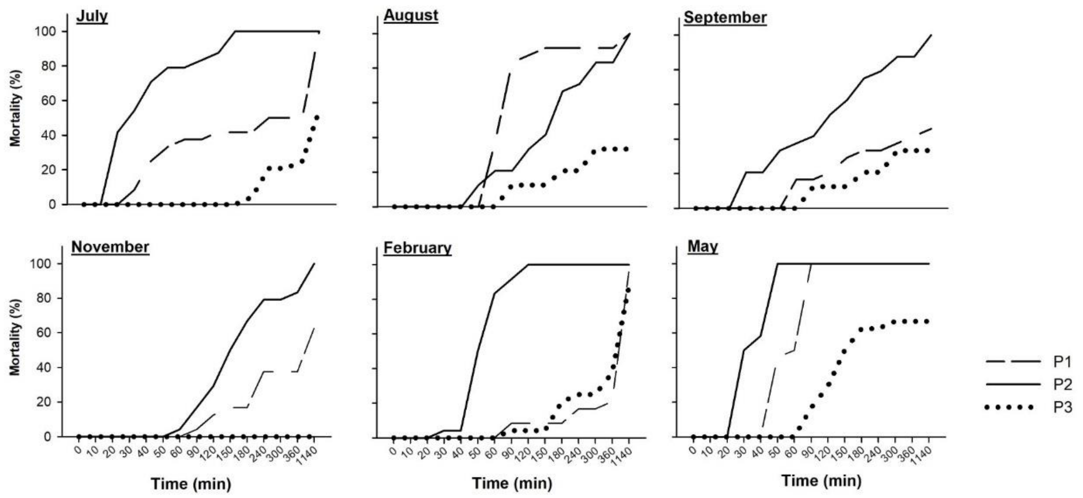

Figure 8 Percentage of mortality observed after different periods of exposure (10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 240, 300, 380, 1140 min) to natural water samples (P1, P2 and P3).

3.2.5 *Daphnia magna* acute immobilization assays

The results obtained in the *D. magna* acute immobilization assays are shown in **Table 8**. No acute toxicity was recorded after exposure to the lowest percentages of natural waters (15, 30, and 50 %) of P1, P2 and P3, while the direct samples (without dilutions) cause 100 % of *D. magna* mortality in all the sampling periods. In addition, 70 % of P2 sample (in February and May) and 90 % of P1 and P2 samples (in all the sampling periods, except November) also caused 100 % of mortality. Water from P3 showed lower acute toxicity to *D. magna* in all sampling periods, causing a mortality inferior to 50 % (except in the highest percentage tested for November and May). Comparing the different sampling periods, November reveals less toxic to *D. magna* since with 90 % of natural waters the mortality values was below of 50 % (**Table 8**). However, the physical and chemical characterization of natural waters (**Table 1**) showed high differences, namely pH values, when compared to the values of the other months there is a slight rise in the value in all the three ponds. The water level of the three ponds also raised in this period and a possible sediment suspension occurred.

The low toxicity in the lowest percentages of natural waters (until 50 %) can be associated with the neutralizing effect of ASTM hard water, which has a strong buffering capacity. As shown in **Table 12**, during the assay the values of pH were noted at 24 hours of observation and 48 hours in each sample and control. And it is possible to observe the strong buffering capacity. Until 50 % the pH value established and didn't suffer much alteration, stagnated around similar values to those which are standardized values for the cultivation of *D. magna*. The results showed that at this point no mortality was registered. On the other hand, at the highest dilutions, the value of pH decreased or maintained, and the results showed an increase in *D. magna* mortality. Moreover, the raise of pH to neutral or alkaline values, promoting the precipitation of some metals like Ni, Cd, and Zn, being not available for the organisms as described by Ferreira et al. (2007). However, Goodfellow et al. (2000) reported the occurrence of a toxic ion imbalance, that is physiologically intolerable for aquatic organisms (e.g. *Daphnia magna*). The present study resembles with the conditions described previously, Gens ponds present acidic values and the lowest pH values were recorded in the same pond where, the concentration of metals are slight superior to the other two ponds. (e.g. P2 in July, see **Table 1**, **Table 2** and **Table 3**). Kenneth et al. (1972) describes acute effect of Zn and Cu on *Daphnia magna,* reporting the respective lethal concentration that affect 50 % of the organism (LC50) were 0.1 and 0.0098 mg/L, respectively. In the here present study, the concentrations of Zn quantified in P1 and P2 in summer period was superior (e.g., 0.6 mg/L in P2 of August). The same happened with concentration of Cu, that in August, September, and February samples (**Table 2**), exceeded the LC50 value (0.0098 mg/L). Khangarot et al., (1989) studied the toxicity of heavy metals to *D. magna* and reported that Cu and Zn are the top four that induce more toxicity (Hg>Ag>Cu>Zn>Cd>Pb). Although the remaining of chemical elements detected in the water samples appear in concentrations lower than LC50 reported in the literature (e.g., (Kenneth and Glenn, 1972) the samples of Gens ponds are complex due to the past mining activities and currently land use in the surrounding areas.

Table 8 Results (%) of acute immobilization assay of *Daphnia magna* after exposure to the different sample percentages (15%, 30%, 50%, 70%, 90% and 100% of samples).

	July			August			September			November			February			May		
Water samples (%)	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**
0 (CTL)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
70	0	10	0	0	0	0	0	0	0	0	0	0	15	100	5	80	100	0
90	100	100	30	100	100	30	100	100	25	45	40	60	100	100	100	100	100	90
100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

3.2.6 *Daphnia magna* feeding rate assays

The feeding rate assay was carried out to better understand if the samples under study could affect the feeding performance of *D. magna*. **Figure 9** shows the results obtained on the feeding rate assay with the natural waters samples with pH adjustment. A significant decrease in the feeding rate was observed in BP1 in July. Also, the water samples collected on May showed a significant decrease of the feeding rate of *D. magna* after exposure of all samples (**Figure 9**).

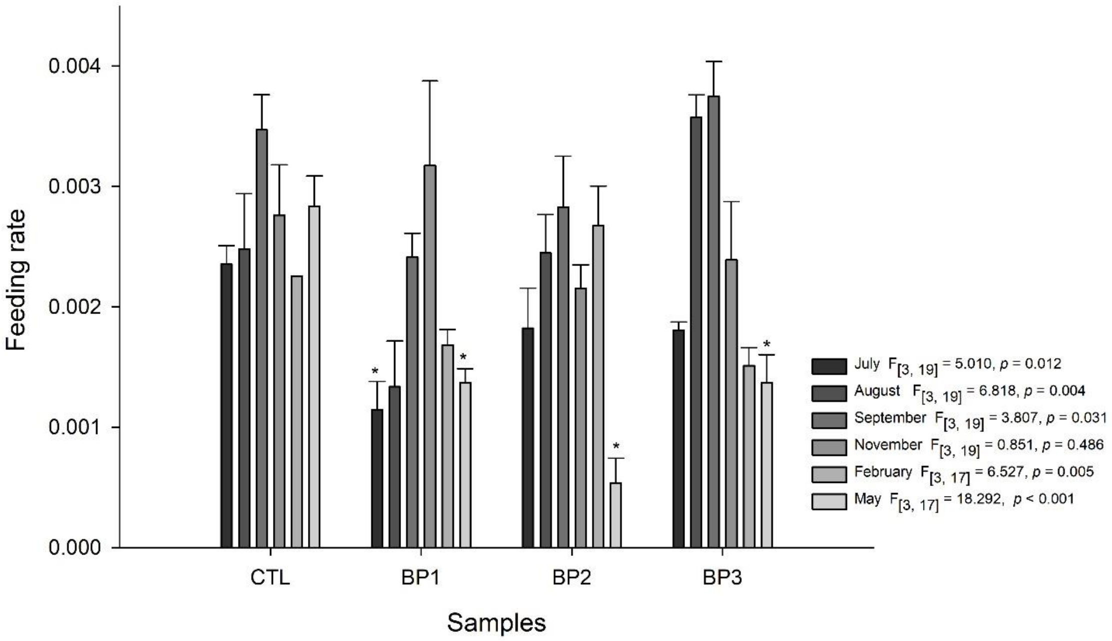

Figure 9 Results of feeding rate assay of *Daphnia magna* after exposure of natural water samples with pH adjustment treatment collected at the different sampling campaigns. *Stand for significant differences between each treatment and control group (Dunnet test, $p < 0.05$).

The results show that even with pH adjustment to the optimal value of the organism the feeding rate capacity of *Daphnia magna* is still affected, this fact may be due to the chemical composition of the water. Gens ponds water samples are complex due to the presence of several metals (**Table 2** and **Table 3**). The environmental metal mixtures have particular interest among aquatic toxicologists due to the toxicity that may apply to the survival and feeding behaviour of aquatic species, namely *Daphnia* (Dedourge-Geffard et al. 2009; Lari et al. 2017). Indeed, several metals (e.g., Cu, Cd, Ni, and Zn) are commonly found in aquatic ecosystems at high concentrations that are already describe able to cause ecotoxicological effects (Komjarova and Blust 2008). Data recorded for feeding rates and food consumption of the organism is considered a sensitive endpoint to assess the toxic effects on primary consumers, such as *D. magna*. Lari et al., (2017) characterize the individual toxicities of Cd, Cu, Ni and Zn at lethal and sub-lethal concentrations on *D. magna* and quantified the interactive toxic effects of these metals by performing feeding rate assays and evaluating the feeding behaviour. The results showed that Cu and Zn are the metals that induce more toxicity (Hg>Ag>Cu>Zn>Cd>Pb). Moreover Lari et al. (2017) described that the combination of Zn-Cu produced a more-than-additive inhibitory effect on food consumption. In addition, the decrease of feeding rates in *Daphnia* leads to a reduction of growth and reproduction, setting the survival of natural populations at risk (Lari et al. 2017).

4. Conclusion

Based on the WFD metrics, for the 3º cycle of planning, the results show that ponds of Gens are characterized as moderate ecological potential, during the entire sampling campaign (**Table 1**, **Table 2** and **Table 3**) taking in consideration the physical and chemical and biological parameters (**Table 4**, **Table 5**, **Table 6** and **Table 7**). These results are due to low values of pH, high values of TDS in P2 in February and May, and high values of total nitrogen in P2 and P3 in July and P1 in February. Regarding the biota of ponds, the macroinvertebrate community showed a high diversity of species in summer, and in general the ponds showed tolerant and resistant species (i.e *Ceratopogonidae* and *Oligochaeta*), although there are some sensitive *taxa* (i.e *Coenagriinidae*). Meanwhile, in the phytoplankton community, in generals the ponds registered a good EQR except in P3 in August and P2 and P3 in September. The combination of the selected biological parameters with macroinvertebrates and phytoplankton community are helpful tools to better understand these types of ecosystems, as they provide valuable ecosystem services that are not described by the current WFD assessment.

The ecotoxicological assays with *Daphnia magna* and *Lemna minor* showed species sensitivity to water samples from the ponds of Gens. After a pH adjustment a decrease of toxicity was observed. However, it is insufficient since, other factors may induce toxic effects. The presence of heavy metals, like Cd, Cu, Ni and Zn cause visible symptoms, like stunted growth, chlorosis, and necrosis that can cause visible damage to *Lemna minor*, effects already described by other authors. *Daphnia magna* was also affected in the survival and the feeding rate performance. The cluster of bioassays performed in this study, reveal that the ponds of Gens display toxicity. The use of ecotoxicological assays show to be a helpful tool to a more in-depth analysis of the toxicity in natural ecosystems.

Further research should be carried out, to complement the whole ecosystem assessment, namely the surrounding landscape should be analysed to better understand the interactions that can exist and may influence the quality of the water sample.

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6. Attachments

Table 9 Quality standards for heavily modified water bodies or artificial water bodies, according with the hydrographic region management plans published in the 1º cycle by the water framework directive.

	Parameters		Unit	Threshold value
General Physical and Chemical	**Colour**		Scale of Pt-Co	--
	Turbidity		NTU	--
	Temperature		ºC	--
	Total suspended solids		mg/L	--
	Depth of secchi		m	--
	Dissolved oxygen		mg O2/L	≥ 5 mg O_2/L
	Biochemical oxygen demand		mg O2/L	--
	Chemical oxygen deficiency		mg O2/L	--
	Oxygen saturation rate		% saturation of O_2	60 % - 120 %
	Conductivity		µS/cm	--
	pH		Scale of Sorensen	6 – 9
	Alkalinity		mg HCO_3/L	--
	Water hardness		mg $CaCO_3$/L	--
	Nitrates		mg NO_3/L	≤ 25 mg NO_3/L
	Nitrites		mg NO_2/L	--
	Ammoniacal nitrogen		mg NH_4/L	--
	Total nitrogen		mg N/L	--
	Total phosphate		mg P/L	≤ 0.05 mg P/L
	Orthophosphate		mg PO_4/L	--
Biological Elements	Phytoplankton	**Concentration of chlorophyll *a***	mg/m^3	2.00
		Total biovolume	mm^3/L	0.36
		% Biovolume of cyanobacteria's	--	0
		AGI	--	0.10

Table 10 Quality standards for heavily modified water bodies or artificial water bodies, according with the hydrographic region management plans published in the 2º cycle by the water framework directive.

	Parameters		Unit	Threshold value
General physical and Chemical	**Dissolved oxygen**		mg O_2/L	≥ 5 mg O_2/L
	Oxygen saturation rate		% saturation of O_2	60 % - 120 %
	pH		Scale of Sorensen	6 - 9
	Nitrates		mg NO_3/L	≤ 25 mg NO_3/L
	Total phosphate		mg P/L	≤ 0.05 mg P/L
Biological Elements	**Phytoplankton**	**Concentration of chlorophyll *a***	mg/m^3	1.70
		Total biovolume	mm^3/L	1.20
		% Biovolume of cyanobacteria's	--	0.02
		AGI	--	2.00

Table 11 Quality standards for artificial water bodies, according with the hydrographic region management plans published in the 3º cycle by the water framework directive.

	Parameters	Unit	Threshold value
General Physical and Chemical	**Temperature**	ºC	10 - 27
	Ammoniacal nitrogen	mg/L NH_4	2.5
	Total nitrogen	mg/L N	8.0
	Biochemical oxygen demand	mg/L O_2	7.0
	Chloride	mg/L	70
	Conductivity	µS/cm, 25º	1100
	Phosphate	mg/L PO_4	1.0
	Total phosphate	mg/L P	0.6
	Nitrates	mg/L NO_3	20
	Nitrites	mg/L NO_2	0.7
	pH	Escala pH	6 a 9
	Total dissolved solids	mg/L	640
	Total suspended solids	mg/L	45
	Sulphates	mg/L	575
Biological elements	**Chlorophyll *a***	µg/L	9.66
Specific pollutants	**Dissolved arsenic**	mg/L	0.05
	Dissolved copper	mg/L	0.0078
	Dissolved chromium	mg/L	0.0047
	Dissolved lithium	mg/L	2.5
	Dissolved zinc	mg/L	0.0078
Other pollutants	**Aluminium**	mg/L	5.0
	Copper	mg/L	5.0
	Manganese	mg/L	0.2
Priority substances	**Dissolved cadmium**	mg/L	0.00025
	Dissolved lead	mg/L	0.0012
	Dissolved nickel	mg/L	0.004

Table 12 Results of the pH values of the samples after 24 h and 48 h of the beginning of acute immobilization assay

		Percentage of samples									
		15 %		30 %		50 %		70 %		90 %	
	Site	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
July	**P1**	7.345	7.701	6.099	6.945	5.789	6.503	5.421	4.989	3.370	2.989
	P2	7.236	7.854	6.389	6.984	5.187	6.398	5.329	4.789	3.567	3.102
	P3	7.521	8.174	7.369	8.056	6.647	7.225	6.342	5.897	5.789	4.558
August	**P1**	7.156	7.468	6.984	7.398	6.658	7.225	6.578	5.748	4.367	3.874
	P2	6.785	7.285	6.427	7.145	6.258	6.985	5.658	4.879	4.623	3.758
	P3	6.978	7.896	5.874	6.431	5.421	6.278	5.105	4.742	4.567	4.105
September	**P1**	6.423	7.543	6.102	7.014	5.235	6.536	5.018	4.569	4.735	4.234
	P2	6.648	7.657	6.645	7.221	5.102	6.742	4.954	4.254	4.655	3.879
	P3	7.123	7.985	5.756	7.099	5.082	6.896	4.741	4.325	4.458	3.982
November	**P1**	6.627	7.452	6.134	6.896	4.879	5.785	4.555	4.125	4.236	3.785
	P2	6.784	7.687	5.865	6.681	5.369	6.123	4.989	4.256	4.587	3.895
	P3	6.109	8.105	6.234	7.013	6.102	6.741	5.423	4.657	4.679	3.745
February	**P1**	5.985	7.539	6.579	7.231	5.784	6.398	5.207	4.752	4.745	3.589
	P2	6.321	8.368	6.245	6.879	5.569	6.237	4.834	4.420	4.346	3.789
	P3	6.754	8.427	6.378	6.985	5.748	6.321	4.876	4.325	4.125	3.410
May	**P1**	6.897	7.654	5.879	6.678	6.102	6.402	5.532	4.555	4.417	3.654
	P2	6.345	7.632	6.125	6.989	6.028	6.587	5.427	4.874	4.579	3.417
	P3	6.714	7.747	6.235	7.012	5.895	6.874	5.369	4.485	4.467	3.875

Table 13 General physical and chemical parameters (Sampling sites: P1, P2 and P3; Sapling periods: July, September, August, November, February, and May).

	Site	O2 mg/L	% O2	Sal	VSS (mg/L)
RV					
July	**P1**	7.97	101.6	0.1	8.5
	P2	7.53	94.4	0.2	17.8
	P3	8.36	103.2	0.1	8.4
August	**P1**	8	95.9	0.1	7.9
	P2	7.8	93.3	0.2	21.4
	P3	7.11	94	0.1	21.02
September	**P1**	8.51	95.1	0	8.87
	P2	8.78	96.8	0.2	10.7
	P3	9.75	108.9	0.1	7.62
November	**P1**	6.44	61.3	0	21.8
	P2	7.89	75	0.1	15.5
	P3	8.1	78	0	24.2
February	**P1**	10.2	95.4	0.1	8.78
	P2	8.69	89.7	0.3	26.8
	P3	9.53	94.4	0.2	15.7
May	**P1**	8.88	99.9	0.1	8.78
	P2	8.22	93.2	0.3	19.4
	P3	8.64	98.7	0.2	12.2

Taxonomic Class	Taxonomic Families	Score
Ephemeroptera	Ephemeridae	10
	Heptagoniidae	10
	Leptophlebiidae	10
	Pothamanthidae	10
	Siphonurridae	10
Plecoptera	Capniidae	10
	Chloroperlidae	10
	Leuctridae	10
	Perlidae	10
	Taeniopteterygidae	10
Hemiptera	Aphelochereididae	10
Trichoptera	Beraecidae	10
	Brachycentridae	10
	Goeridae	10
	Lepidostomatidae	10
	Leptoceridae	10
	Mollanidae	10
	Odontoceridae	10
	Phyrgancineidae	10
	Sericostomatidae	10
Ephemeroptera	Caenidae	7
Plecoptera	Nemouridae	7
Trichoptera	Rhyacophilidae	7
	Polycentropodidae	7
	Limnepphilidae	7
Mollusca	Neritidae	6
	Viviparidae	6
	Ancylidae	6
	Unionidae	6
Trichoptera	Hydroptilidae	6
Crustacea	Corophiidae	6
	Gammaridae	6
	Paleamonidae	6
Polychaeta	Nereidae	6
	Nephthyidae	6
Odonata	Plaqthycnemididae	6
	Coenagriidae	6
Hemiptera	Mesovelidae	5
	Hydrometridae	5
	Gerridae	5
	Nepidae	5
	Naucoridae	5
	Notonectidae	5
	Pletidae	5

Taxonomic Class	Taxonomic Families	Score
Coleoptera	Corixidae	5
	Haliplitidae	5
	Hygrobiidae	5
	Dytiscidae	5
	Gyrinidae	5
	Hydrophilidae	5
	Helobidae	5
	Dryopidae	5
	Eliminthidae	5
	Chyssomelidae	5
	Curcuionidae	5
Phyrgancineidae	Hydropsychidae	5
Diptera	Tipulidae	5
	Simullidae	5
Planaria	Planariidae	5
	Dendrocoelidae	5
Ephemeroptera	Baeitilidae	4
Megaloptera	Sialidae	4
Hirudinea	Piscicolidae	4
Mollusca	Valvatidae	3
	Hygrobiidae	3
	Lymnaeitidae	3
	Physidae	3
	Planorbidae	3
	Sphaeriidae	3
Hirudinea	Erpobdellidae	3
	Glossiphonidae	3
	Hirudidae	3
Crustacea	Asellidae	3
Diptera		
Oligochaeta		
Others	Alderfly	4
	Shrimps	6
	Hoglice	3
	Blackfly	5
	Cranefly	5
	Madgse	2
	Worms	1

BMWP = sum of score of organism found
ASPT = divided by the number of group found

5 – 5	excellent
4 – 4.5	good
3 – 3.5	moderate
2 – 2.5	poor
1 – 1.5	very poor

Figure 10 Biological Monitoring Working Party (BMWP) Average Score per Taxon (ASPT) Scoring System