INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

Ecotoxicological impacts of olive mill wastewaters on freshwater fauna of River Tua (NE Portugal)

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Porque sozinha nunca seria capaz…

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ABSTRACT

Portugal stands out as one of the main olive oil producing countries, with the Trás-os-Montes region being particularly relevant due to its intense olive growing activity. Olive oil extraction generates significant volumes of liquid effluent, known as Olive Oil Mill Wastewater (OMWW). The predominant extraction system is two-phase, considered more environmentally sustainable as it produces smaller volumes of wastewater. According to Portuguese legislation, OMWW can be stored in evaporation ponds. However, although this practice is economically advantageous, it does not ensure effective environmental protection, particularly in aquatic ecosystems due to water contamination. To date, there are few studies evaluating the effects of OMW on macroinvertebrate and fish communities, particularly related to bioindicator changes (e.g. diversity, abundance, biotic indexes), as well as sublethal effects in terms of physiological biomarkers in sensitive native fish species, such as *Luciobarbus bocagei*. Given the risk of surface runoff or infiltration of these wastewaters into the aquatic environment, either by accidental discharges or by leaching processes, it is imperative to investigate their environmental impacts. In this context, the main objective of the present study was to evaluate the bioecological and toxicological impacts of wastewater from olive oil mills of 2 olive pomace oil extraction companies, located near Mirandela, on the aquatic fauna of the Tua River (Northeast Portugal). For the bioecological evaluation, 8 sampling sites were selected, grouped according to the degree of disturbance: 1) LOW, sites located upstream (T1, T2); 2) HIGH, sites located immediately downstream of the effluent entry point from both settling ponds (T3 and T6); and 3) INTERMEDIATE, sites located further downstream (T4, T5, T7 and T8). The methodologies defined by the Portuguese Environment Agency (APA), within the scope of the Water Framework Directive (WFD), were used to evaluate biological elements, such as macroinvertebrate and fish communities, as well as physical-chemical elements of the water, considering both the winter and summer sampling periods of 2024, As well as the subacute toxicity assay with *Luciobarbus bocagei*.

The results of the bioecological assessment showed a significant decrease in water quality (high conductivity, phenols, oxidability and low pH), especially in the sampling sites located under the direct influence of the discharges of both olive pomace oil extractors (i.e., T3 and T6). The impact is also well expressed in several bioindicator

metrics (e.g., richness, abundance, diversity, biotic indices IPTIN, F-IBIP) reinforced by statistical analyses confirming the biodiversity loss and substantial changes in the invertebrate and fish communities, particularly in native fish species, such as *L. bocagei*, suggesting a high sensitivity of this endemic species to water contamination.

The results of the acute toxicity tests with *Artemia franciscana* revealed high toxicity of OMW, regardless of the season. Subsequently, additional acute toxicity tests were performed with *Luciobarbus bocagei*, using OMW samples collected in summer and winter. Exploratory tests with summer samples showed that concentrations between 0.5% and 5% induced significant toxic effects in exposed individuals. In a second test of a subacute nature, physiological responses were evaluated by quantifying plasma electrolytes (Na^+, Ca^{2+} and K^+) after 24 and 48 hours of exposure. Plasma electrolytes were selected as biomarkers due to their high sensitivity to disturbances in ionic homeostasis, often caused by environmental pollutants. This choice is justified by the central role of the gills in osmoregulation, since they are in direct contact with the aquatic environment and represent one of the first organs to react to chemical stress. Changes in plasma sodium, calcium and potassium concentrations may therefore indicate impairment of essential physiological functions and ion regulation capacity.

The results showed significant changes in potassium (K^+) levels, while calcium (Ca^{2+}) and sodium (Na^+) levels did not show relevant variations. This lack of response may be due to technical limitations, such as the reduced blood volume available resulting from the small size of the specimens (13–14 cm) and the decline in phenolic compound concentrations observed throughout the test in the test tanks. It is important to emphasize that the absence of variation in some parameters does not imply the absence of stress, since L. bocagei can activate compensatory mechanisms of physiological adaptation to adverse environmental conditions.

Keywords: Olive mill wastewater, ecological-impacts, bioindicator, biomarkers, toxicity- tests, *Luciobarbus bocagei, Artemia franciscana.*

RESUMO

Portugal destaca-se como um dos principais países produtores de azeite, sendo a região de Trás-os-Montes particularmente relevante devido à sua intensa atividade olivícola. A extração do azeite origina volumes significativos de efluente líquido, designado por Águas Residuais de Lagares de Azeite (ARL). O sistema de extração predominante é bifásico, considerado mais sustentável do ponto de vista ambiental por produzir menores volumes de águas residuais. De acordo com a legislação portuguesa, as ARL podem ser armazenadas em lagoas de evaporação. No entanto, embora esta prática seja economicamente vantajosa, não assegura uma proteção ambiental eficaz, nomeadamente nos ecossistemas aquáticos devido à contaminação da água. Até ao momento, são escassos os estudos que avaliem os efeitos das ARL nas comunidades de macroinvertebrados e peixes, nomeadamente no que se refere a alterações em bioindicadores (e.g., diversidade abundância, índices bióticos) bem como aos efeitos subletais ao nível de biomarcadores fisiológicos em espécies piscícolas nativas sensíveis, como o *Luciobarbus bocagei*. Dado o risco de escoamento superficial ou infiltração destas águas residuais no meio aquático, seja por descargas acidentais ou por processos de lixiviação, é imperativo investigar os seus impactos ambientais.

Neste enquadramento, o principal objetivo do presente estudo foi avaliar os impactes bioecológicos e toxicológicos das águas residuais de lagares de azeite provenientes de 2 empresas de extração de óleo de bagaço de azeitona, situadas na proximidade de Mirandela, na fauna aquática do rio Tua (Nordeste de Portugal). Para a avaliação bioecológica foram selecionados 8 locais de amostragem agrupados de acordo com o grau de perturbação: 1) BAIXO, locais situados a montante (T1, T2); 2) ALTO, locais situados imediatamente a jusante do ponto de entrada do efluente proveniente de ambas as lagoas de decantação (T3 e T6); e 3) INTERMÉDIO, locais situados mais a jusante (T4, T5, T7 e T8). Recorreu-se as metodologias definidas pela Agência Portuguesa do Ambiente (APA), no âmbito da Diretiva Quadro da Água (DQA), para avaliação de elementos biológicos, caso das comunidades de macroinvertebrados e de peixes e ainda elementos físico-químicos da água, considerando ambos os períodos de amostragem do inverno e verão de 2024, assim como o ensaio de toxicidade subaguda com *Luciobarbus bocagei*,

Os resultados da avaliação bioecológica mostraram um significativo decréscimo na qualidade da água (valores elevados de condutividade, fenóis, oxidabilidade e decréscimo do pH), em especial nos locais de amostragem situados sob influência direta das descargas de ambas as extratoras de óleo de bagaço de azeitona (i.e., T3 e T6). O impacte está também bem expresso nas diversas métricas bioecológicas calculadas (e.g., riqueza, abundância, diversidade, índices bióticos IPTIN, F-IBIP) e as análises estatísticas reforçaram a perda de biodiversidade e alterações substanciais nas comunidades de invertebrados e peixes, nomeadamente nas espécies piscícolas nativas, caso de *L. bocagei*, sugerindo uma elevada sensibilidade desta espécie endémica à contaminação.

Os resultados dos ensaios de toxicidade aguda com *Artemia franciscana* revelaram elevada toxicidade das ARL, independentemente da estação do ano. Subsequentemente, foram realizados ensaios adicionais de toxicidade aguda com *L. bocagei*, utilizando amostras de ARL recolhidas no verão e no inverno. Os ensaios exploratórios com amostras estivais mostraram que concentrações entre 0,5% e 5% induziram efeitos tóxicos significativos nos indivíduos expostos. Num segundo ensaio, de natureza subaguda, foram avaliadas as respostas fisiológicas através da quantificação dos electrólitos plasmáticos (Na^+, Ca^{2+} e K^+) após 24 e 48 horas de exposição. Os electrólitos plasmáticos foram selecionados como biomarcadores devido à sua elevada sensibilidade a perturbações da homeostase iónica, frequentemente causadas por poluentes ambientais. Esta escolha justifica-se pelo papel central das brânquias na osmorregulação, uma vez que estão em contacto direto com o meio aquático e representam um dos primeiros órgãos a reagir ao stress químico. Alterações nas concentrações plasmáticas de sódio (Na^+), cálcio (Ca^{2+}) e potássio (K^+) podem indicar comprometimento das funções fisiológicas essenciais e da capacidade de regulação iónica. Os resultados evidenciaram alterações significativas nos níveis de K^+, enquanto os níveis de Ca^{2+} e. Na^+ não apresentaram variações relevantes. Esta ausência de resposta pode dever-se a limitações técnicas, como o reduzido volume sanguíneo disponível resultante do pequeno porte dos espécimes (13–14 cm) e o declínio das concentrações de compostos fenólicos ao longo do ensaio observados nos tanques de ensaios. É crucial salientar que a ausência de variação em alguns parâmetros não implica inexistência de stress, uma vez que *L. bocagei* pode ativar mecanismos compensatórios de adaptação fisiológica a condições ambientais adversas.

Palavras-chave: Águas residuais de lagares de azeite, impactos-ecológicos, bioindicadores, biomarcadores, testes-de-toxicidade, *Luciobarbus bocagei*, Artemia *franciscana.*

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LIST OF ABBREVIATIONS

ANOSIM - Analysis of Similarities

EC - Electric Conductivity

EPT- Ephemeroptera, Plecoptera, Trichoptera

F-IBIP – Fish biotic integrity index

ASPT - Average Score by Taxon

IASPT - Iberian Average Score by Taxon

IBMWP - Iberian Biological Monitoring Working Party

INAG - National Institute of Water

IPtI$_N$ - Portuguese Index of North Invertebrates

IUCN - International Union for Conservation of Nature

nMDS - Non-metric Multi-Dimensional Scaling Ordination

TDS- Total Dissolved Solids

WFD – Water Framework Directive in Portugal

LC$_{50}$ – **(**Lethal Concentration 50%)

LD$_{50}$ – (Lethal Dose 50%)

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1. STUDY FRAMEWORK

Olive oil mill wastewater (OMWW) is a byproduct generated during olive oil production. Despite being produced seasonally, it poses a significant environmental pollution threat due to its harmful effects (Al-Bsoul et al., 2020). The composition of OMWW largely depends on the extraction method used and the olive cultivar (Cuffaro et al., 2023). Generally, OMWW is typically characterized by strong acidity and a high content of phenolic compounds (Zahi et al., 2022). It is considered a regional environmental problem because of its negative impacts on aquatic life. The toxicity of OMWW can lead to unpleasant odours and the formation of an oily layer on the surface of natural water bodies, which hinders oxygen transfer by blocking sunlight (Duman et al., 2020). Among aquatic negative impacts, due to high organic load and relatively acidic pH, phenolic compounds present in OMWW are toxic to many aquatic organisms, including fish and invertebrates, even at low concentrations (Babić et al., 2019).

The biomarkers are promising indicators that show toxicants that have entered organisms, been distributed throughout tissues, and are causing toxic effects on critical targets (McCarthy and Shugart, 1990). Fish gills serve as the initial point of impact when it comes to environmental pollutants, highlighting their sensitivity to both physical and chemical changes in aquatic environments (McDonald and Wood, 1993). In this context, ATPases—specifically Na^+/K^+-ATPase and Ca^{2+}-ATPase - are essential membrane-bound enzymes that facilitate the transport of ions across cell membranes. These enzymes are vital for maintaining osmotic pressure and membrane permeability (Evans, 2008). Notably, a decrease in plasma sodium can be viewed as a valuable indicator of environmental stress, as it signals a reduction in the activity of branchial Na^+/K^+-ATPase (Mazon et al., 2002). Understanding these relationships can help us better assess the health of aquatic ecosystems and the effects of pollution on aquatic life.

Portugal is amongst the main producers of olive oil worldwide, and Trás-os-Montes e Alto Douro region is the second national producer. The OMWW produced could be contained in open-air lagoons, legally permitted as a form of treatment for this type of wastewater. These open-air lagoons, usually presents high volume of OMWW, which may flow into the river and affect the biota. Although it is known that OMWW presents high organic load, namely phenolic compounds, that could be highly polluting, deeper knowledge is necessary to understand how it can impact the ecological integrity of freshwater ecosystems, and particularly the native threatened fish fauna.

Based on the research conducted so far, there are no studies evaluating the impact of olive mill wastewater (OMWW) on the ecological composition of river systems or on the aquatic fauna communities, particularly in the Tua River. Furthermore, no studies have assessed fish survival when exposed to environments contaminated with OMWW from open-air lagoons.

1.1. Objectives

The present study aims to evaluate the toxicological impact of olive mill wastewater from open-air lagoons on the freshwater fauna of the Tua River and also on their ecological integrity. Several bioindicators and biomarkers were employed, particularly focusing on the Iberian barbel (*Luciobarbus bocagei*), which serves as an effective experimental model for assessing the toxicological mechanisms of contaminants.

A. Bioecological Evaluation of Tua River (Douro basin)

The evaluation of the biological and ecological state of Tua River, was made considering sites located upstream and downstream of olive mill wastewaters companies and open-air lagoons, near Mirandela, Trás-os-Montes e Alto Douro:

- Evaluation of the biological and ecological state, using fish (particularly Iberian barbel, *Luciobarbus bocagei*) populations and community of macroinvertebrates, considering 8 sampling sites in the Tua River located upstream and downstream of an industry and open-air lagoons.
- Characterization of several physical and chemical parameters of the water river (temperature, conductivity, total suspended solids, dissolved oxygen (DO), % DO saturation, pH, nitrates and total phenols).

B. Toxicity Evaluation

Since the composition of OMWW in open-air lagoons depends, among others, on season, production and summertime, tests will be done in two different times. The evaluation of the toxicological impact of OMWW from open-air lagoons was conducted using *Luciobarbus bocagei* as a model organism:

- Characterization of some physico-chemical parameters of the OMWW from open-air lagoons (total phenols and pH).
- Assessment of LC_{50} values of OMWW, using microcrustaceans.
- Acute toxicity tests with OMWW, using plasmatic electrolytes as biomarkers (potassium K^+, sodium Na^+ and calcium $Ca^{2+)}$ in *Luciobarbus bocagei*.

The impact of OMWW on plasmatic electrolytes will be evaluated after 24 and 48 hours of exposure, with the aim of verifying possible changes in the plasma levels of the electrolytes potassium (K^+), calcium (Ca^{2+}) and sodium (Na^+) depending on the exposure time. Plasmatic electrolyte was selected as biomarkers due to their high sensitivity to environmental contaminants and their ability to rapidly reflect disturbances in ionic homeostasis. This balance is primarily regulated by the gills, which are key organs in fish osmoregulation and among the first to be affected by waterborne pollutants.

2. LITERATURE REVIEW

2.1. Olive Mill Wastewater

Olive mill wastewater (OMWW) is widely recognized as a toxic pollutant for both terrestrial and aquatic ecosystems due to its complex chemical composition, which includes high concentrations of organic matter, phenolic compounds, and nutrients (Cuffaro et al., 2023; Al-Bsoul et al., 2020; Pardo et al., 2017; Gómez-Caravaca et al., 2014; Justino et al., 2012; S’habou et al., 2009; Giannoutsou et al., 2004). The quantity and composition of by-products can vary based on the olive oil processing system used. The olive oil production process consists of four main steps: 1) washing and defoliation; 2) crushing and malaxation; 3) separation of oil from solid pomace; and 4) separation of oil from the liquid phase (Issa et al., 2023) as presented in Figure 2.1. Olive phenols have a wide range of bioactivities, such as antioxidants, antimicrobial, radical scavenging properties, role as an uncoupling agent, anticarcinogenic and metal chelating properties that have beneficial effects on humans. Nevertheless, phenols present in OMWW also contribute, inversely, to the toxicity of OMWW and their potential environmental impact (Pardo et al., 2017, Gómez-Caravaca et al., 2014; Justino et al., 2012). OMWW has low pH and high polyphenol content and when placed near aquatic systems or spread onto arable land, it leads to contamination of surface and groundwater and soil pollution (S'habou et al. 2009).

The principal olive oil-producing countries are Spain, Italy, Greece, Turkey, Tunisia, Portugal, Morocco, and Algeria (Al-Qodah et al., 2022). Advances in olive oil extraction technology have led to increased efficiency, with the majority of extraction now utilizing a two-phase system, which minimizes wastewater generation compared to traditional three-phase processes. Khdair and Abu-Rumman (2020) reported that the annual production of 10 million m^3 of olive mill wastewater (OMWW) is equivalent to the wastewater generated by 20 million people, with 1 m^3 of OMWW exerting an environmental impact comparable to that of 100–200 m^3 of domestic sewage. It is estimated that approximately 30 million m^3 of OMWW is produced annually in the Mediterranean region. The management practices for OMWW have evolved and vary by region. Frequently, olive oil producers dispose of OMWW through land application, open-air lagoons, sewage systems, or direct discharge into water bodies, all of which are considered environmentally detrimental practices (Al-Qodah et al., 2022; Issa et al., 2023).

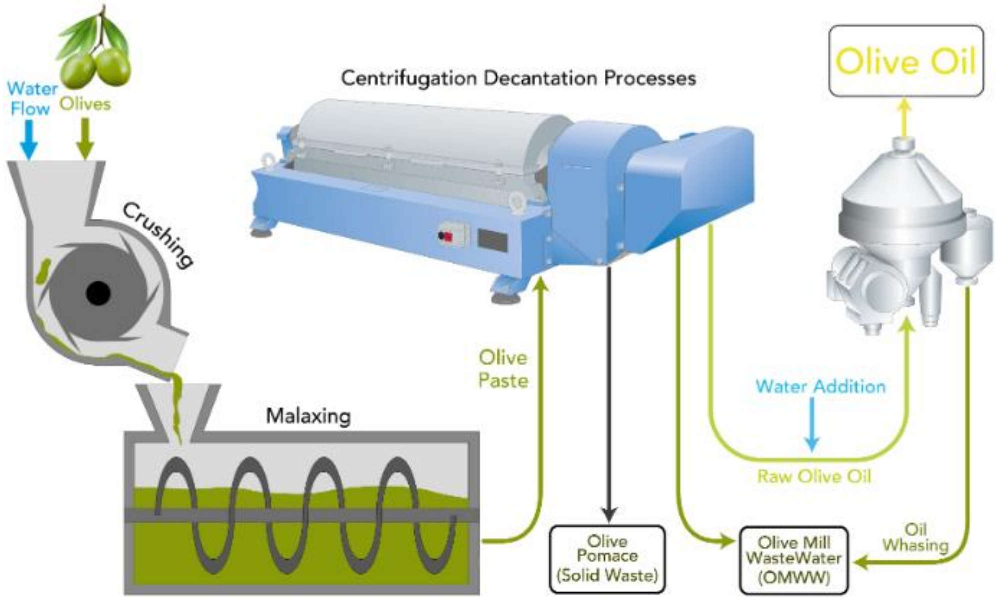

Figure 2.1. Flowchart of obtaining olive oil through the three-phase procedure yielding three fractions: olive oil, olive pomace (solid residue), and OMWW (from Ahmed et al., 2019)

Olive mill wastewater (OMWW) primarily comprises vegetative water from olives, wash water, and additional water introduced during the malaxation and pressing processes. The chemical composition of OMWW (Table 2.1) can exhibit considerable variability due to several factors, including meteorological conditions, olive cultivar, fruit maturity, and the specific oil extraction technique employed (Aktas et al., 2001; Niaounakis and Halvadakis, 2006; Khdair et al., 2019).

The main inconvenience of the three-phase system, besides an increase of water consumption and therefore generation of large quantities of OMWW during the process, is that is a very polluting effluent, comparing with two-phase (Albuquerque et al., 2004; Tsagaraki et al., 2007). To minimize the environmental impact of OMWW a two-phase centrifugation system for olive oil extraction was developed during the early nineties (Albuquerque et al., 2004; Giannoutsou et al., 2004). The main advantages of this process are the higher olive oil yield and lower energy consumption and the decreased of produced waste and its contaminant load (Albuquerque et al., 2004; Giannoutsou et al., 2004; Tsagaraki et al., 2007).

Table 2.1. Characteristic composition of OMWW (adapted from Zbakh et al., 2012*).

* 1. (Andreozzi et al., 1998); 2. (Paredes et al., 1999); 3. (Vlyssides, Loizides and Karlis, 2004); 4. (Ben-Sassi et al., 2006); 5. (Paraskeva et al., 2007); 6. (Asses et al., 2009); 7 (Karpouzas et al., 2010)8 (El-Abbassi, Khayet and Hafidi, 2011)

Parameters	**Authors**								**Range**
	1	2	3	4	5	6	7	8	
pH	5.10	5.17	4.80	4.85	5.20	5.10	5.70	5.30	4.7-5.7
Conductivity (mS/cm)	-	5.5	12	13-14	5	–	11	24	5-24
COD (g O2/L)	121.8	–	93	97-190	16.5	95	48	156	16.5-190
BOD (g O2/L)	–	–	46	–	–	–	–	–	41.3-46
Dry matte (g/L)	102.5	71.9	63.5	–	11.5	84.2	–	90	11.5-102.5
Organic matter (g/L)	81.6	46.5	–	–	–	–	26	–	26 - 81.6
Lipids (g/L)	9.8	3.1	1.64	–	–	–	–	7	1.64 - 9.8
Polyphenols (g/L)	0.002	6.2	1.6	10.7	7-11.5	0.8	4.82	8.8	0.002 – 8.8
Sugars (g/L)	–	8.79	–	–	1.3	–	–	4.3	1.3 -8.79
Total N (g/L)	0.6	0.63	0.76	–	0.06-0.3	–	0.90	–	0.06 – 0.90

In Portugal, DL No. 209/2008 mandates that facilities generating over 300 tons of olive oil daily, or 60 tons in sensitive areas, must undergo an environmental impact assessment, regulated by DL No. 151-B/2013, which also oversees emissions monitoring and pollution prevention programs under DL No. 173/2008. Current practices for disposing of olive mill wastewater often involve open-air lagoons, which reduce waste volume but do not treat the pollutants. Regulation nº143/2018 (March 6) stipulates that the user is responsible for the pre-treatment of wastewater to decrease pollutant loads and make it suitable for discharge into the public drainage system. Additionally, according to Portuguese Order No. 3123/2023 (March 8), alternative solutions to open-air lagoons must be evaluated for OMWW disposal, especially when land application is not allowed, ensuring compatibility with the seasonal nature of the activity.

Open-air lagoons (OL) can serve as a solution for industrial effluent, where wastewater is directed into large ponds and gradually evaporated using direct sunlight (Saad et al., 2022). The OL has gained popularity in most countries with high solar radiation levels as one of the most cost-effective, simple, and cheaper engineering approaches to treating wastewater. This system has been used to treat highly concentrated and toxic wastewaters produced from several industries, e.g., pesticides, oil, and gas production (Izady et al., 2020).

2.2 Ecological integrity of rivers

Ecological integrity is relevant topic in biology, natural resource management, and environmental legislation. Biotic integrity, for example, refers to an ecosystem's ability to support and maintain a community of organisms that closely resembles a reference condition regarding species composition, diversity, and functional organization. Thus, ecological integrity encompasses chemical, physical, and biological integrity, representing a holistic approach to ecosystem management (Karr and Dudley, 1981).

One of the main factors affecting the ecological integrity of aquatic ecosystems is human activity. Human influence is increasing with population, economic, and technological growth. River ecologists have attempted to assess the impacts of multiple stressors (e.g., nutrient levels, toxic contaminants, and habitat alteration) that could impair the inherent structure and functions of riverine ecosystems (Kim et al., 2015; Chen et al., 2019). Traditionally, river health monitoring has been based on water chemistry assessments (Halstead et al., 2014) however, recent investigations have revealed that this approach does not yield critical information about a river's ecological health status (Heatherly et al., 2007). Therefore, innovative techniques (e.g., integrated methods) were developed by incorporating physical, chemical, and biological processes. Such integrated approaches provide a multifaceted snapshot of the ecological health status of rivers and other water bodies (Kim et al., 2015; Chen et al., 2019).

The European Community (EC) has established a legal framework for the protection and management of freshwater resources through a holistic ecosystem-based approach, known as the Water Framework Directive "WFD" (Directive 2000/60/EC). The main objectives of the WFD are the prevention and reduction of pollution, promotion of sustainable use, protection and enhancement of the aquatic environment and mitigation of the effects of floods and droughts, in order to ensure the achievement of a good ecological status of freshwaters ecosystems (Calisi, 2023). For these reasons, the implementation of methodological approaches to assess the ecological risk posed by different stressors (e.g., organic and emergent pollutants) has deserved particular attention (Suter & Norton, 2019). The need to detect the biological effects of different stressors implies the use of complementary biomarker and bioindicator tools for environmental monitoring, hazard assessment and remediation measures for aquatic environments (Lomartire et al., 2021; Dondero & Calisi, 2015). Some of Europe's rivers have undergone strong structural modifications, including engineering modifications for navigation purposes, flood protection, or the use of hydroelectric power (Auerswald et al., 2019). Despite their critical value, freshwater ecosystems - and notably streams and rivers - are facing a growing environmental crisis (Reid et al., 2019). An unprecedented scale of environmental degradation and biodiversity loss in these ecosystems, both past and present, is the result of numerous human activities. Threats, including widespread land-use conversion, resource extraction, waste disposal, fragmentation by transportation infrastructure and the withdrawal or storage of water in reservoirs, collectively exacerbate flows of pollutants and sediments, alter hydrology and disrupt fluvial geomorphological processes (Grill et al., 2019, Chen and Olden, 2020).

2.3. Bioindicators

The bioindicator term refers to all kinds of organisms that reflect the presence of an environmental stressor (e.g., organic and mineral pollutants, nutrient enrichment) through physical, chemical, and/or behavioural responses (Hee, 1993). Bioindicators can provide qualitative and/or quantitative data about the presence and effects of the different stressors in the ecosystem (Gerhardt, 2002). Animals (e.g., fish, birds, macroinvertebrates), plants and fungi (e.g., mosses, lichens, tree rings), and microorganisms (e.g., algae, diatoms) are commonly used as bioindicators in environmental assessment studies (Bonanno et al., 2020). However, regardless of the environment, geographic region and type of organism and disturbance, a good bioindicator must be abundant and commonly distributed, possess an indicator ability, and be a well-studied organism (Holt & Miller, 2011).

The selection of a bioindicator depends on the objectives of the study (Han et al., 2015). However, an *ideal* bioindicator must have: 1) taxonomic knowledge and wide distribution; 2) low mobility; 3) well-known ecological characteristics; 4) suitability for laboratory experiments and high sensitivity to environmental and anthropic stressors; 5) economic, cultural, and social value; and 6) possibility to quantify and standardize their characteristics (Li et al., 2010). Aquatic macroinvertebrates and fish are the most common type of bioindicators used in freshwater ecosystems. **Aquatic macroinvertebrates** are a diverse group of small animals (e.g., insects, crustaceans, bivalves, snails, and worms) extremely important in terms of food webs, representing links between the lower and higher trophic levels (Nieto et al., 2017; Collier et al., 2018). Invertebrates are particularly vulnerable to: 1) low levels of dissolved oxygen; 2) nutrient excess and eutrophication phenomena; 3) extreme pH levels; 4) removal of riparian vegetation; and 5) seasonality: in winter, can occur a reduction on food sources decreases (Juvigny-Khenafou et al., 2021; Lin et al., 2020). Fish are abundant in many aquatic environments, and very important for humans (Elliott, 2010). As consumers at different levels, fish can reflect the integrated trophic conditions in aquatic environments (Anderson & Cabana, 2007). Fish are an important food source for humans, and monitoring their trace levels is important to ensure food safety. In fact, several studies were made about bioaccumulation (i.e., the gradual accumulation of toxic substances - pesticides and heavy metals, for example) in the tissue of living organisms (Van der Oost *et al.*, 2003; Fernandes et al., 2008a) and biomagnification or bioamplification (i.e., the increased of the concentration of these toxic substances at successively higher levels in the food chain) phenomena in fish (Fernandes et. al, 2007a; Alonso et al., 2008; Daley et al., 2014). Several disturbances can negatively impact fish communities: 1) eutrophication and decrease of dissolved oxygen levels; 2) invasive non-autochthonous species and the displacement of native species; 3) pollution and the increase of toxic substances (heavy metals, pesticides); and 4) water flow modifications inducing changes to physical habitat, nutrient distribution, and community composition (Wilson et al., 2010).

Biomonitoring involves observing individual organisms and/or communities to see how they respond to physical or chemical changes in their environment over time. It can provide a qualitative assessment through the observation and recording of these changes, or a quantitative evaluation by measuring substance concentrations in the organisms' tissues. Several biomonitoring methods including biotic indices, diversity indices, multimetric indices, multivariate approaches, functional feeding groups, multiple biological traits and, more recently, DNA-Metabarcoding methodologies (Deiner et al., 2017; Friberg et al., 2011) can be used:

Biotic indices: a score (numerical value) is normally obtained to represent the tolerance of organisms to an environmental stressor (e.g., organic pollution). Typically, biotic indices assign different types of indicator species to different levels of environmental disturbance, based on the principle that most sensitive species tend to disappear, and more tolerant species to increase in abundance (Burger, 2006). Several biotic indices were developed, such as: 1) Trent Biotic Index (Cairns & Pratt, 1993); 2) Belgian Biotic Index (1983) (De Pauw *et al.*, 1986; Gabriels *et al.*, 2005); 3) Hilsenhoff Biotic Index (Hilsenhoff, 1988), and one of the most commonly used 4) the Biological Monitoring Working Party Score System (BMWP, 1980) (Bartram & Ballance, 1996) and their evolution to Iberia (IBMWP) by Alba-Tercedor & Sanchez-Ortega (2000). Some of them have been standardized and recommended for the ecological assessments of river ecosystems by the Water Framework Directive in Europe.

Diversity indices: Univariate diversity indices were the most used indices in the past (Cairns & Pratt, 1993). However, nowadays, univariate diversity indices are combined with other type of metrics to obtain more accurate approaches for aquatic ecosystem assessments. Some examples are: 1) Shannon-Wienner Index (Strong, 2016); 2) Simpson Index (Somerfield *et al.*, 2008); 3) Margalef Index (1958) (Gamito, 2010), and are based on community structure indicators like abundance (total number of individuals), evenness (uniformity in the distribution of individuals of different species), and richness (number of species present).

Multimetric indices: in this case the biomonitoring and assessment use quantitative tools to evaluate the ecosystem integrity (Burger, 2006) based on diverse community structural and functional metrics or variables (e.g., abundance, equitability, tolerance to pollution, and richness) (Hering et al., 2006). Some examples are: 1) Biotic Integrity Index (Karr, 1981), and the Portuguese Invertebrate index - IPTIn (INAG, 2008a).

Multivariate indices: are statistical models designed to predict the biota that should be present at a river environment unexposed to anthropogenic stress. The model is based on reference environmental parameters (e.g., Reference Condition Approach) (Bowman and Somers, 2005) and then the modelled results compared with the observed biota at the study site (Burger, 2006). Finally, if the observations are like the biota predicted, then the site is in “good condition” and vice versa. To accurately calibrate the model through knowledge of the biota, seasonal distribution and reference conditions in the survey area are an important prerequisite. The River Invertebrate Prediction and Classification System (RIVPACS) was the first large-scale application of a multivariate model for a biomonitoring assessment (Wright et al., 1998).

Multiple Biological Traits: the status of an ecosystem can be quantified through the functional diversity of communities (Nock et al., 2016). Biological traits can be species physiology, morphology, life history, and behaviour, using both interspecific interactions and the relationship between species and their environment. Multiple biological traits (e.g., size, number of descendants per reproductive cycle, parental care, and mobility) can be combined with multimetric approaches to identify different types of human impact (Dolédec et al., 1999).

Finally, traditional biomonitoring assessments (e.g., biotic and diversity indices) are based on direct observations, which have been proven to be expensive and require a lot of resources and time (Baird & Hajibabaei, 2012). However, a next generation of biomonitoring with new approaches based on molecular analysis have been developed in recent years, such as: **DNA-Metabarcoding:** High-throughput amplicon sequencing (HTS), known as DNA-Metabarcoding, is an emerging technology of environmental biomonitoring, allowing the complete identification of species. Analysis can be performed in parallel from many samples at the same time. DNA can be analysed from living cells (e.g., diatoms) and tissue samples (e.g., from fish), as well as from water samples or sediments (environmental DNA or eDNA) (Baird & Hajibabaei, 2012).

Integrative methods: the most sophisticated approaches for aquatic ecosystems quality assessments (Burger, 2006) are based on the integrated analysis of multiple bioindicator organisms, including macroinvertebrates, periphyton and fish, to evaluate the status of the ecosystem (Markert, 2007). An example consists in the Water Framework Directive in Europe that has proposed an integrated assessment system for freshwater bodies based on physicochemical analysis, hydromorphological characteristics, and bioindicators elements (Birk & Hering, 2006b).

2.4. Biomarkers

Biomarkers could be assessments in body fluids, cells or tissues that may indicate biochemical or cellular modifications due to the presence and magnitude of toxic substances or the host response (NRC, 1987). Biomarkers can be classified into three categories: exposure, effect and susceptibility (Who, 1993, NRC, 2006). For instances, histopathological biomarkers provide valuable insights into the condition of specific target organs, such as gills, gonads, and liver, which are critical for essential physiological functions. Due to their sensitivity and specificity, these biomarkers serve as effective tools in environmental monitoring programs aimed at assessing the health of aquatic ecosystems (Gernhofer et al., 2001).

The use of biomarkers aims to relate the presence of toxic substances in the environment with effects on the organism (fish), the effects can generally depend on the degree of toxicity of the substance (concentration) and the degree of exposure. Biomarkers have been used widely, but there are some limitations to their applicability that include the complexity and cost of methodology, as well as the varying degrees of specificity (Fernandes et. al, 2008a). Assessing the impacts of contaminants on the ecosystem and aquatic organisms has been a challenge due to the presence of multiple stress factors and the complexity of ecosystems (Van et al., 2023; Handy et al., 2003). Certain biomarkers can be used to indicate only physiological changes in aquatic animals exposed to a polluted environment. Other biomarkers are useful for monitoring biochemical levels (Stara et al., 2022).

The gills of most teleost fish are the main site of physiological exchange with the surrounding environment, participating in gas exchange, ion acquisition, acid-base regulation, nitrogenous waste excretion, osmoregulation and hormone production (Evans et al., 2005). They also represent an important barrier to pollutants between the external and internal environment and are a primary target for pollutants (Mallatt, 1985; Perry & Laurent, 1993). The basic functional unit of the gill is the filament, which supports rows of plate-like lamellae. The lamellae are designed for gas exchange, with a large surface area and a thin epithelium surrounding a well-vascularised core of pillar cell capillaries. The lamellae are positioned so that the blood flow is counter-current to the water flow over the gills (Evans et al., 1999; Evans et al., 2005).

The gills (Figure 2.2) are large surface area and very thin water-blood diffusion distance favour xenobiotic uptake in freshwater fish due to the great water volume flowing onto the gill lamellae to obtain the necessary O_2 for aerobic metabolism (Fernandes and Moron, 2020).

Osmoregulation is a fundamental process in living systems and is of importance concerning respiration, digestion and reproduction. Osmoregulation processes are those that allow a fish to maintain the composition and volume of its cellular fluid. This is of critical importance because the protein processes of cells are sensitive to cytoplasmic ionic concentrations and cell membranes tolerate relatively small deviations in cell volume (they either collapse or explode) (Evans, 2011). Osmoregulation in fish occurs in structures such as the gastrointestinal tract, kidneys and gills (Evans et al., 1999). The osmoregulatory process is an important adaptation of freshwater fish to overcome salinity changes in natural and farmed conditions. ATPases (Na+/K^+-ATPase, Ca^{2+}-ATPase) are membrane-bound enzymes responsible for ions transport through membranes and, thus, they help in the regulation of osmotic pressure and membrane permeability (Evans, 2008; Atli and Canl, 2011).

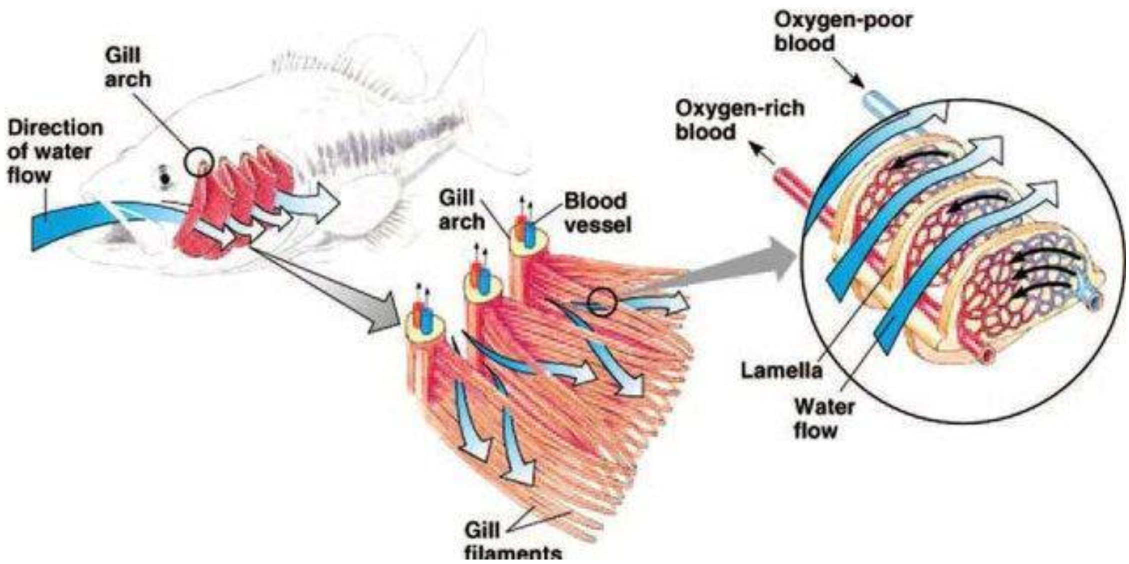

Figure 2.2. Schematic teleost fish gill (source: Evans et al., 2005)

The concentration of electrolytes offers significant information regarding the impact of stress on fish and status of diseases. Electrolytes are a part of the biochemical composition of blood and values change in some conditions, such as stress, environmental condition, bacterial infection, nutrition and season. Blood and body fluids contain several electrolytes. As the concentration of electrolytes in the body fluid of freshwater fish is much higher than the surrounding water, osmoregulation is essential to maintain correct fluid-electrolyte balance in the body fluids of fish (Wurts & Stickney, 1989).

Gills serve as the initial point of impact when it comes to environmental pollutants, highlighting their sensitivity to both physical and chemical changes in aquatic environments (McDonald and Wood, 1993). In this context, a decrease in plasma sodium can be viewed as a valuable indicator of environmental stress, as it signals a reduction in the activity of branchial Na^+/K^+-ATPase (Mazon et al., 2002). Understanding these relationships can help us better assess the health of aquatic ecosystems and the effects of pollution on aquatic life.

The effect of pollutants on the function of osmoregulation in aquatic animals in general has been sparsely investigated (Depledge et al., 1995, Monserrat et al., 2007). When osmoregulatory impairment has been studied, the focus was on the osmoregulation-related enzymes Na^+, K^+-ATPase and carbonic anhydrase (reviewed in Monserrat et al., 2007). In fish, measurement of major electrolytes (Na^+, K^+) under stressful conditions can be used as sensitive biomarkers of chemical exposure and its effects on the ion-regulating tissues (Fernandes et al., 2007a). The biomarkers are promising indicators that show toxicants have entered organisms, been distributed throughout tissues, and are causing toxic effects on critical targets (McCarthy and Shugart, 1990).

2.5. Fish and Microcrustaceans under Study

The Iberian barbel, *Luciobarbus bocagei*, is an endemic cyprinid of the Iberian Peninsula, with a very wide distribution, which occupies almost the entire watershed of Portugal (Magalhaes, 1992). It shows resistance and resilience in adverse conditions due to its easy adaptation to lentic waters and low levels of dissolved oxygen. It is considered a non-threatened species in the Iberian Peninsula (Doadrio, 2001; Alexandre et al., 2015; Magalhães et al., 2023). The species has a generalist diet made up of plant material and detritus, and predominantly invertebrate larvae (e.g., Diptera and Ephemeroptera). It is considered a potamodromous species, as it carries out seasonal movements along the river that may be related to the search for suitable sites for reproduction, zones with greater availability of food with greater refuge potential (Lobón-Cerviá & Fernandez-Delgado, 1984; Magalhães, 1992). The breeding season takes place between March and June, a period in which these animals move to suitable sites for spawning, typically shallower depth and higher current speed, with gravel substrate and turbulent flow, which provides greater oxygenation of the water (Ferreira et al., 1999). Also, in this period, the species adopts a rheophilic behaviour, i.e, facing strong water currents, traveling for reproductive purposes (Baras and Cherry, 1990; Baras et al., 1994; Lucas and Batley, 1996) and females can travel greater distances than males. The latter generally present nuptials at this critical time.

Iberian barbel is medium-sized fish, with a slightly convex head profile and a lower mouth with two pairs of barbels. The posterior barbels reach the midline of the eye. The dorsal fin has an ossified radius 2/3 the height of the dorsal fin, and the posterior profile of the fin is almost linear and oblique to the dorsal profile of the body. The upper lip is large and thick, with the lower lip slightly retracted. The dorsal region is greenish-brown, the ventral region white or reddish (Figure 2.3).

Figure 2.3. Example of the species *Luciobarbus bocagei* (www.fishbase.org)

The brine shrimp *Artemia franciscana (*formerly *Artemia salina*) (Figure 2.4) is a species of saltwater microcrustacean, which has a very efficient osmoregulatory system and is found with a wide geographic distribution in high salinity environments such as oceans and salt lakes (Sorgeloos et al., 1986). The literature reports the wide use of this microcrustacean in toxicity tests for a diverse range of substances, as they present advantages, such as: short life cycle, possibility of keeping organisms stored for a long time in the form of dormant eggs (cysts) and obtaining viable individuals easily and at any time of the year, and because they are small in size, which facilitates the reduction of materials and space required for maintaining cultures and carrying out tests (Meyer et al., 1982; Sorgeloos et al., 1978; Kalčíková et al., 2012).

Figure 2.4. Example of the species *Artemia franciscana* (www.biodiversity4all.org/taxa/214217-Artemia-franciscana).

3. METHODOLOGY

3.1. Bioecology Evaluation of Tua River

3.1.1. Study Area

River Tua is one of the main tributaries of Douro River basin in Portugal, located in Northeastern Portugal, crossing the city of Mirandela in the middle section. The Tua River is formed from the junction of Rabaçal and Tuela tributaries, 4 km upstream from the city of Mirandela, reaching approximately after 40 km the Douro River (Gomes et al., 2021).

Eight sampling sites were selected and distributed along the longitudinal axis of River Tua (T1 to T8), considering the potential impact of two olive pomace industries, the Aucama and Mirabaga companies located, respectively, up and downstream of Mirandela city, and closely to the villages of Eixes and Frechas (Figure 3.1).

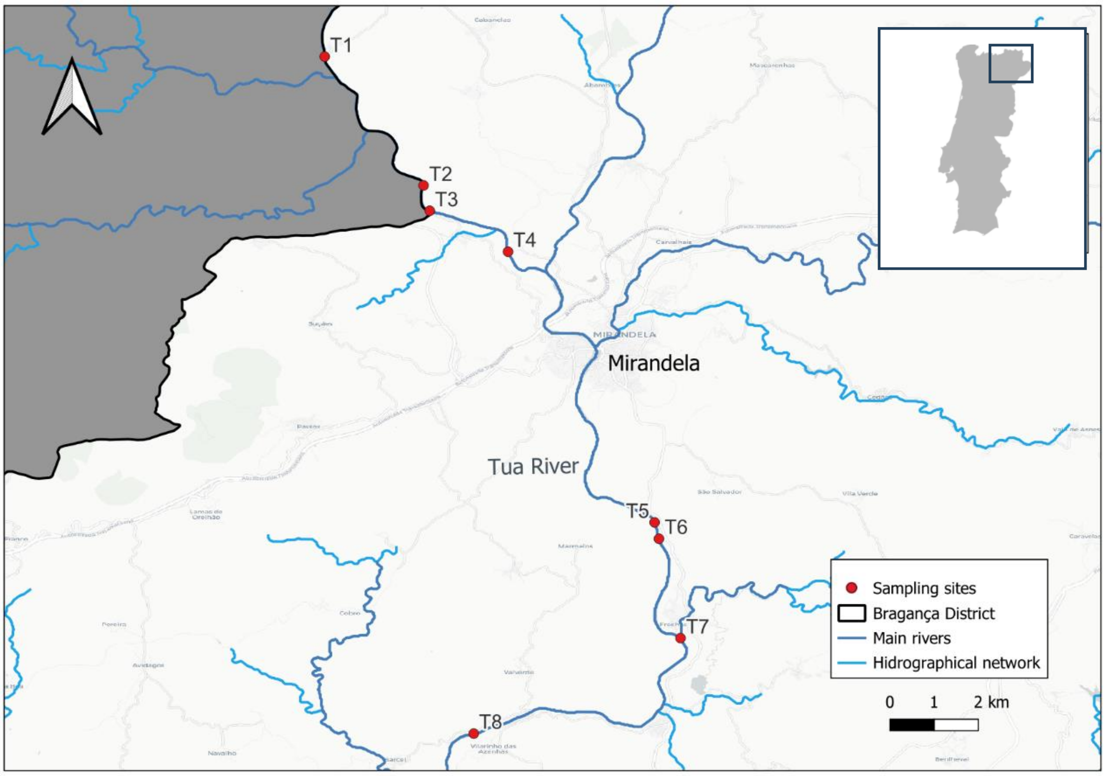

Figure 3.1.Map of sampling sites located in river Tua basin, near Mirandela (NE Portugal).

The bioecological evaluation was made considering 3 sectors, with different level of disturbance: 1) Low disturbance - sampling sites located upstream of both companies (T1 and T2); 2) High disturbance - sampling sites located immediately downstream of both open-lagoons (T3 and T6) and 3) Intermediate disturbance – sampling sites located downstream of main impact, but more far way (T4, T5, T7, T8) (see Annex I).

3.1.2. Biological elements: Macroinvertebrate communities

Sampling and capture of benthic macroinvertebrate followed the protocol established by the Environment Portuguese Agency (APA), based on the implementation of the Water Framework Directive (WFD) in Portugal (INAG, 2008a). In summary, the protocol establishes for each sampling site a selection of a 50 m section, representing the different habitats (e.g., riffle, pool and run) and microhabitats (e.g., fine and coarse inorganic materials, leaves, aquatic plants), considering both presences, if possible, of erosion (turbulent flow) and adjacent sedimentation units (laminar flow). The operator must collect a global sample composed by six subsamples, in upstream direction removing the substrata of the river bottom (1-meter extension), using a handnet (25*25 cm dimensions and 500 µm of mesh size of the net) (Figure 3.2).

Figure 3.2. Sampling procedures of benthic invertebrate collection (summer 2024) : a) Collection of invertebrate; b) The invertebrates are placed in a bucket filled with water from the river

Attached invertebrates were also collected from coarse substrate (e.g., gastropods, caddisflies, mosquito flies) and immediately preserved in ethanol 96% in polyethylene bottles to posterior laboratory processing of the samples.

In the laboratory, the macroinvertebrates were sorted, counted, and identified using a stereomicroscope SMZ10 with 10-132*x* zoom magnification, and recurring mainly to appropriate dichotomous keys (e.g. Tachet et al., 1981, 2010) (Figure 3.3). The taxonomic level of identification was Family level. However, Oligochaeta and Acari groups were only identified until the Subclass level.

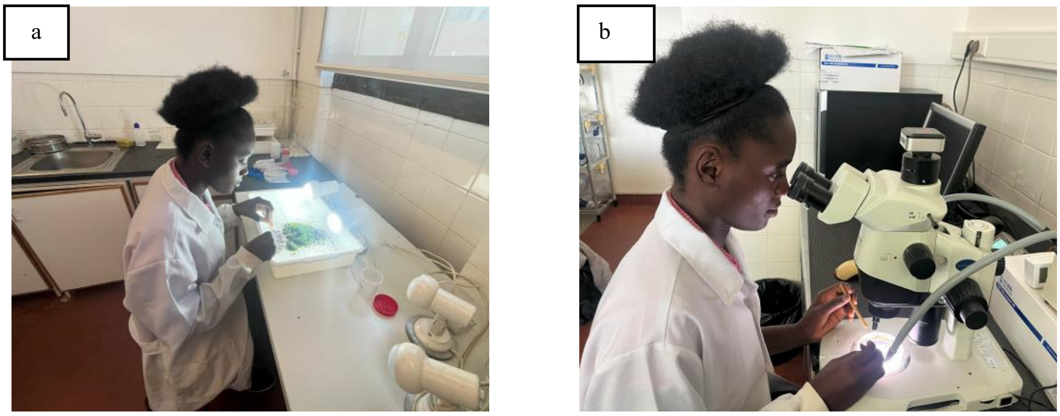

Figure 3.3. Laboratorial procedures: a) Macroinvertebrate sorting; b) Macroinvertebrate identification.

The biological quality based on macroinvertebrate communities was calculated using the AMIIB@ (*http://dqa.inag.pt/implementacao_invertebrados_AMIIB.html*) software, and several metrics determined: 1) Number of individuals (N) and number of *taxa* (S); 2) Diversity (*e.g.* H' Shannon-Wienner index); 3) Evenness (*e.g.* Pielou J' index); 4) Relative abundance of Ephemeroptera, Plecoptera and Trichoptera (% EPT); 5) Biotic Index IBMWP (Alba-Tercedor, 1996), and 6) Portuguese Northern Invertebrate Index- $IPtI_N$ (INAG, 2009).

The Portuguese Northern **multimetric Index ($IPtI_N$)** is calculated using the following formula:

$$IPtI_N = N^o\ taxa \times 0.25 + EPT \times 0.15 + Evenness \times 0.1 + (IASPT - 2) \times 0.3 + \text{Log}\ (\text{Sel. ETD}+1) \times 0.2$$

where:

EPT: Nº families belonging to Ephemeroptera, Plecoptera, Trichoptera orders

Evenness: Defined as Pielou index or Evenness and calculated by the formula:

$$E = H'/(LnS)$$

where:

H' - diversity of Shannon-Wienner
S - number of present *taxa*
Ln - natural or neper logarithm

The **H' Shannon-Wienner Index** is calculated by the formula:

$$H' = -\sum \mathbf{p_i \, Ln \, p_i}$$

where:

$\mathbf{p_i = n_i/N}$
$\mathbf{n_i}$- nº of individuals of each *taxon i*
N- total nº of individuals present in sample

- **IASPT**: Iberian ASPT, corresponding to IBMWP (Alba-Tercedor, 2000) divided by the number of families present in the sample.
- **Log (Sel. ETD+1)** - Log_{10} of (1 + abundance of Heptageniidae, Ephemeridae, Brachycentridae, Odontoceridae, Limnephilidae, Goeridae, Polycentropodidae, Athericidae, Dixidae, Dolichopodidae, Empididae, Stratiomyidae);

In the Table 3.1 reference values and boundary values for the quality class of N2 is presented, according to the Management Plan of River Basin in 2016/2021 (APA, 2016).

Table 3.1.Reference values and boundaries for the river type of the study (APA, 2016).

River Typology	Reference Values	Excellent	Good	Moderate	Poor	Bad
Alto Douro Rivers of Medium-Large Dimension (N2)	1.01	≥0.83	[0.69 – 0.83[	[0.41 – 0.69[	[0.20 – 0.41[	[0 – 0.20[

3.1.3. Biological elements: Fish communities

The sampling of the fish fauna was based on the Manual for the Biological Assessment of Water Quality in River Systems, according to the WFD - Protocol for sampling and analysis for fish fauna (INAG, 2008b). Fish was captured in two seasons, Winter (January) and Summer (July) of 2024, using an electrofishing equipment (Hans Grassl ELT 60II GI ©, DC direct current, 1.5A and 600 v) (Figure 3.4).

Figure 3.4. During Summer 2024, fish fauna sampling using electrofishing was conducted, including: a) collection of specimens along the left riverbank; b) collection along the right riverbank.

The electrofishing method was carried out by wading the channel in a zig-zag way, from down to upstream, adjusting the device to the water conductivity, to increase fishing efficiency and simultaneously avoid mortality and fish injuries. Representative habitats were selected, including the riffle/pool sequence and several microhabitats. The sampled area corresponded to 20 times its width, with a length of no less than 100 meters, and a capture effort (CPUE) of 30 minutes.Captured fish was identified, recurring to Collares-Pereira et al. (2021) and Magalhães et al. (2023). Biometric data registered were total length, using an ichthyometer (accuracy of 0.1 cm), and biomass, using a digital balance (accuracy of 0.01 g) (Figure 3.5). After the data collection fish was immediately released in the river.

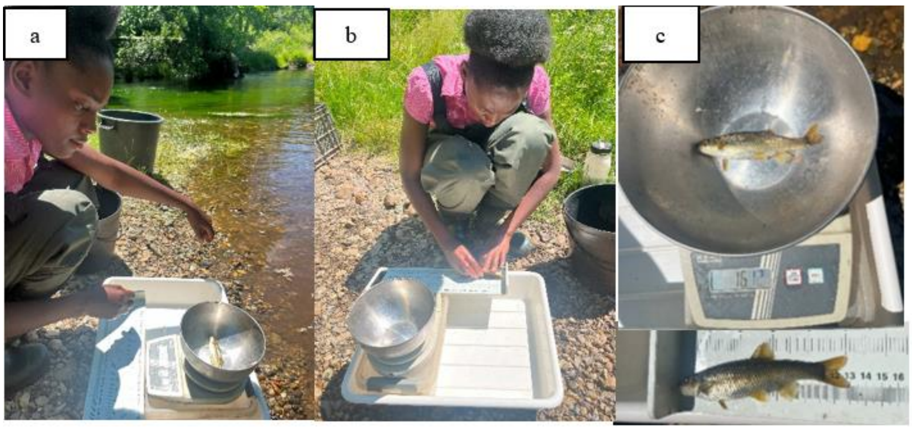

Figure 3.5. Biometric data collection and fish fauna identification were conducted in Summer 2024: a) fish weighing; b) fish total length; c) example of data recording.

The biological quality, based on fish fauna, was evaluated though the application of the F-IBIP - Fish Biotic Integrity Index for wadable Rivers of Portugal (INAG & AFN, 2012), This index uses several metrics to reflect basic structural and functional characteristics of the fish communities in lotic systems of Portugal (Oliveira et al., 2007, 2010). These metrics can decrease or increase according to the intensity of the anthropic disturbance and are included in two major groups: richness and specific composition (e.g., number of native species, percentage of exotic individuals) and ecological factors (related, for example, to food or reproduction). The F-IBIP score is obtained through the arithmetic mean of the metrics considered in each fish group. The final score of F-IBIP varies between 0 (zero), corresponding to bad quality, and 1 (one) corresponding to excellent quality (Table 3.2). The F-IBIP index was determined using the software http://www.isa.ulisboa.pt/wproj/fibip/.

Table 3. 2. Variation values of the F-IBIP quality classes

Score (Ecological Quality Ratio)	Quality Classes
[0.850 – 1.000]	Excellent
[0.675 – 0.850[	Good
[0.450 – 0.675[	Moderate
[0.225 – 0.450[	Poor
[0 – 0.225[	Bad

3.1.4. Physical and Chemical elements: Water quality

To assess water quality, several physical and chemical parameters were evaluated. Some of them were measured *in situ* (Figure 3.6), using a portable probe (HACH model 40d ©), namely: 1) Dissolved Oxygen (mg O_2/L); 2) Temperature (°C); 3) Electrical Conductivity EC25 (µS/cm), and 4) pH.

Figure 3.6. In situ evaluations of water quality in the River Tua. (Summer): a) measurement of water parameters using a multiparameter probe; b) results reading.

Other variables were determined in the laboratory, after the collection of water samples (1.5 L), and their transportation in coolers (to maintain the temperature of 4°C). The following variables were evaluated in the laboratory: 1) Nitrates (mg NO_3^-/L); 2) Phosphates (mg PO_4^{3-}/L); 3) Oxidability (mg O_2/L); 4) Total Acidity (mg HCO_3^-/L) and 5) Phenols (mg eq. gallic acid/L) contents.

All procedures were performed and determined according to the APHA (2005). Physical and chemical parameters were measured in the spring season and the analyses based on Portuguese legislation (DL 236/98 of 1 August).

3.2. Toxicological Evaluation of OMWW from Open-air Lagoons

Toxicity evaluation of OMWW from open-air lagoons was performed in *vivo* by two steps: Acute toxicity tests for LC_{50} evaluation, using microcrustaceans, and electrolytes levels evaluation, using *Luciobarbus bocagei.*

3.2.1. Sampling of OMWW

The olive mill wastewater (OMWW) was sampling in December 2023 and July 2024 at the open-air lagoons from Mirabaga-Mirandela company (Figures 3.7 and 3.8). The samples were collected in 1.5 L plastic bottles and transported under refrigeration to the ESA agro-industrial laboratory. One part was taken to the chemistry laboratory to determine total phenols and the other part was frozen until the toxicity test. Mirabaga (Indústria e Comércio Alimentar S.A., Latadas, Frechas, Bragança) is an anonymous company whose headquarters are Tapada da Estação 7430-143 Crato (Portalegre), it carries out various activities, including the production, refining, packaging and marketing of edible oils and olive oil.

Figure 3.7. Overview of the Mirabaga company, downstream of the Tua River (Google maps).

3.2.2. Total phenolic compounds

The determination of the total phenolic compound content (in water River, OMWW, and subacute toxicity tests) was conducted using spectrophotometry in accordance with the Folin-Ciocalteu method, as described by Singleton et al. (1965) and Scalbert (1989). In summary, 10 mL of samples (filtered in the case of OMWW from open-air lagoons, as well as samples from subacute toxicity tests) were pipetted into a falcon tube and 1 mL of Folin-Ciocalteu reagent and 1 mL of saturated sodium carbonate solution (Na_2CO_3) were incorporated into each sample. The mixtures were then incubated in the dark for 30 minutes. Following this incubation period, the samples were analysed using a spectrophotometer set to a wavelength of 760 nm.

The blank control was prepared using all reagents combined with distilled water, excluding the sample. A calibration curve was established utilizing gallic acid as a standard reference. The results are expressed in terms of gallic acid equivalents (GAE), mg GAE/L.

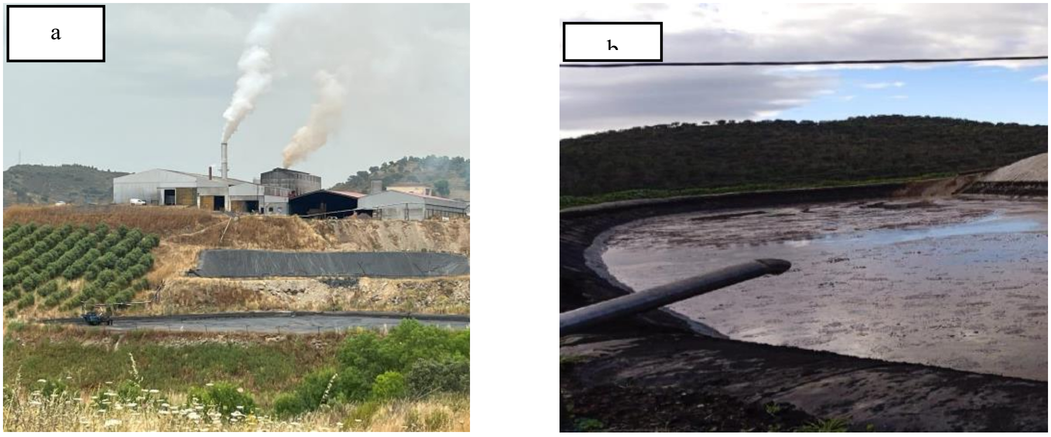

Figure 3.8. Open-air lagoons: (a) Mirabaga company; and (b) entrance tube of OMWW.

3.2.3. Acute toxicity test with Artemia franciscana

Artemia franciscana (former *A. salina*) cysts were obtained from ArtoxKit M (MicroBio Tests, Gent, Belgium). Using a 60 µm mesh, cysts were washed and hydrated for approximately 3 hours in distilled water until they obtained a round shape. The incubation medium was prepared according to Sorgeloos et al. (1986). The hydrated cysts were transferred to an Erlenmeyer flask with 500 mL of incubation water under aeration and lighting (3000 – 4000 lux) at a temperature of 24-26 ºC until hatching in order to obtain instar II-III nauplii (metanauplii).

First, an exploratory test was performed in a range of different concentrations of olive mill wastewater, with winter OMWW sample.

Acute toxicity tests were performed with different OMWW solutions (at least 6 different concentrations *per* test) prepared with incubation medium, in a range of concentrations of 0.2% to 1.4%. The tests were applied to different OMWW seasons, i.e., winter and summer sampling. A stock solution of potassium dichromate was prepared (K2Cr2O7; 1000 mg/L) from which concentrations of 100, 56, 32, 18 and 10 mg/L were diluted with incubation medium and used as assay sensitivity control (negative control). The positive control was incubation medium.

Nauplii were collected, and transferred to the test wells, ensuring an observation group to at least 20 individuals *per* concentration tested. The plates were kept in the dark, at a temperature of 24-26 °C, and the number of immobilized/dead individuals was counted at 24h, 36h, and 48 hours of the test.

Results are expressed as LC_{50}, meaning that the percentage of effluent that immobilized/dead 50% of the *A. franciscana* population exposed, considering data on the percentage of deaths *per* concentration tested, through linear regression of the concentration Log_{10} as a function of the Probit values and presented. Also, the LD50 value was used as mg/L of GAE, taking to account the total phenolic content in OMWW.

3.2.4. Subacute Toxicity Test with *Luciobarbus bocagei*

The sampling of the fish, *Luciobarbus bocagei*, was by electrofishing, according to previously described methods. Before toxicity tests, fish was acclimatised/quarantine in an Aquaculture System (Aquaneering Systems ®) (Figure 3.9), under a 16h/8h photoperiod, constant temperature of 20ºC and fed once a day with pellets, until 48h before bioassays. This is a system of artificial aquariums, composed of 40 tanks of 16L each, with water recirculation system with physical filters, activated carbon, and UV for maintenance of a good water quality, temperature control system, and photoperiod.

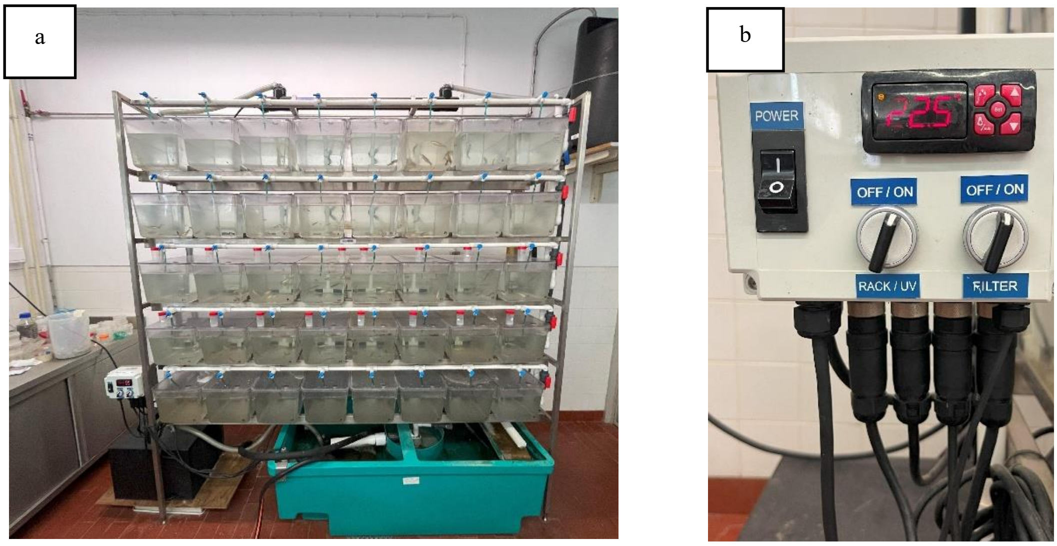

Figure 3.9. Aquaneering System (Aquaculture Laboratory -ESA) used for acclimation of Luciobarbos bocagei: (a) general aspect; (b) water parameter control.

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River water was also collected from Tua river, for toxicity tests with fish. This water was oxygenated by aeration using pumps (aquarium type) until tests.

First, an exploratory subacute test was performed using different concentrations of OMWW. Toxicological tests were performed with *Luciobarbus bocagei*, for 2 days in a static system, without feeding the fish, at room temperature and in present of natural light (photoperiod approximatily16h:8h). After the acclimatization period, at least 6 fish were distributed among the tanks, according to the scheme in figure 3.10, and being exposed for 48 hours. As an experimental criterion, a minimum proportion of 1 L of water *per* fish was adopted. Plastic tanks with a capacity of 32L (work volume of 15L) were prepared with different OMWW concentrations in river water, namely 0.5%, 1.7%, 2.5%, 5%. OMWW used in this test was sampling in summer. Control Tank was the river water.

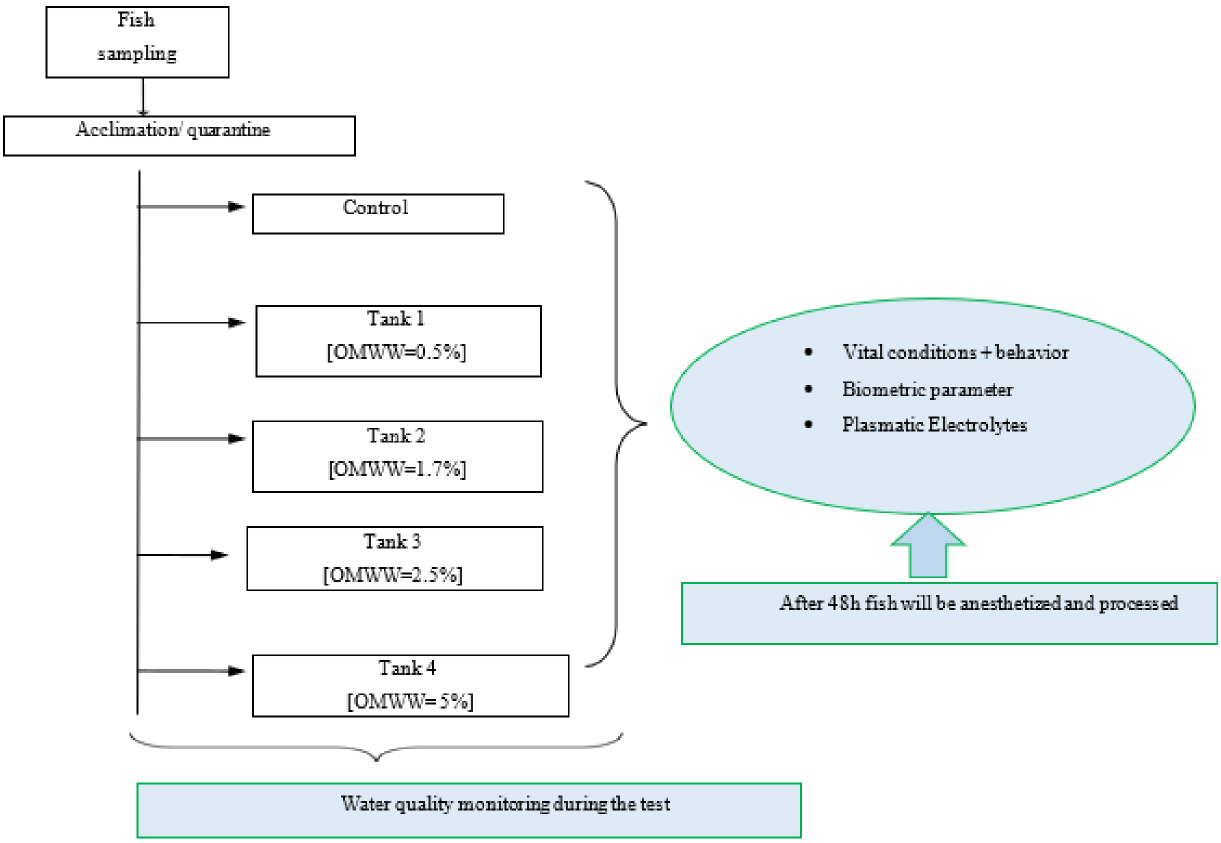


Figure 3.10. Scheme of the exploratory subacute toxicity test developed with summer OMWW using *Luciobarbus bocagei*.

Several parameters were evaluated, namely**:**

Vital conditions and behavior- During the experiments, fish in all tanks were observed, assessing their balance and swimming and respiratory problems. If in a tank there are signs of total absence of movement in fish, or other obvious signs of stress, the test will be terminated and the fish euthanized with anesthetic, and blood sample collected.

Plasmatic Electrolytes - Fish were anesthetized (ether ethylene glycol monophenyl, MERCK) and then blood was drawn from the caudal vessels with heparinized syringes, to Eppendorf tubes (Figure 3.12) subsequently used to determine electrolyte concentrations, namely K^+, Na^+ Ca^{2+}.

Biometric parameters- Fish are weighed and measured to determine their condition factor (*k*), taking into account:

$$k = \text{weight (g)} / (\text{length (cm)})^3 \times 100).$$

Based on the results from the fish behaviour in this exploratory test, the second subacute test was performed with lower concentration OMWW. This test was performed using OMWW sampling in winter (previously, there were no differences in LC_{50} between seasons). Once again, toxicological tests were performed with *Luciobarbus bocagei*, captured and acclimated as described previously. The test was performed over 2 days in a static system, with a controlled laboratory temperature of 21±0.5 ºC, without feeding fish during the test. OMWW concentrations, ranging from 0.5% to 1%, were diluted in river water. As before, control tank was river water.

Here, fish will be exposed for 24 and 48 hours, 12 fish in each tank (work volume of 20L) and vital conditions, fish behaviour, plasmatic electrolytes and biometric parameters was evaluated at two different times: 24 and 48 hours (Figure 3.11).

These subacute toxicity tests with sublethal concentrations of OMWW were approved by the IPB ethics committee.

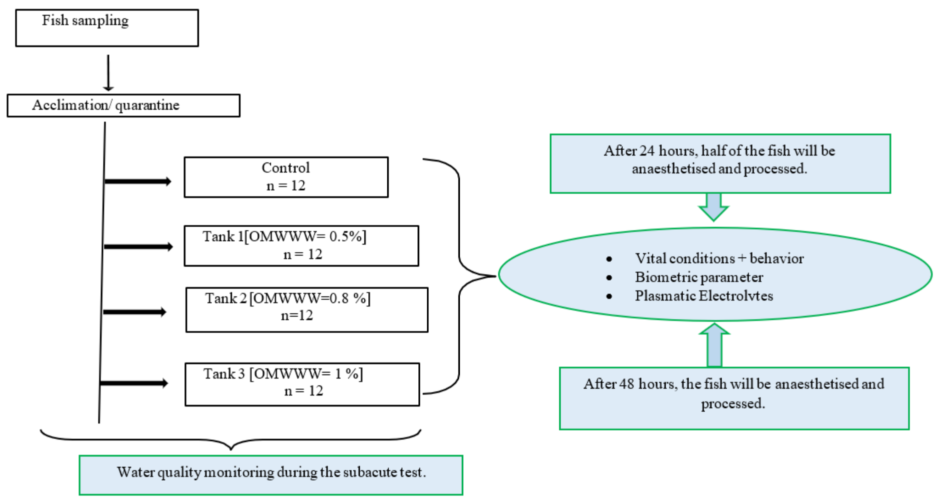


Figure 3.11. Scheme of the subacute toxicity test developed with winter OMWW using *Luciobarbus bocagei* .

3.2.4.1. Electrolytes Quantification

Blood samples were centrifuged at 4 ºC for 15 minutes at a maximum speed of 13,200 rpm. The resulting plasma was diluted with a 5% nitric acid solution and stored at -20 ºC until analysis (Figure 3.12).

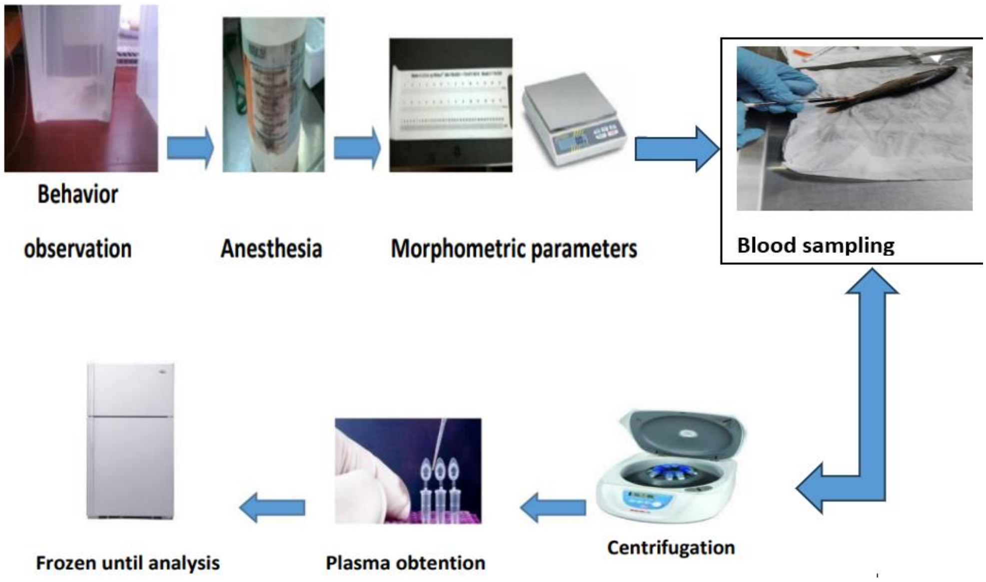


Figure 3.12. Biological collection and sample procedure.

The concentrations of electrolytes, potassium (K^+), sodium (Na^+), and calcium (Ca^{2+}), were determined by atomic absorption spectrometry (PinAAcle900T), using a calibration curve, with results expressed in ppm.

Water quality parameters, during toxicity tests, were monitored, namely pH, temperature and oxygen, using a HACH HQ2200 © multiparameter probe. For the second subacute toxicity test the content of total phenolic compounds in the tanks, was also measured with time of exposure, T0, T24h and T48h, using the mentioned method.

3.3. Data Analyses

Data from biological and ecological assessments of the river Tua was made through univariate and multivariate analysis. For water quality analysis, the data were previously normalised to perform the dbRDA analysis for environment conditions, i.e., to sort the different sites according to the abiotic variables. The 8 sampling sites were previously distributed by disturbance groups for macroinvertebrate and fish abundances and non-metric multidimensional scaling (nMDS) performed. Prior to analysis, data were log transformed [Log (x + 1)] to reduce the influence of abundant taxa and to overcome the unity-sum constraint (Clarke and Gorley, 2015). Analysis of similarity (ANOSIM) was used to assess differences in macroinvertebrate and fish communities between river typology groups. Similarity Percentage (SIMPER) analysis was used to identify the macroinvertebrate and fish taxa with the highest contribution to river typology dissimilarity. The Richness (S), abundance (N), Shannon-Wiener diversity index (H') and the Pielou's evenness (J'), differences among the 3 considered groups for the invertebrate and fish analyses were performed using nonparametric Kruskall-Wallis and Mann-Whitney (for seasons) tests, since normality or homogeneity of variance were not met. The analyses were performed using PRIMER 7 & PERMANOVA+ and STATISTICA 7.

Results of total phenolic compounds and the ones related to fish, were expressed as M ± SD. Firstly, the Shapiro-Wilk test was conducted to assess the normality of the parameters. The parameters of fish exposed to different concentrations of OMWW, at different exposure times (24h and 48h) were compared using ANOVA for length and the Wilcoxon test for total phenolic compounds and fish weight and condition factor (*k*). Wilcox was used to analyze the effects of the different treatments on electrolyte levels, namely Na^+, K^+, and Ca^{2+}. A significant level of 5% was considered. Statistical analyses were performed using RStudio, version 2024.12.0+467 Copyright (C) 2024.

4. RESULTS AND DISCUSSION

4.1. Bioecological Evaluation of Tua River

The OMWW impacts on bioecological metrics measured for Tua River are presented in terms of 1) biota, i.e., benthic invertebrates and fish communities and 2) physical and chemical water quality elements, considering the 3 defined groups.

4.1.1. Biological Quality – Macroinvertebrate communities

The total number of individuals distributed by the 8 sampling sites, for the winter and summer seasons 2024, can be visualized in Figure 4.1. A total of 6,331 individuals of invertebrates were identified in both surveys, belonging to 48 faunistic groups.

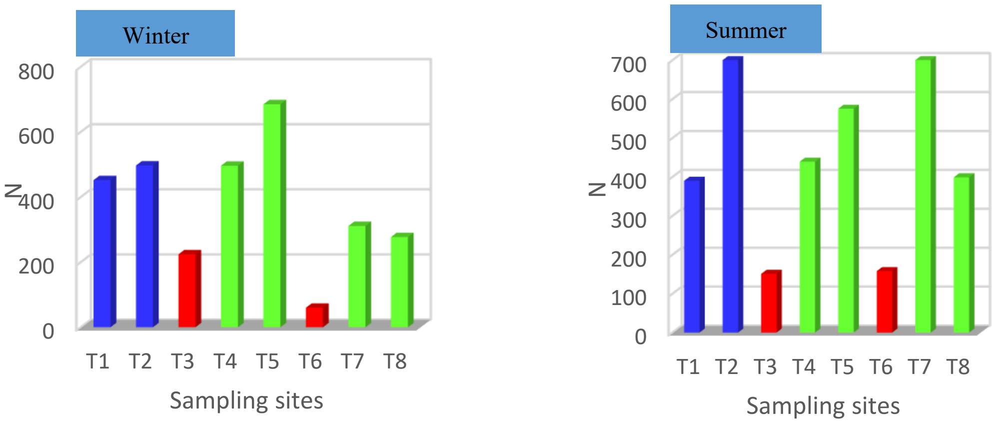


Figure 4.1.Number of individuals in sampling sites of Tua River (winter and summer 2024). Blue bar in the graph represents the low, the red bar represents the high, and the green bar represents the intermediate disturbance.

The most disturbed sampling sites (i.e., T3, T6), i.e., immediately downstream of open lagoons (OMWW), presented the lowest number of individuals (N), ranging between 60 to 224 individuals. The same tendency was observed for the number of taxa (S), ranging from 8 to 25 between high and low disturbed sites, respectively (Figure 4.2).

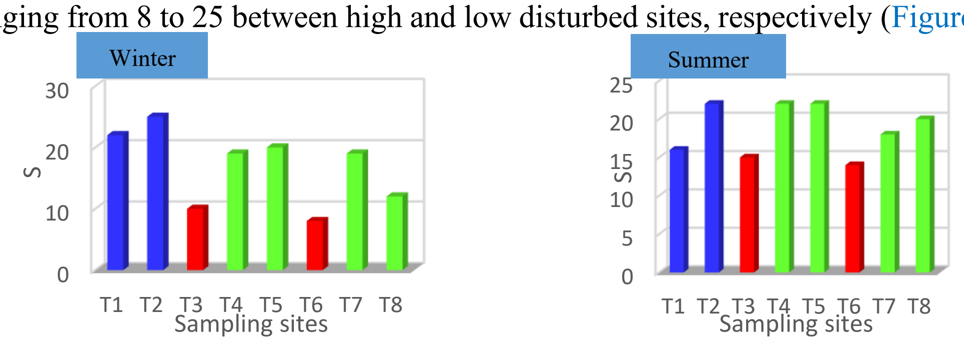


Figure 4.2. Number of taxa in sampling sites of Tua River (winter and summer 2024). Blue bar in the graph represents the low, the red bar represents the high, and the green bar represents the intermediate disturbance.

The variation of alpha-diversity indexes, i.e., Shannon-Wienner diversity (H'), Pielou Equitability (J') are presented in Figure 4.3, considering the 3 defined groups.

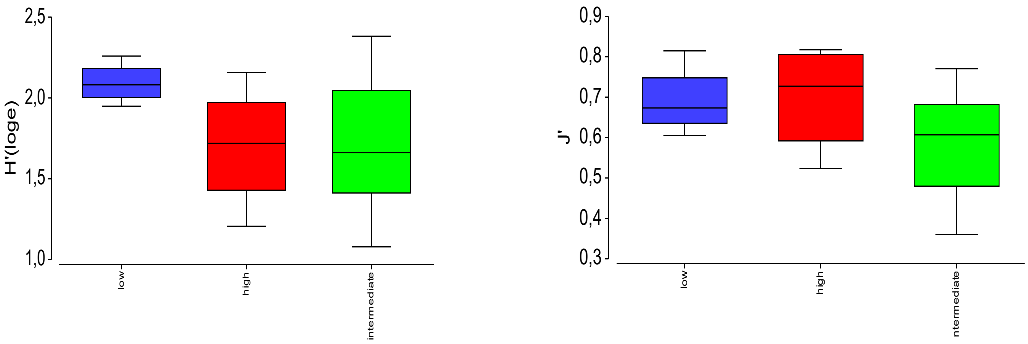


Figure 4.3.Variation of Shannon-Wienner diversity (H') and Pielou evenness (J') Margalef (d) and Fisher indexes (winter and Summer 2024). Blue bar in the graph represents the low, the red bar represents the high, and the green bar represents the intermediate disturbance.

The highest H' Shannon-Wienner index were obtained for the low disturbance zones (T1, T2). No significant differences were detected between seasons (U Mann-Whitney tests, $P > 0.05$) for all metrics (i.e., S, N, H' and J' indexes). However, significant differences (Kruskall-Wallis tests H (2, N=16); $P < 0.05$) were detected among disturbance groups, except for J' evenness ($P > 0.05$).

The faunal composition showed to be sensible to the main impact studied, i.e., water polluted by OMWW. In fact, faunistic groups more sensible, i.e., Plecoptera, Ephemeroptera and Trichoptera Insecta orders, almost disappear from the high disturbed sites (T3, T6), where resistant taxa to pollution dominate, namely Diptera, Oligochaeta, Heteroptera and Crustacea (Figures 4.4 and 4.5).

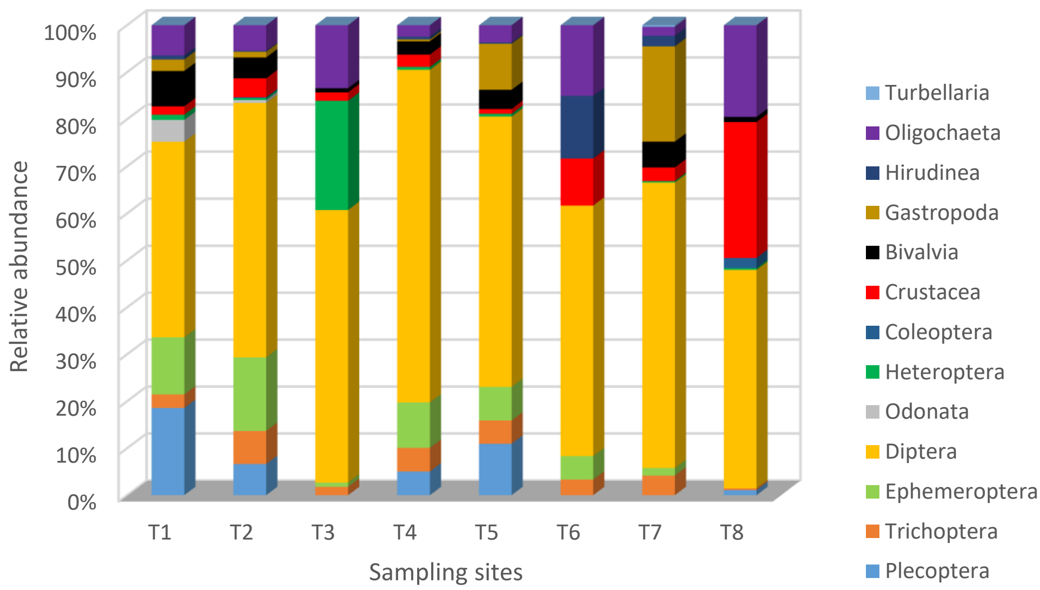


Figure 4.4. Faunal composition of invertebrates in river Tua (Winter 2024).

Several issues can be highlighted, considering the analyses for all sampling sites:

- Dominant families are Chironomidae (40.4%), Simuliidae (8.6%) (Diptera), Baetidae (13.8%) (Ephemeroptera), Oligochaeta (4.9%) Hydropsychidae (3.7%) (Trichoptera) and Atyidae (2.5%) (Crustacea).
- Higher proportion of EPT (Ephemeroptera, Plecoptera, Trichoptera) during summer, suggesting more harsh environmental conditions during winter season.
- Presence of invasive alien species, such as *Procambarus clarkii* (Cambaridae, Crustacea) and *Corbicula fluminea* (Corbiculidae, Bivalvia), increasing their proportion in more disturbed sites.
- Detection of native threatened species in low disturbed sites, such as *Unio delphinus, Potomida littoralis* and *Anodonta anatina* (Unionidae, Bivalvia).

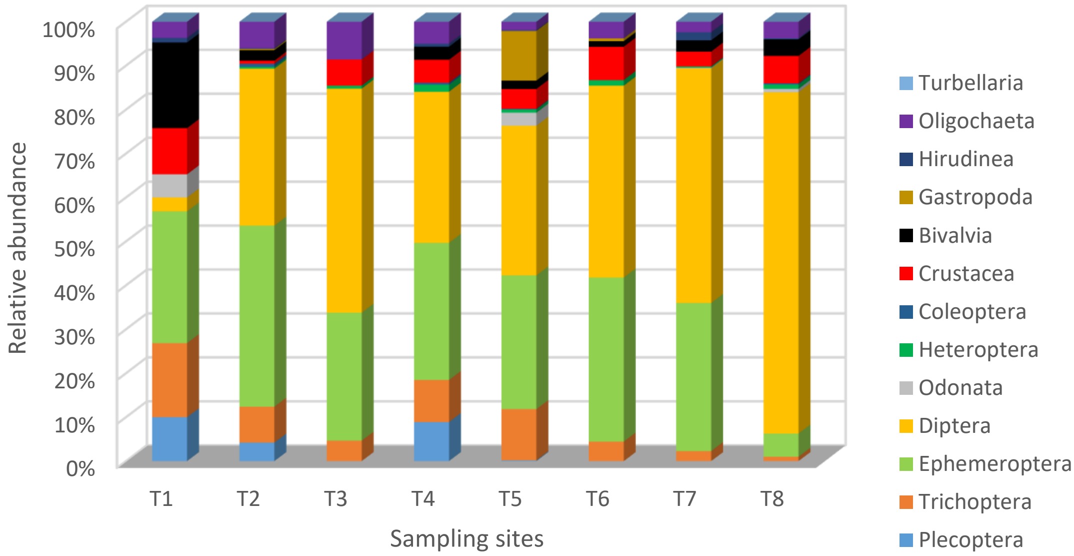


Figure 4.5. Faunal composition of invertebrates in river Tua (Summer 2024).

The biomonitoring results, based on macroinvertebrate communities, showed a strong decrease in terms of abundance, taxa richness and diversity, not only near the release of olive mill wastewater in the Tua River, but also in downstream zones. According with Karaouzas et al. (2014), OMWW, even highly diluted, had dramatic impacts on the aquatic fauna and to the ecological status of the receiving stream ecosystems. In fact, several studies revealed a spatial and temporal structural deterioration of the aquatic and semiaquatic communities due to OMWW pollution and a reduction of the river capacity for reducing the effects of polluting substances through internal mechanisms of self-purification (Karaouzas et al., 2014; Smeti et al., 2019; El Alami & Fattah, 2020).

The results of the official Portuguese IPtI$_N$ index identified a lower biological condition in high and intermediate disturbed sites. In fact, a moderate condition was commonly observed for both seasons, although in winter 2024 the T6 sampling site have been reached a poorest condition (Table 4.1). Karaouzas et al. (2014) found, the same tendency, i.e., upstream sites that were used as control, presented good and high ecological status whereas the ecological status of the sites affected from OMWW pollution ranged from moderate to bad.

Table 4.1.IPtIN: Classification of sampling sites in Tua River (Winter and Summer 2024).

IPtIN	T1	T2	T3	T4	T5	T6	F7	T8
Score	0.709	0.806	0.471	0.747	0.590	0.363	0.533	0.530
Winter	Good	Good	Moderate	Good	Moderate	Poor	Moderate	Moderate
Score	0.611	0.888	0.683	0.867	0.823	0.673	0.635	0.551
Summer	Moderate	Excellent	Moderate	Excellent	Good	Moderate	Moderate	Moderate

The non-metric MDS analysis (2D stress - 0.13, indicator of a good two-dimensional representation), showed, clearly, the separation between seasons (Figure 4.6), and between high and low disturbed sites (Figure 4.7) confirming the effects of OMWW on the spatial and temporal composition of macroinvertebrate communities.

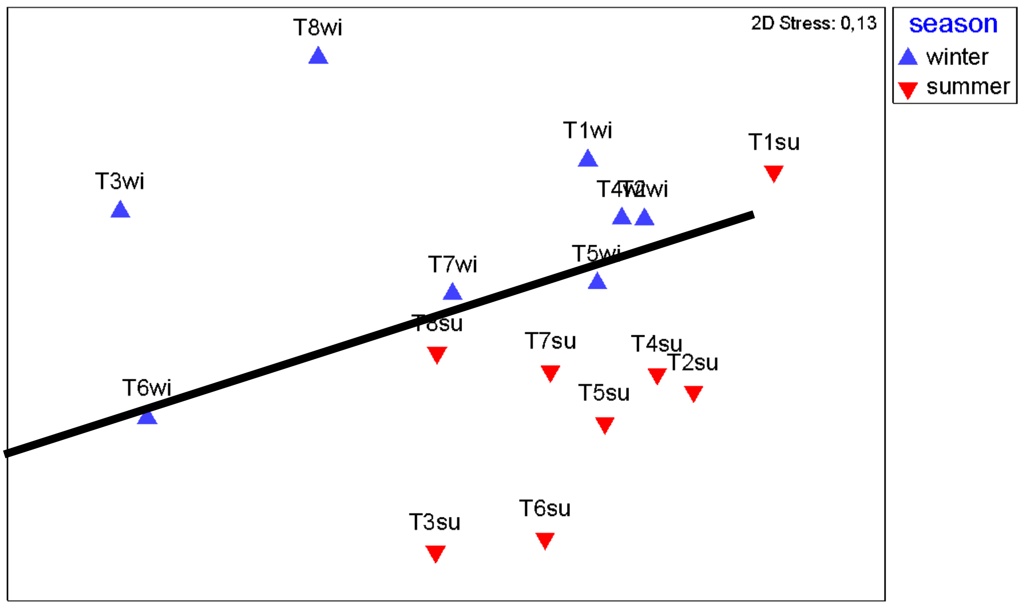


Figure 4.6. Non-MDS ordination of sampling sites, based on invertebrate communities, considering winter and summer 2024.

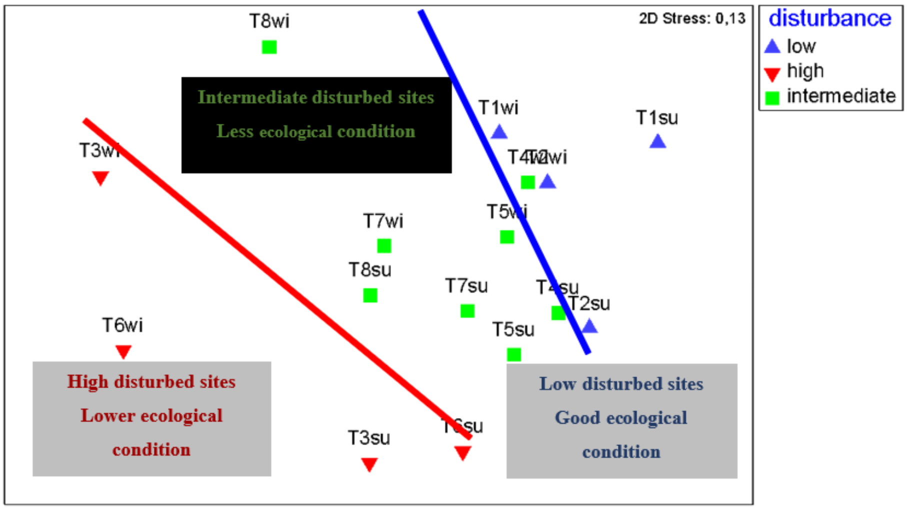


Figure 4.7. Non-MDS ordination of sampling sites, based on invertebrate communities, considering the 3 disturbance groups: low, intermediate and high disturbed sites.

ANOSIM pairwise similarity (one-way) tests identified significant differences (P <0.01) between seasons (Winter vs, Summer) and between **low and high disturbed sites** (T1, T2 vs. T3, T6) and **low and intermediate disturbed sites** (T1, T2 vs T4, T5, F7, T8).

The non-MDS analysis (2D stress - 0.15; good two-dimensional representation) of the macroinvertebrate communities can be observed in Figure 4.8.

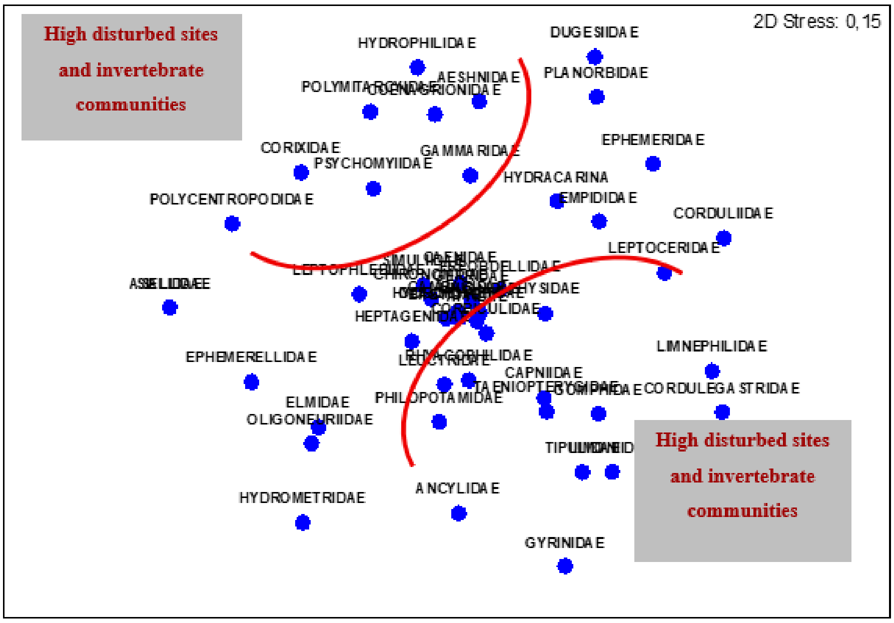


Figure 4.8. nMDS Ordination of macroinvertebrate communities of Tua River.

Analysing the nMDS ordination, it is possible to identify the separation between macroinvertebrates more adapted to degraded conditions, such as Oligochaeta, Corixidae (Heteroptera), Planorbidae (Gastropoda, Mollusca) and also Gammaridae e Cambaridae (Crustacea), in opposition to more stenobiont taxa belonging to Cordulegasteridae (Odonata), Heptageniidae (Ephemeroptera), Limnephilidae (Trichoptera) and Capniidae (Plecoptera) (Figure 4.8). The SIMPER analysis identified the taxa most contributing to dissimilarity between **Low and High disturbed sites** (59.5% average dissimilarity) were Corbiculidae (6.4%), Baetidae (6.2%), Leuctridae (5.3%) and Atyidae (5.2%) while between **Low and Intermediate disturbed sites** (44.4%) were Simuliidae (6.2%), Corbiculidae (5.0%) and Baetidae (5.0%).

4.1.2. Biological Quality – Fish communities

A total of 3,085 individuals distributed by 10 species, 5 native and 5 non-natives, were captured and identified in the 8 sampling sites of Tua River, considering both winter and summer sampling seasons (Figures 4.9 and 4.10).

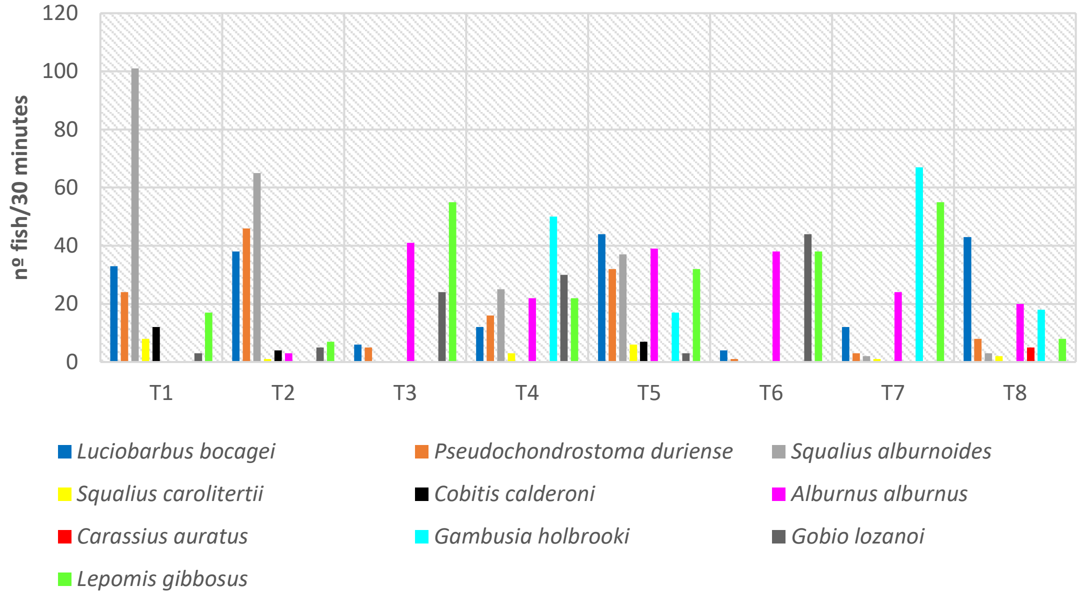


Figure 4.9. Fish fauna: CPUE (nº fish /30 minutes) in River Tua (Winter 2024).

The native fish species, all Iberian endemic species, belonged to the following 3 families: 1) Leuciscidae: *Pseudochondrostoma duriense, Squalius carolitertii, Squalius alburnoides*; 2) Cyprinidae: *Luciobarbus bocagei*; 3) Cobitidae: *Cobitis calderoni*. Non-native species captured belongs to 4 families of distinct sources: 1) Centrarchidae: *Lepomis gibbosus* (North America); 2) Leuciscidae: *Alburnus alburnus* (Europe and Asia); 3) Gobionidae: *Gobio lozanoi* (North Spain and South France) 4) Poecilidae: *Gambusia holbrooki* (North America); and 5) Cyprinidae: *Carassius auratus* (Asia).

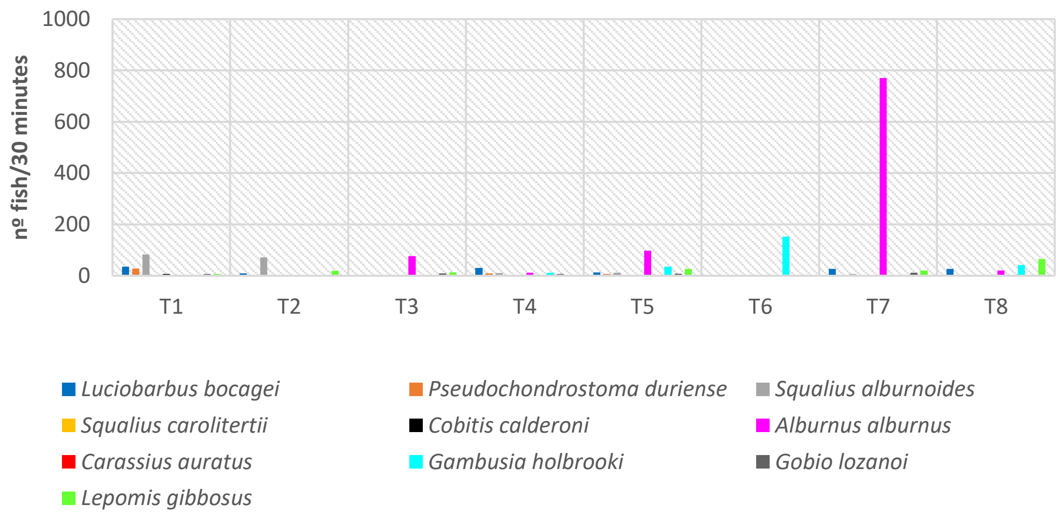


Figure 4.10. Fish fauna: CPUE (nº fish /30 minutes) in River Tua (Summer 2024).

The relative abundance of dominant non-native species (ranged from 21.3% to 78.7% in summer season) is evident in Tua River, except for the two low disturbed sampling sites. In fact, pollution and limnophilic environments favoured the density and biomass of exotic species, more evident near the releasing zone of olive oil wastewaters of both companies, i.e. T3 and T6 sampling sites (Figure 4.11).

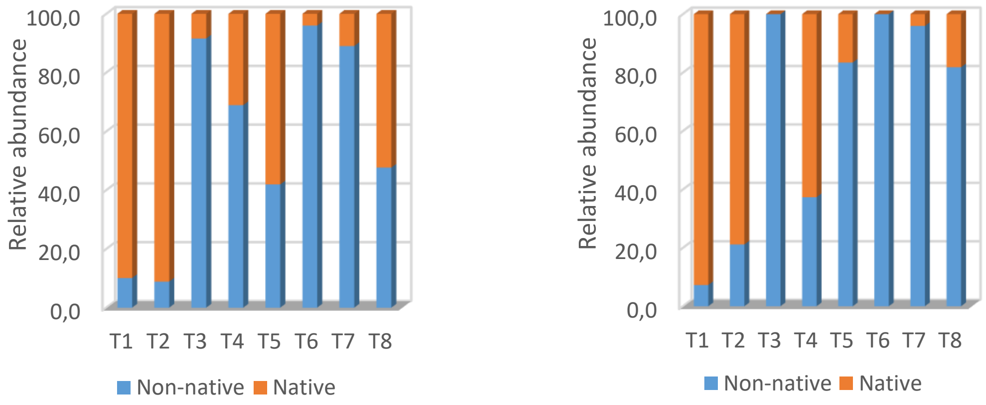


Figure 4.11. Relative abundance in Tua River (Winter and Spring 2024).

The results for the number of individuals (N), number of taxa (S) can be observed in Figure 4.12 in a summarized way, considering the 3 defined groups.

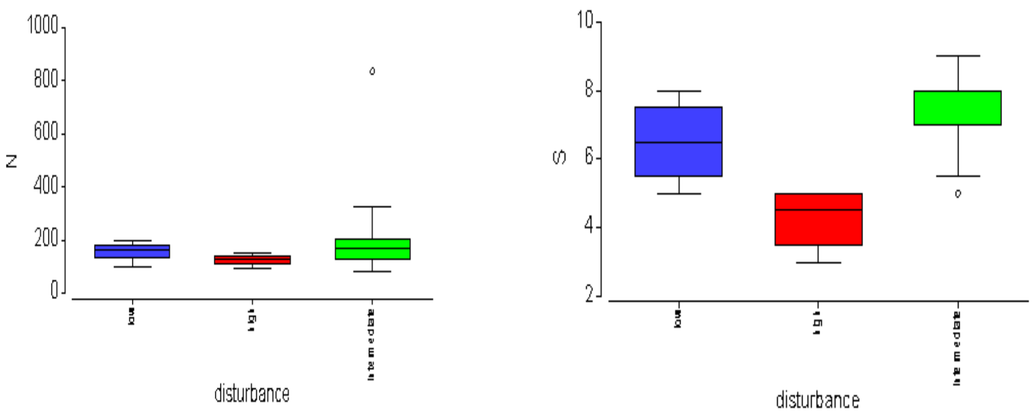


Figure 4.12.Number of individuals (N) and Number of taxa (S), considering the 3 disturbance groups: Low, High and Intermediate (Winter and Summer 2024). Blue bar in the graph represents the low, the red bar represents the high, and the green bar represents the intermediate disturbance.

The alpha-diversity variation of Shannon-Wiener diversity (H’), and evenness Pielou (J’) indexes, considering the 3 defined disturbed groups are presented in Figure 4.13.

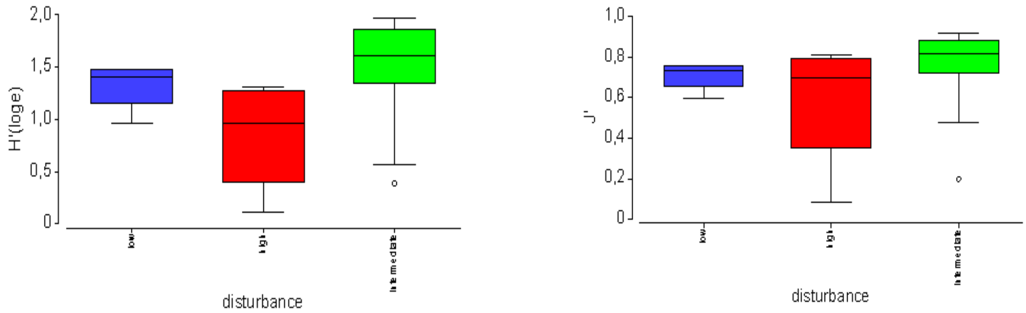


Figure 4.13. Shannon-Wienner diversity (H’) index for fish communities (Summer 2024). Blue bar in the graph represents the low, the red bar represents the high, and the green bar represents the intermediate disturbance.

The lowest diversity, represented by the Shannon-Wienner H’ index was obtained for the High disturbed zones (T3, T6), similarly to invertebrate communities’ tendency. No significant differences were detected between seasons (U Mann-Whitney tests, P > 0.05) for all metrics (i.e., S, N, H’, and J’). However, significant differences (Kruskall-Wallis tests H (2, N=16); P< 0.05) were detected among defined groups for S, and H’ indexes.

Several studies showed an increasing freshwater fish biodiversity loss in worldwide, and in particular in Mediterranean zones due the synergic effects of water scarcity (climate change) pollution, such as from olive oil raw, invasive alien species, habitat fragmentation and degradation, among other pressures from human activities (Clavero et al., 2004; Karaouzas et al., 2018; Dudgeon, 2019; Barbarossa et al., 2020). Indeed, freshwater fish represent 40% of all fish diversity and 25% of all vertebrates (Tedesco et al., 2013) and are one of the most threatened groups of animals worldwide (Clavero et al., 2010).

Relatively to fish biotypology, expressed by the nMDS ordination (2D, stress - 0.06, excellent representation), no separation was detected for seasonality (Figure 4.14).

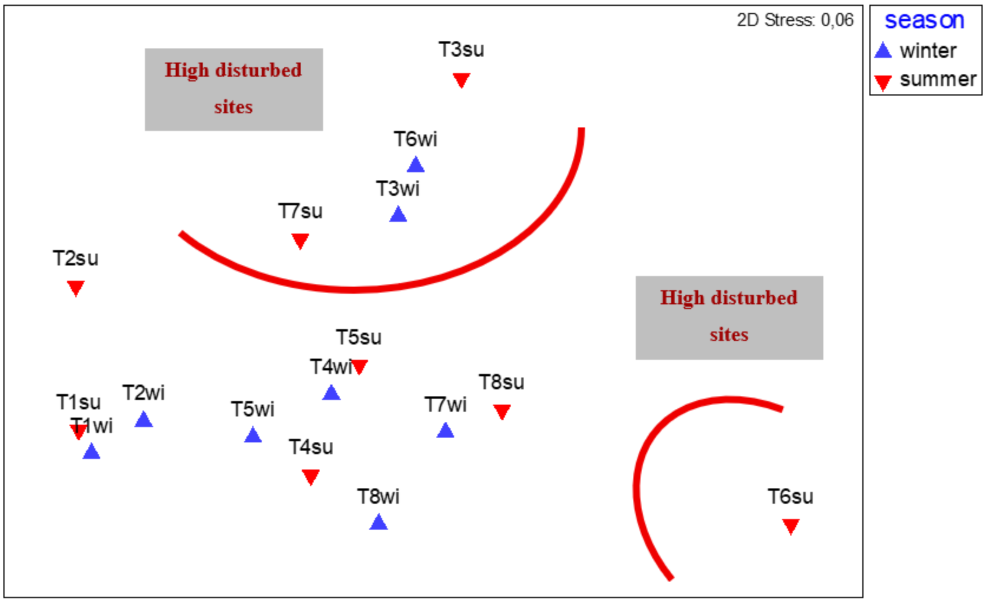


Figure 4.14.Non-MDS ordination of sampling sites, based on fish communities, considering winter and summer seasons 2024.

However, the same pattern for disturbance was observed for fish communities comparing to other biological communities, i.e. macroinvertebrates. In fact, the organization of fish communities seemed to be highly influenced by the olive oil wastewaters (Figure 4.15).

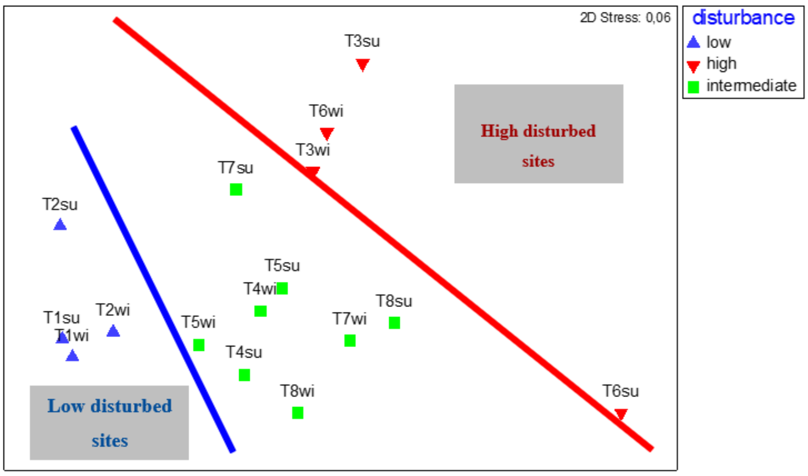


Figure 4.15. Non-MDS ordination of sampling sites, based on fish communities, considering winter and summer seasons 2024.

The ANOSIM pairwise similarity (one-way) tests did not detect significant differences (P >0.05) between seasons (Winter vs. Summer). However, significant differences (P< 0.01) were found between all groups (low, intermediate and high disturbed sites).

The non-MDS analysis (2D stress - 0.03; excellent two-dimensional representation) of the fish communities can be observed in Figure 4.16. Some differentiation was found between species colonizing the Tua River in zones of good ecological condition (such as T1 and T2), represented by *Cobitis calderoni* and *Squalius carolitertii* in oosition to more impacted zones (such as T3 and T6), represented for example for high density of *Lepomis gibbosus, Alburnus alburnus* and *Gambusia holbrooki*.

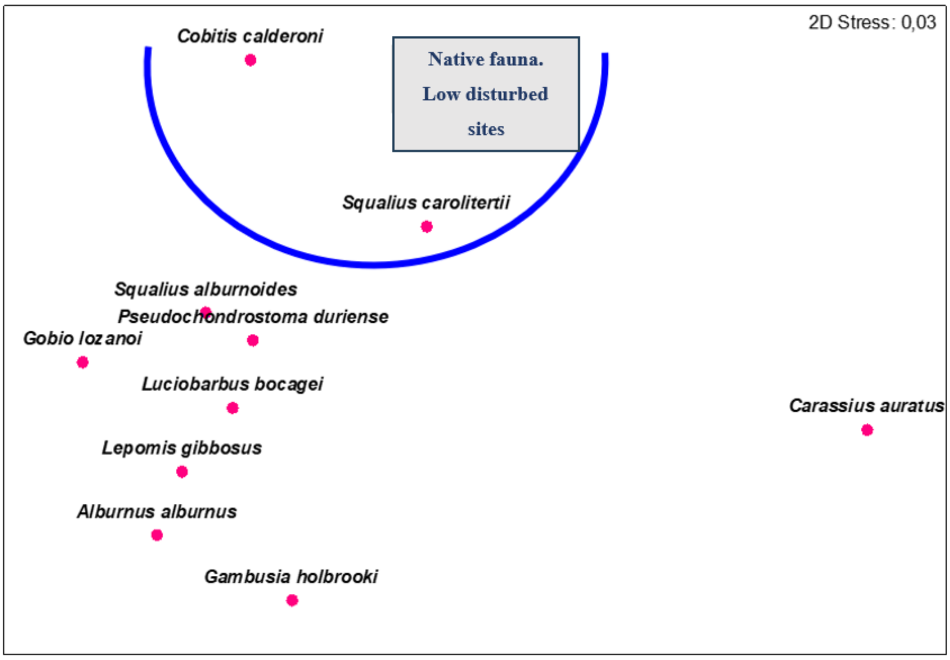


Figure 4.16.Non-MDS ordination of macroinvertebrate communities, considering the 3 disturbance groups: low, intermediate and high disturbed sites. Blue bar in the graph represents the low, the red bar represents the high, and the green bar represents the intermediate disturbance.

The SIMPER analysis identified the taxa most contributing to dissimilarity between **Low and High disturbed sites** (67.5% average dissimilarity) were *S. alburnoides* (24.1%), *A. alburnus* (15.3%), *L. bocagei* (13.3%), and *P. duriense* (12.1%) while between **Low and Intermediate disturbed sites** (42.9%) were *A. alburnus* (21.7%), *G. holbrooki* (19.0%), *S. alburnoides* (15.5%), and *P. duriense* (10.8%). Finally, between **Intermediate and High disturbed sites** (42.9%) the fish species which most contributed to the observed dissimilarity (47.9%) were *G. holbrooki* (18.7%), *L. bocagei* (16.4%), *S. alburnoides* (13.3%), G. lozanoi (11.5%) and *A. alburnus* (11.2%).

The evaluation of the biological quality of the fish fauna, based on the Biotic Integrity Fish Index for Wadable Rivers of Portugal (F-IBIP), can be observed in Table 4.2.

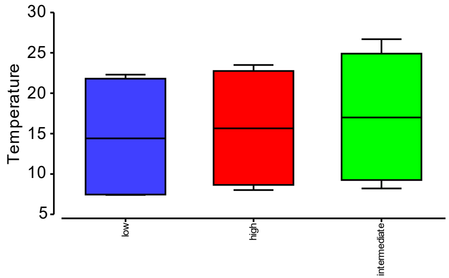
Table 4.2.F-IBIP: Classification of for sampling sites in Tua River (Winter and Summer 2024).

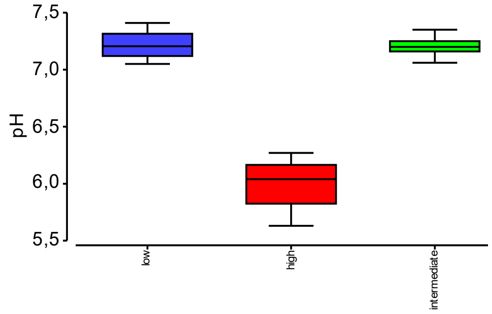
F-IBIP	T1	T2	T3	T4	T5	T6	T7	T8
Score	0.888	0.901	0.000	0.556	0.667	0.000	0.556	0.556
Winter	Excellent	Excellent	Bad	Moderate	Moderate	Bad	Moderate	Moderate
Score	0.808	0.376	0.000	0.556	0.556	0.000	0.556	0.000
Summer	Good	Poor	Bad	Moderate	Moderate	Bad	Moderate	Bad

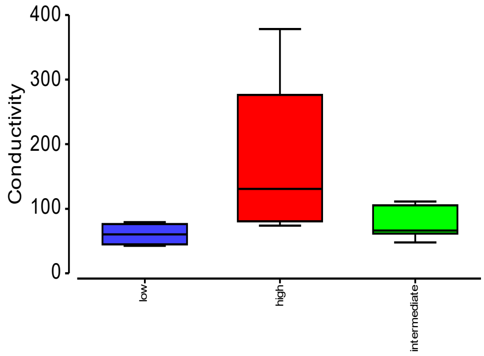
The Bad fish quality classification obtained through F-IBIP was found in high disturbed sites (T3, T6) and is strongly influenced for the worst water and the dominance of non-native species. In fact, native fishes are more vulnerable to environmental stressors. Adakole (2011) found symptoms of toxicity observed in the fish include various behavioural responses like rapid and erratic swimming, uncoordinated movement, vertical darting, with occasional agitated movement, swimming alternately on the lateral and ventral sides and rapid opercula movement. Prior to mortality recorded at some concentrations, there was negative thigmotropism with prolonged gaping of jaws. The toxic action of the oil mill company wastewater appeared to be a joint consequence of precipitation of mucus on the gills and stress.

4.1.3. Environmental influence: Physical and chemical water quality

The water quality of the 3 disturbance groups: Low (T1, T2), High (T3, T6), a intermediate (T4, T5, T6, T8) of River Tua can be observed in Figures 4.17 and 4.18.







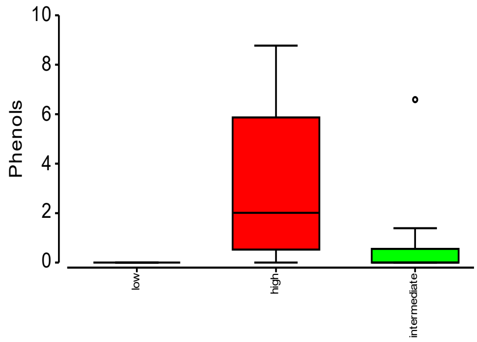


Figure 4.17. Temperature (ºC), pH, conductivity (µS/cm), phenols variations (mg AGAE/L), considering the 3 defined groups, in the Tua River (winter and summer 2024). Blue bar in the graph represents the low, the red bar represents the high, and the green bar represents the intermediate disturbance.

The impact of olive mill wastewaters on the water quality (i.e., temperature, pH, conductivity, phenols, DO and BDO_5, oxidability, acidity, nitrates and phosphates) can be identified by the high content of phenols and reduction of pH and dissolved oxygen.

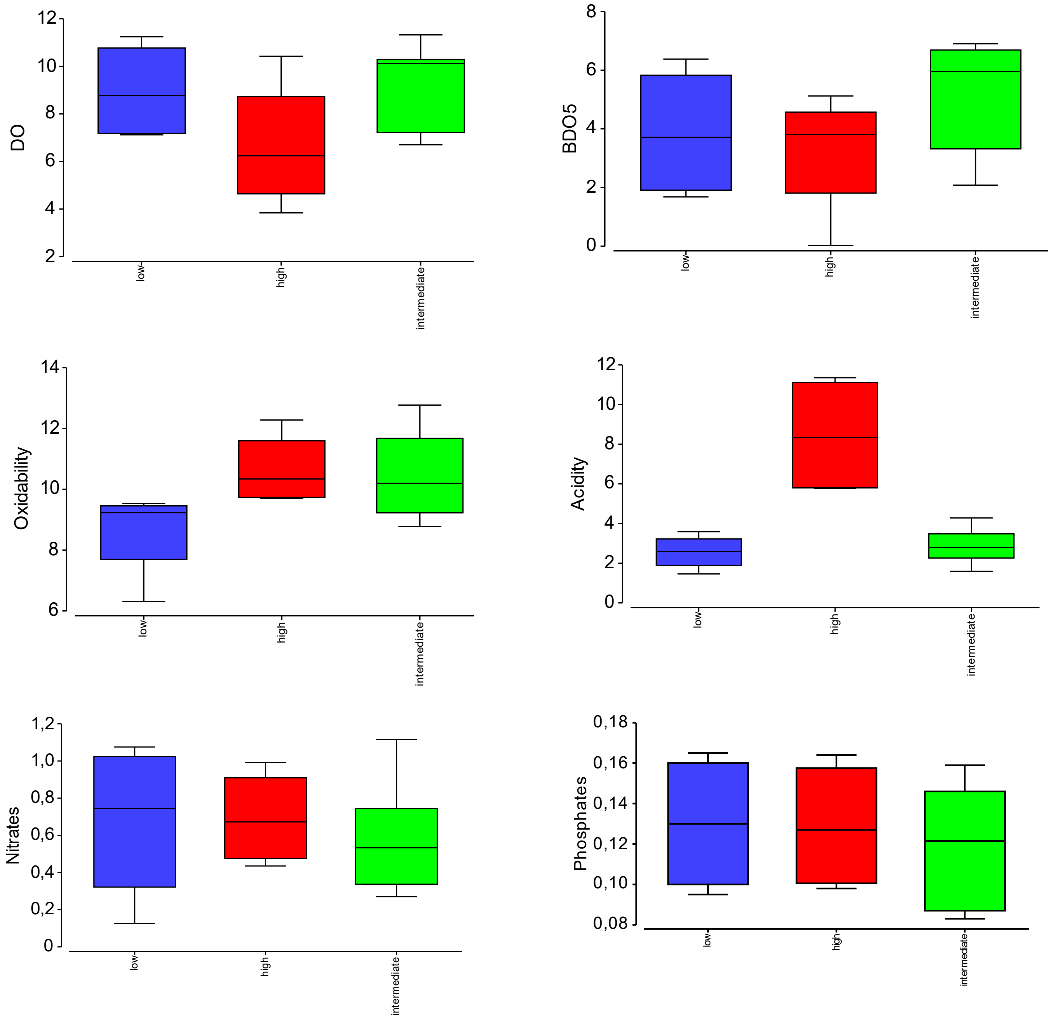


Figure 4.18.Dissolved oxygen (mg O2/L), BDO5, oxidability (mg O2/L), total acidity (mg HCO3-/L), nitrates (mg NO3-/L) and phosphates (mg PO43-/L) in Tua River (winter and summer 2024). Blue bar in the graph represents the low, the red bar represents the high, and the green bar represents the intermediate disturbance.

No substantial differences were observed for nutrients, i.e. nitrates and phosphates, while oxidability increased downstream of the releasing point of olive mill wastewaters from both companies (i.e., T3 and T6, high disturbed sites). Significant differences (Kruskall-Wallis tests H (2, N=16); $P < 0.05$) were detected, among the 3 defined groups, namely for the variables of pH, conductivity, phenols, DO, BDO_5 and total acidity.

The dbRDA analysis (72,8% of total variation explained by the first 2 axes) showed a spatial and temporal segregation between low (T1, T2) and intermediate (T4, T5, T7, T8) relatively to high (T3, T6) disturbed sites. The decrease of water quality must be highlighted near OMWW releases (i.e., T3, T6), and conductivity, nitrates, phosphates acidity and phenols, were the main variables justifying the discrimination between highly disturbed sites (T3, T6, both seasons) and the remain sampling sites (Figure 4.19).

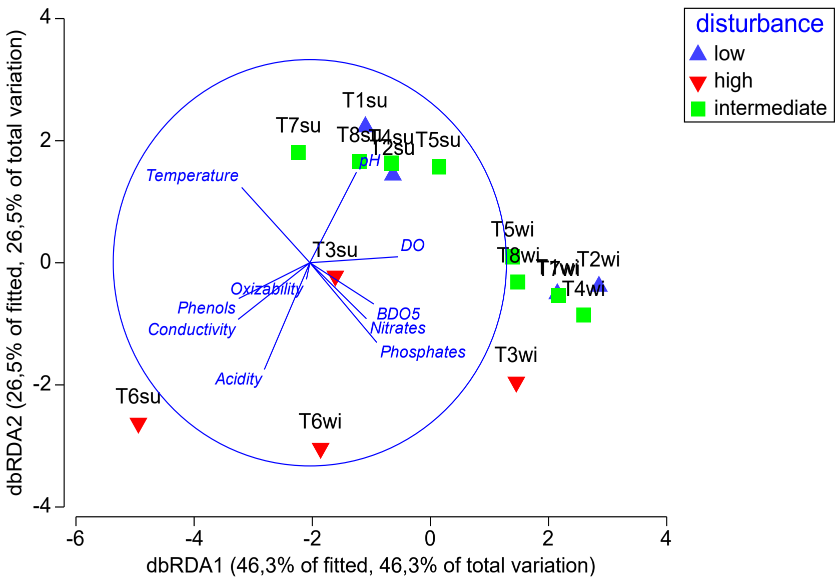


Figure 4.19. Distance-based redundancy analysis (dbRDA) for abiotic variables and the 3 defined typologies considering the 8 sampling sites and 2 season periods (Winter and Summer 2024).

The uncontrolled disposal and treatment of OMWW has been also reported in other studies and is the cause of severe environmental water contamination, due to the high organic COD, BDO_5 and Total Suspended Solids (TSS) concentrations and phenolic compounds, and a decrease in pH, inhibiting the microbial growth, and so their degradation (Basheer et al., 2004; Khdair and Abu-Rumman, 2017; Fedorov et al., 2022) justifying the development of measures to reduce the environmental impact (Khatib et al., 2009; Gagol et al., 2020; Al Bsoul et al., 2020; Vaz et al., 2024).

4.2. Toxicological Evaluation of OMWW from Open-air lagoons

4.2.1. Acute toxicity test with *Artemia fransciscana*

The OMWW was characterized by determining total phenol compounds (TPC), and pH, and results from two different sampling seasons from open-air lagoons are in Table 4.3. Results showed that total phenolic compounds in summer are higher than winter. This indicates the importance of considering seasonal variations in assessments. This variability can be attributed to differences in climatic conditions, olive cultivar varieties, fruit ripeness, and the methodology used for oil extraction, as discussed by Khdair et al. (2019). Also, other factors such as dilution by rain, oxidation from air and light exposition can lead to a decrease on TPC in winter. Nevertheless, TPC of both summer and winter OMWW samples are higher than the value of 10.82 ± 0.11 mg GAE/mL reported by

Gueboudji et al. (2022) in OMWW from olive oil extraction from Khenchelain region, eastern Algeria. Also, Shabir et al. (2022) reported a total phenolic content (TPC) of 8.650 mg GAE/L on OMWW generated from a two-phase olive oil extraction process in Islamabad. This value is greater than those reported for TPC in both seasonal samples analyzed in the current study. The summer sample showed pH value of 4.62 and winter sample with a higher value of 5.57. According, OMWW has an acidic pH ranging from 3 to 6, depending on extraction method (Niaunakis and Halvadakis, 2004; Azzam et al. 2015). In this study, the pH values of both OMWW samples also fell within this range.

Table 4.3. Total Phenolic compound (TPC) of OMWW in two different seasons (M±SD)

Sample	[TPC] mg GAE/L	pH
Summer (n =3)	159.01 ± 18.34^{a}	4.62 ± 0.2^{a}
Winter (n=3)	97.78 ± 2.20^{b}	5.57 ± 0.1^{b}

*Different letters in column indicate differences in mean values.

Acute toxicity tests on *Artemia franciscana* were performed in the dark at temperatures between 24 and 26 ºC, using at least 150 individuals *per* test, in a total of two different tests *per* season sample. The LC_{50} (%) values obtained after 24, 36, and 48 hours of exposure are presented in Table 4.4. According to OECD (2004) and Kalcíková et al. (2012), LC_{50} is the percentage of contaminant that immobilized/dead 50% of the *A. fransciscana* population exposed and the lower LC_{50} values obtained here demonstrate an impact of OMWW due to the higher toxicity. No significant differences in LC_{50} values for 24h of exposition between summer and winter OMWW samples were observed, neither between 24 hours of summer OMWW and 36 hours of exposure of winter OMWW samples. Considering there was no difference between winter OMWW samples at 24 and 36 hours, we extended exposure time to 48 hours for summer OMWW samples, and, in this case, differences were detected. Nevertheless, all the samples (winter and summer OMWW) remained toxic, even after 48 hours of exposure. In fact, both samples, regardless of the seasons, according to the scale proposed by Clarkson et al. (2004), are classified as extremely toxic, since they match as substances within a range of $LC_{50} \leq 100$ µg/mL.

Table 4.4. LC_{50} values of winter and summer OMWW samples (M±SD)

Samples	LC_{50} %	LC_{50}% (M±SD)	
Winter - 24h	3.96	$3.47±0.68^a$	Classified as extremely toxic (Clarkson et al. 2004)
	2.99		
Summer -24h	2.06	$2.15±0.12^a$	
	2.23		
Winter - 36 h	3.22	$3.17±0.07^a$	
	3.12		
Summer- 48h	4.76	$4.47±0.42^b$	
	4.17		

*Different letters in the same row and column indicate differences in mean values.

A study conducted by Elnabris (2014), concerning toxicity evaluation of OMWW includes sampling at different times, during 2012–2013 extraction seasons. The olive season in the Gaza Strip starts in early October and ends in late December. The OMWW was tested using four marine invertebrate species, including *Artemia salina*, *Balanus Amphitrite* (Acorn barnacle), Mytilus *sp.* (Mollusca, mussel) and *Brachionus plicatilis (*Rotifera, rotifer), and results showed similar values as the present work. In fact, LC_{50}, 24h, was approximately 5%, for *Artemia salina*, whereas here LC_{50}, for 24h, values range between 2.06% and 3.96%, (Table 4.4)**.** Also, Karaouzas et al. (2010) evaluated the toxicity of OMWW on two species, the amphipod *Gammarus pulex* (Amphipoda, Crustacea) and the caddisfly larvae of *Hydropsyche peristerica* (Hydropsychidae, Trichoptera). They reported LC_{50} values (24h) ranging from 2.64% to 3.36% for *G. pulex* and from 3.62% to 3.88% for *H. peristerica*. Once again, these values are not different than the ones obtained in the present study.

Toxicity of winter OMWW samples is due to recent post-harvest oil production and resuspension of unconsolidated sediments caused by harsh winters (Li et al., 2007). In turn, summer OMWW samples were expected to be also toxic, since prolonged storage can result in the formation of partially dried and toxic sediments or sludge (Kavvadias et al., 2017), leading to potentially pollutant compounds concentration, in addition to a higher pollutants concentration in the water. In this sense, concentration of total phenolic compounds is higher in summer than winter (Table 4.3).

Although two-phase extraction systems are now the prevailing trend in olive oil mills in Portugal, the open-air lagoons receive OMWW from both, two-phase and three-phase extraction processes. Additionally, OMWW from the three-phase extraction system is more toxic than the two-phase system. The toxicity of OMWW, besides oil extraction method, is also influenced by various factors, including the profile of the olive cultivar and the prevailing climatic conditions, all of which contribute to variations in its chemical composition and environmental impact (Khdair et al., 2019).

In turn, the effects of a toxic substance, in this case OMWW, depends on several factors, such as duration of exposure, concentration and composition of toxicant, temperature, type of organisms tested (Rozman and Doull 1998; Rozman et al 2000). Temperature can influence the toxicity of a substance, either increasing or decreasing it, but generally increasing temperature increase toxicity. Nevertheless, some effluents become more toxic at certain temperatures, while others become less toxic or maintain the same level of toxicity (Hooper et al 2013; Weston et al 2009; Zakrzewski (1919).

Results expressed as LD_{50} (Lethal Dose 50%), in mg GAE/L, are shown in Figure 4.20. There are no differences between values of LD_{50} for 24h of exposition, but when time of exposition is increased to 48h, for the summer samples, it was observed difference, in line with LC_{50} trends. Gueboudji et al. (2022) assessed the LD_{50} values of OMWW from olive oil extraction from Khenchelain region, Argelia, using *Artemia salina*, after 24 hours of exposure. They expressed also the LD_{50} in total phenolic content and reported a value of 23.72 ± 0.1 µg/mL, which is higher than those observed in this study, for both summer and winter samples (Table 4.5). The lower LD_{50} values in this study indicate that both, summer and winter OMWW samples, are more toxic.

Table 4.5. LD50 values of winter and summer OMWW samples (mean ± SD)

Samples	LD_{50} (GAE mg/L)	LD_{50} (GAE mg/L) (M±SD)
Winter-24h	3.86	$3.41±0.19^{a}$
	2.92	
Summer-24h	3.27	$3.39±0.66^{a}$
	3.54	
Winter-36h	4.65	$4.36±0.40^{a}$
	4.07	
Summer-48h	7.57	$7.09±0.66^{b}$
	6.31	

*Different letters in the same row and column indicate differences in mean values.

4.2.2. Subacute Toxicity Test with Fish

Based on the LC_{50} values obtained for OMWW samples tested on *A. franciscana*, a subacute exploratory test on fish was performed. Fish are more complex organisms and more resilient to adverse environmental conditions and pollutants than *A. franciscana*. Therefore, a concentration range between 0.5% to 5%, was tested. Also, no differences in LC_{50} values were observed between winter and summer samples, therefore, tests were performed using summer OMWW.

In the first 12 hours, the behavior of the fish in all tanks was observed, and it was noticed that fish in Tanks 2 (1.7%), 3 (2.5%), and 4 (5%), with high concentrations of OMWW, exhibited typical acute stress behaviours. For ethical reasons, these fish were euthanized with an anesthetic, but it was impossible to collect blood. Based on these results, from exploratory test, it was decided to conduct the second subacute test with lower concentrations and use summer OMWW.

Nevertheless, experiment in tank 1 (0.5%) and control tank, was pursuit. Fish were collected after 48 hours of exposure, and their measurements and weights were taken to determine biometric parameters. Following this, blood samples were collected to analyse concentrations of plasmatic electrolytes. Moreover, results obtained in this exploratory subacute test demonstrated that summer samples were very toxic for the fish under study.

During this test water quality was assessed and oxygen, temperature, and pH are present in table 4.6. Regarding pH levels, both the control tank and tank 1 remained within the range recommended by OECD (1992), which is between pH 6.0 and 8.5. The remaining tanks, however, exhibited pH values below 6.0. This decrease in pH is attributed to the increasing concentration of OMWW. Oxygen saturation levels exceeded the reference value of 80%, established by OECD (1992), in all test tanks, including the control. This indicates that the fish were not exposed to hypoxic stress, thereby maintaining the integrity of the exploratory subacute test with sublethal concentrations of OMWW.

Table 4.6. Water quality in tanks test during the exploratory subacute test

Tanks	TºC	O2 %	pH
Control (River water)	19.5	103.1	7.13
Tank 1 [OMWW=0.5%]	17.8	104.5	6.68
Tank 2 [OMWW=1.67%]	16.2	104.4	4.41
Tank 3 [OMWW=2.5 %]	16.1	107	4.23
Tank 4 [OMWW=5%]	15.6	96.3	3.94

The subacute toxicity test, as previously mentioned, was conducted using lower OMWW concentrations and winter OMWW sampling. The experiment was carried out over a two-day period in a static system maintained at a controlled temperature of 21±0.5 ºC and OMWW concentrations ranging from 0.5% to 1%. As before, a control tank containing only river water was also included. In an attempt to assess time of exposure, here, fish were exposed for 24 and 48 hours. At both times, vital signs, behavioral responses, plasma electrolyte levels, and biometric parameters were evaluated.

During this toxicity tests, water samples were collected from each test tank, placed in plastic bottles, and stored for later determination of total phenols. As expected, it was observed differences in TPC levels among tanks 1, tank 2, and tank 3 at initial time, i.e, an increase with concentration of OMWW, as well as after 24 hours and 48h hours of exposure. A decrease in TPC levels was observed in each tank with time of exposition (Table 4.7). This fact probably leads to the absence of differences among TPC levels between tank 1 and tank 2 after 24 and 48 hours of exposure. This decrease could be associated with oxidation, resulting from agitation, and photodegradation, due to photoperiod and natural light exposition in the tanks. Silva (2014) conducted a study on bioremediation OMWW using microalgae *Chlorella vulgaris* in agitated and aerated reactors, under a controlled photoperiod. The author was observed that agitation/aeration, combined with the photoperiod, effectively reduced concentration of total phenolic compounds. In fact, it was observed a decrease in phenolic compounds in samples when exposed to light intensity of 4500 lux (Gro-Lux fluorescent lamps) and 16:8 h light: dark. Aeration includes a filtration system, comprising sterile filtration units of 22 µm and 50 mm diameter (Millex ™). This reduction was associated with the oxidation of phenolic compounds in the presence of oxygen (due to agitation) and photodegradation (related to the photoperiod and natural light exposure). Veneziani et al. (2023) analyzed effect of different concentrations of oxygen, added during olive crushing process, on reducing the concentration of phenolic compounds in olive oil. In the same study, authors observed that, in the presence of oxygen, concentrations of phenolic compounds decrease, which suggests that phenolic compounds oxidize.

Table 4.7. Monitoring of total phenol concentrations in water tanks (mg/mL) from subacute test with winter OMWW samples (n=3; M±SD).

	Total Phenolic Content (TPC mg/L)		
Time (hours)	**T1 [OMWW=0.5%]**	**T2 [OMWW=0.8%]**	**T3 [OMWW=1%]**
Initial (0 h)	15.43±1.40^{a}	29.87±1.78^{c}	48.82±7.79^{e}
t1 (24 h)	8.83 ±0.32^{b}	15.18±1.93^{d}	21.53±2.91^{c,d}
t2 (48 h)	6.59±1.40^{b}	8.27 ±0.55^{d}	---

*Different letters in same columns and row correspond to different values.

The oxygen, temperature, electrical conductivity, and pH, in each tank is shown in Table 4.8. Regarding pH, once again the values observed in this study fell within the range recommended by the OECD (1992), between 6.0 and 8.5. The pH values in the control tank and tank 1 remained around 7.5, within the recommended limits, ensuring suitable conditions for the fish. However, in tanks 2 and 3, the pH dropped below 6.0 due to the increased concentration of OMWW. The oxygen saturation levels in all test tanks, including the control, remained above 80%, in accordance with the guidelines set by the OECD (1992).

Table 4.8. Water quality in tanks test during the subacute test (M±SD).

Samples	T (C°)	O2 %	EC (µS/cm)	pH
Control	19.33±0.59	95.07±6.84	62.80±19.13	6.92±0.81
Tank1 (0.5%)	19.13±0.32	99.3±2.40	91.40±2.96	6.52±1.32
Tank2 (0.8 %)	19.20±0.29	93.50±0.71	117.03±9.15	5.85±0.85
Tank3 (1.0%)	19.25±0.35	99.70±0.70	166.45±25.53	5.33±1.17
Range	**18.90 – 19.40**	**80-99.70**	**60-167**	**5 -7**

Regarding electrical conductivity (EC), the control tank, along with tanks 1 and 2, recorded mean EC values ranging from 62.80 to 117 µS/cm, while tank 3 exhibited a mean higher EC value of 166 µS/cm. According to Giraldo-Buitrago (2022), river waters with EC values below 121 µS/cm are considered optimal for aquatic species. These results suggest that only the control tank, as well as tanks 1 and 2, fall within the ideal EC range. It is expectable a direct relationship between OMWW increase in tanks and EC values. Regarding temperature, the average ranged from 19.13 ± 0.32 ºC to 19.33 ± 0.59 ºC in tanks 1, 2, 3, and the control. According to Baras (1995), temperatures between 10ºC and 20ºC are optimal for barbels during the summer, promoting their comfort and overall health. Moreover, Souchone and Tissot (2012) estimated the optimal temperature for *Luciobarbus bocagei* to range between 14 and 24 ºC. These results suggest that the temperature during the toxicity test closely matched their natural habitat.

Concerning the biometric parameter, namely condition factor (*k*), no significant differences were observed between the fish in the control tank and those in tanks 1, 2, and 3 after 24 hours of exposure, as well as in tanks 1 and 2 after 48 hours. These results suggest that different concentrations of OMWW did not affect the condition factor of fish, regardless of the duration of exposure (Table 4.9). According to Bernet and Segner (2004), a *k* value between 0.8 and 1.2 is indicative of a normal physiological status, suggesting that the fish are not experiencing significant stress, malnutrition, or pathological alterations. In this study, the *k* values obtained ranged from 0.91 to 1.00, indicating that the fish were in good health before the subacute exposure, with no evident deviation from normal condition after exposure.

Table 4.9. Biometric parameter of the *Luciobarbus bocagei* in the subacute toxicity test with winter OMWW (M±SD).

	Weigth(g)		**Length (cm)**		**k**	
	24h	48h	24h	48h	24h	48h
Control	32.3±10.65^a	32.6±8.72^a	13.5±1.32^a	13.3±6.32^a	0.95±0.04^a	0.96±1.10^a
Tank1 [OMWW=0.5%]	24.16±8.24^a	23.04±4.64^a	13.72±1.60^a	13.30±1.05^a	0.91±0.14^a	0.97±0.08^a
Tank2 [OMWW=0.8%]	22.48±4.04^a	27.25±7.32^a	13.48±0.89^a	14.00±1.28^a	0.91±0.10^a	0.97±0.07^a
Tank3 [OMWW=1%]	31.91±8.35^a	-----	14.65±1.31^a	-----	1.00±0.13^a	-----

*Same letters in the same row and column indicate no differences in mean values.

Fish age can be estimated using several calcified structures, such as otoliths, scales, and other skeletal components (Folkvord et al., 2000; Panfili et al., 2002). The use of age data is a fundamental aspect of contemporary fisheries science (Hilborn and Walters, 1992). It is widely recognized that age could influence the stress response of a fish, and regarding fish length, no differences were observed between fish in the control tank and those in tanks 1, 2, and 3. These results suggest that the fish had approximately the same age. Roberts and Britton (2018) classified barbels into 3 life stages based on previous data: +0 (< 3.8 cm), juveniles (8.6 – 23.1 cm), and adults (more than 38.6 cm). In this study, the fish length ranged from 13 - 14 cm, falling within the juvenile stage.

During the acclimatization period, no fish mortalities or abnormal behaviours were observed, establishing a baseline for comparison. As expected, changes in fish behaviour were anticipated in tank 3, which had the highest concentration of OMWW. Fish behaviour in Tanks 1 and 2 remained similar to that of the control group after 12 hours. However, after 12 hours of exposure, one fish in tank 3 was found dead and after 24 hours, all fish exhibited signs of acute stress. Due to ethical concerns, the test in tank 3 was terminated, and the remaining fish were euthanized for blood collection (Table 4.10).

Table 4.10. Fish behavior observation during second subacute toxicity test.

	Control	**Tank 1 [OMWW=0.5%]**	**Tank 2 [OMWW=0.8%]**	**Tank 3 [OMWW=1%]**
6h	Normal swim Normal Equilibrium Good reflex	Normal swim Normal Equilibrium Good reflex	Normal swim Normal Equilibrium Good reflex	Normal swim 1 fish already present balance problem
12 -24h	Same as before	Same as before	Same as before	Balance problems Swim with circular rhythm Every fish swim in different directions Some fish stay quite Some fish trying to get the surface Some fish straggle to breath 1 fish dead
24- 48h	Same as before	Same as before	Same as before	-----

For the electrolyte quantification, after 24h of exposure, at least 6 fish were collected and anesthetized with 1% of ether ethylene glycol monophenyl (MERCK) and then, blood was drawn from the caudal vessels with heparinized syringes, to Eppendorf tubes and subsequently, frozen (-23ºC). Following 48 hours of exposure, the remaining fish were similarly collected and processed as before. Due to ethical considerations, all the fish from tank 3 were processed after 24 hours of exposure. No significant differences were observed between the control tank after 24 and 48 hours of exposure for all measured electrolytes, namely K^+, Ca^{2+}, and Na^+. Therefore, a mean value for each electrolyte was used for comparison purposes (Table 4.11).

Regarding plasma potassium (K^+) levels after 24 h of exposure, it was observed an increase level in fish from tank 2 and tank 3, comparing to fish control, and results also showed an increase in with OMWW concentration of exposure. This trend, an increase in K^+ levels comparing to control, is also observed after 48h of exposure, nevertheless without differences in OMWW concentrations of exposition (Table 4.11). As mentioned before, there are no fish in tank 3 during 48h of exposure. A similar increase in potassium levels in fish was observed by Monteiro (2012) and Fernandes et al. (2007a, 2009). They investigated the effects of copper exposure on plasma electrolyte balance in *Luciobarbus bocagei* and *Liza saliens.* According to Fernandes et al. (2007a, 2009), an increase in plasma K^+ is associated with osmotic adjustment, as it acts to compensate for the decrease in other plasma serum components. Na^+/K^+-ATPase (NKA) is a membrane-bound enzyme. This enzyme is responsible for the active transport of Na^+ out and K^+ into animal cells. It plays an important role in maintaining intracellular homeostasis and also provides a driving force for many transport systems in a variety of osmoregulatory epithelia, including fish gills (McCormick, 1995). Present results suggest that OMWW-induced alterations in plasma K^+ levels, likely mediated through disruptions in Na^+/K^+-ATPase activity, may compromise osmoregulatory function in fish, highlighting the enzyme's critical role in maintaining ionic balance under environmental stress. That is also supported by fish behaviour at tank 3, with high exposure OMWW concentration. After 24 hours and 48 hours of exposure, significant differences in plasma K^+ levels were observed between the control group and tank 1 and tank 2, suggesting that even moderate concentrations of OMWW can alter plasma potassium levels with prolonged exposure, highlighting a time-dependent effect of OMWW on electrolyte balance.

Table 4.11.Plasmatic electrolytes of *Luciobarbus Bocagei* after 24 and 48 hours of exposure.

	24 hours of exposure			48 hours of exposure		
	K^+	Na^+	Ca^{2+}	K^+	Na^+	Ca^{2+}
Control	**92±23^a**	**6235±709^a**	**139±24^a**	**92±23^a**	**6235±709^a**	**139.38±24.18^a**
Tank-1 (n= 4)	328±182^a	6794±1373^a	138±37^a	422±238^b	7922±1483^{a,b}	143.80±71.53^a
Tank-2 (n=5)	214 ± 72^b	5547±724^a	98±52^a	185.63±56^b	7458.±1434 ^{a,b}	110.82±60.29^a
Tank-3 (n=5)	417±391^c	6001±883^a	158±26^a	-	-	-

*Different letters in same columns correspond to different values.

According to the plasma sodium Na^+ levels, no differences were observed between the control group and tanks 1, 2 and 3 after 24h and 48h of exposure (Table 4.11). Also, no differences were observed for Ca^{2+} levels after 24h and 48h of exposure.

An increase in K^+ levels was expected to coincide with a decrease in Na^+ and Ca^{2+} levels, as previously reported by Monteiro (2012) and Fernandes et al. (2007a, 2009) and Jeney and Jeney 1995). However, in the present study, no reduction in Na^+ and Ca^{2+} levels was observed between 24 and 48 hours of exposure. This result may be related to the decrease in TPC levels, observed during time of exposition, as previously noted. Another important limitation is the reduced blood volume available for electrolyte analysis, due to the small size of the fish, which constrains the collection of larger samples. In addition, the absence of variation in Na^+ levels may reflect an absence of environmental stress response. According to Mazon et al. (2002), a decrease in plasma sodium is commonly used as an indicator of environmental stress, given the crucial role of branchial Na^+/K^+-ATPase in osmoregulation.

Additionally, according to Jeney and Jeney (1995), exposure to various stressors typically results in decreased calcium levels in cyprinid species. However, in this study, results suggest that *Luciobarbus bocagei* did not exhibit a typical stress response, possibly due to species-specific adaptations or because the osmoregulatory dysfunction was mitigated by the low levels of TPC in the test tanks. The gill is the primary organ responsible for the active uptake of and therefore plays a crucial role in maintaining calcium ion homeostasis (Pinto et. al. 2010). Ca^{2+}-ATPase, also known as the calcium pump, plays a critical role in transporting Ca^{2+} across the membrane, thereby maintaining intracellular calcium ion homeostasis. The enzyme effectively sustains the steep Ca^{2+}

gradient across the membrane with remarkable precision (Di Leva et al 2008). Therefore, the stable calcium levels observed in *Luciobarbus bocagei* may also be attributed to efficient regulation by gill-associated Ca^{2+}-ATPase activity, suggesting a potential adaptive mechanism. According to Flik et al. (1995), fish increase their branchial Ca^{2+} influx and thus the net uptake of Ca^{2+} when adapted to environments with reduced calcium concentrations in freshwater. This physiological response serves to compensate for the increased Ca^{2+} losses via the gills under such conditions.

5. CONCLUSIONS

Two olive pomace industries and their open-air ponds near the Tua River raise concerns about water contamination by OMWW. In fact, the wastewater can enter in the aquatic ecosystem through accidental discharges, surface runoff during heavy rainfall, or leaching, which transports toxic compounds into both surface and groundwater systems. Due to its diffuse and intermittent nature, this type of pollution poses a serious threat to water quality and ecological river quality. The main conclusions of the present study to assess the bioecological and toxicological impacts on freshwater fauna of Tua River, near Mirandela (NE Portugal), can be summarized in the following topics:

Drastic spatial and temporal changes in the composition and abundance of macroinvertebrate communities subject to the impact of olive mill wastewater. Significant reduction (> 50%) in terms of abundance, taxonomic richness and diversity in the sampling sites located immediately downstream (i.e., T3 and T6) of the effluent input point in the river from the open-air lagoons of both olive pomace industries. Decrease in biological quality, based on the official Portuguese index, IPtIN, with all impacted sites, i.e. high and intermediate groups (MODERATE or POOR classification) below the requirements of the Water Framework Directive, i.e., good/excellent ecological status. Multivariate analyses effectively signed the differentiation among the defined groups and identify the dominance of euribiont taxa, belonging to faunal groups of Oligochaeta, Corixidae, Planorbidae, Gammaridae and Cambaridae, some of them showing an invasive character, linked to the loss of diversity and low ecological integrity;

Almost exclusive dominance of exotic fish (e.g., *A. alburnus, L. gibbosus, G. lozanoi, G holbrooki*) in sites located immediately downstream of the OMWW industries (T3 and T6) in contrast to stenobiont taxa, represented by native rheophilic species, such as (*S. alburnoides, S. carolitertii, C. calderoni, P. duriense* and *L. bocagei*). Like the invertebrate community tendency, substantial decreases in taxonomic richness and diversity were observed in the most disturbed sites. The F-IBIP, an official index for assessing biological quality based on fish, also showed a BAD classification for the sites under direct influence of the OMWW effluent and MODERATE in the sites of intermediate disturbance.

The detected decrease in water quality clearly supports the variations observed in the biological elements under analysis. In fact, remarkable increase was recorded in the values of conductivity, phenols, oxidability and total acidity in the sites located immediately downstream of the OMWW plants (T3 and T6). This trend appears marked for both periods under analysis, i.e. winter and summer, although a temporal segregation was also observed for the other groups (i.e. low and intermediate disturbance) (visible in the dbRDA analysis).

This study evaluated the impact of OMWW on plasma electrolytes, namely potassium (K^+), sodium (Na^+) and calcium (Ca^{2+}) in *Luciobarbus bocagei* specimens after 24 and 48 hours of exposure to three different concentrations: 0.5%, 0.8% and 1%. The results demonstrated a significant effect on K^+ levels, while Ca^{2+} and Na^+ did not show variations. However, this absence of response should not be interpreted as a absence of stress, as it may be related to the proximity between the concentrations tested or to individual variability of the fish. The progressive reduction in total phenolic compounds (TPC) throughout the test suggests that the organisms may have activated mechanisms of physiological adaptation to chemical stress. It is important to emphasize that fish are not sessile organisms; they have mobility and the ability to evade, which allows them to abandon areas with adverse environmental conditions. This characteristic can mitigate the acute effects of pollution, but does not eliminate the risk of chronic impacts, which are often silent and cumulative, especially when contamination episodes occur repeatedly.

Based on the results obtained, it is recommended to carry out new tests with larger fish (25–30 cm), using the same concentrations or longer intervals, and with extended exposure time. In addition, it is essential to compare the effects of OMWW collected in summer and winter, in order to better understand the influence of seasonality on toxicity. For a more comprehensive assessment of ecotoxicological risks, it is essential to consider that the ecological integrity of the Tua River is influenced by several factors, including other types of wastewaters of industrial and agro-industrial origin, which, together with OMWW, contribute to the degradation of the river ecosystem.

The absence of response in plasma levels may be attributed to the narrow range of concentrations tested, which may have been insufficient to elicit distinct physiological effects, as well as to interindividual variability in baseline plasma values. Nonetheless, the progressive decline TPC during the exposure period suggests the activation of physiological adaptation mechanisms by the fish in response to chemical stress.

It is important to highlight that fish are not sessile organisms; they possess mobility and the ability to evade, which allows them to leave areas with adverse environmental conditions. This ability can mitigate the immediate effects of pollution but does not eliminate the risk of chronic impacts, which are often silent and cumulative, especially when contamination events are recurrent. Based on the results obtained, future investigations are proposed using the same OMWW concentrations (0.5%, 0.8%, and 1%), but with adjustments to the experimental design, namely the use of larger fish specimens (25–30 cm), extended exposure periods, and the comparison between OMWW collected in summer and winter.

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ANNEXES

Annex I: Portfolio of images of the 6 sampling sites (Tua River, summer 2024)

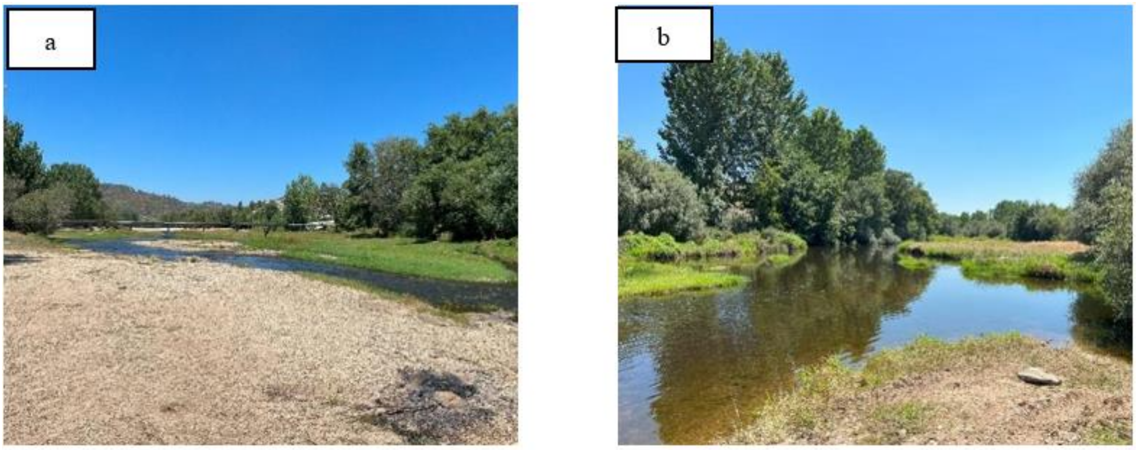

Figure A.1. Sampling site T1 in River Rabaçal, Tua Basin (near Miradeses).

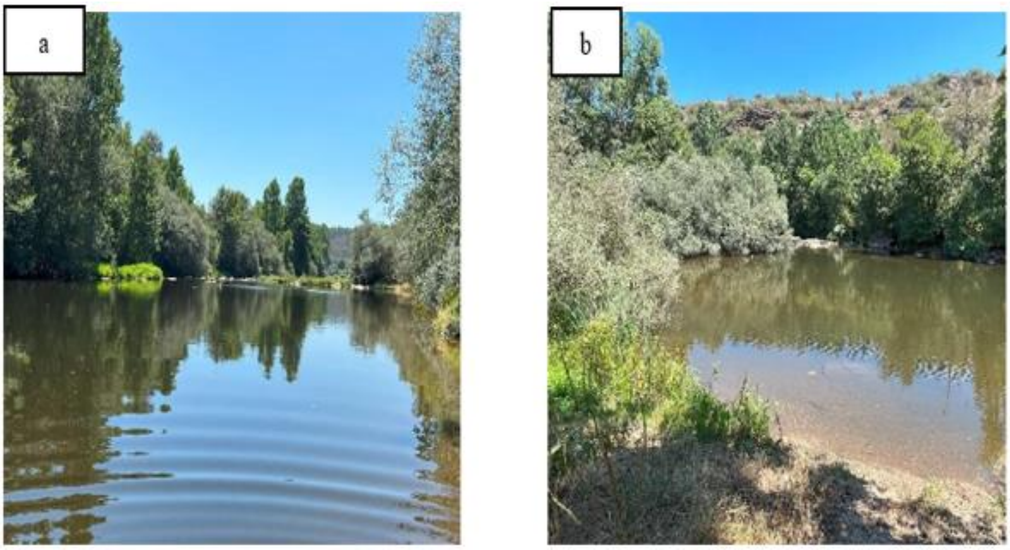

Figure A.2. Sampling site T2 in River Rabaçal (near Eixes, upstream of Rabaçal Company).

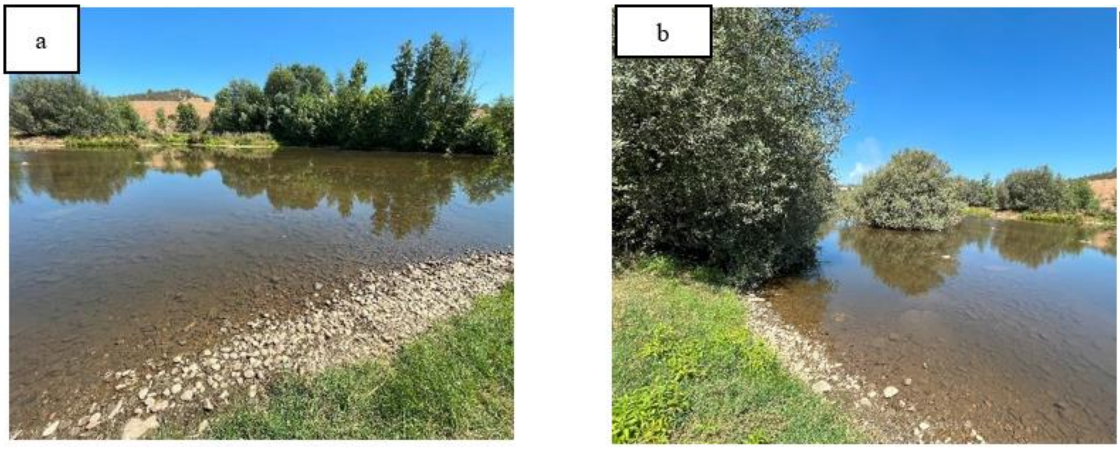

Figure A.3. Sampling site T3 in River Rabaçal (near Eixes, downstream of Rabaçal Company).

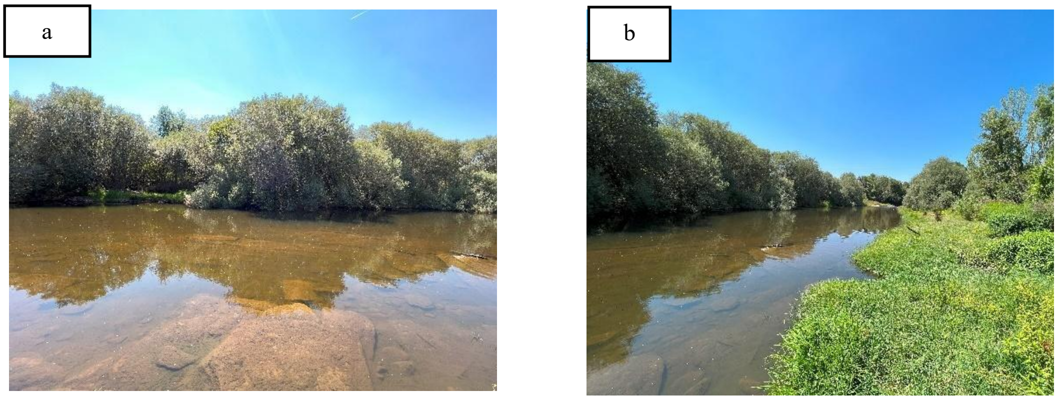

Figure A.4. Sampling site T4 in River Rabaçal (near Chelas village).

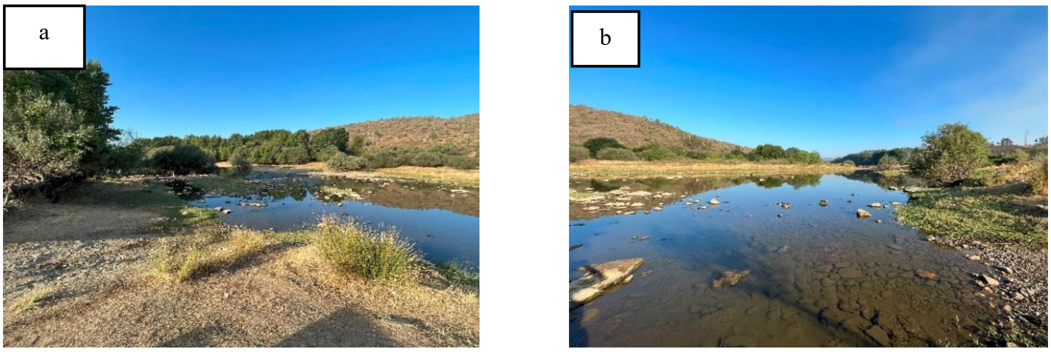

Figure A.5. Sampling site T5 in River Tua (near Frechas, upstream of Mirabaga Company).

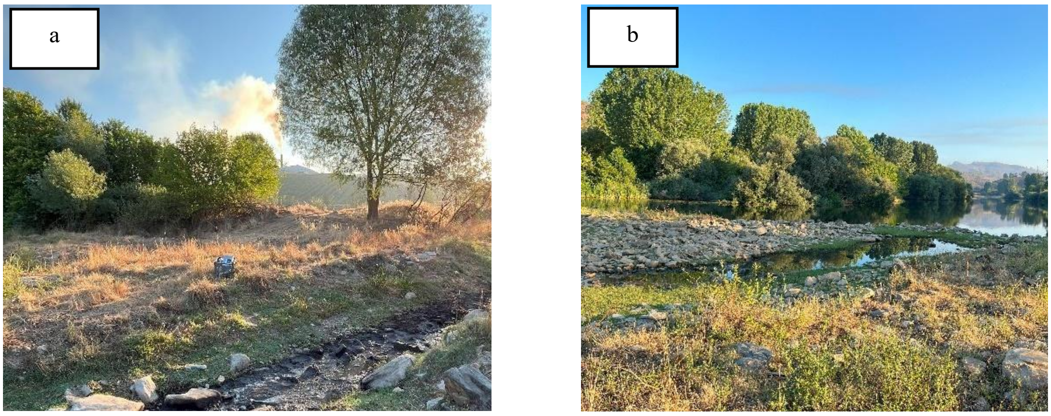

Figure A.6. Sampling site T6 in River Tua (near Frechas, downstream of Mirabaga Company).

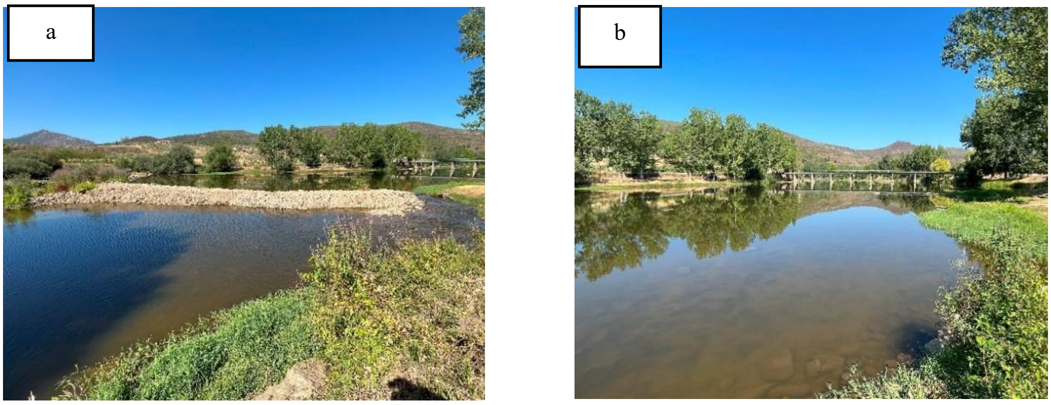

Figure A.7. Sampling site F7 in River Tua (downstream Frechas village).

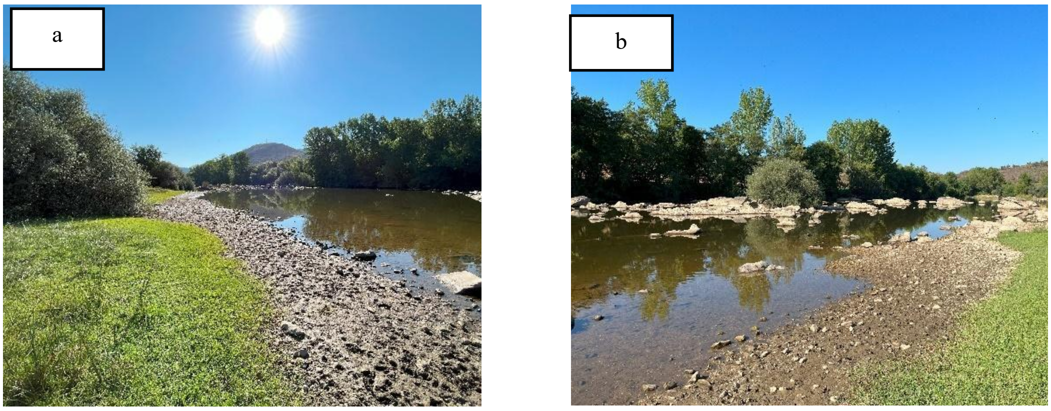

Figure A.8. Sampling site T8 in River Tua (near Vilarinho das Azenhas village).