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Freshwater and estuarine diatom composition and seasonal variation: influence of environmental factors and its forensic application

Mariana Torres Portugal Ramirez

Dissertation Leading to the Degree of Master in Forensic Sciences

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Work carried out under the supervision of Professor Cláudia Maria Rosa Ribeiro and Co-supervisors Professor Cristina Maria Cavadas Morais Couto and Professor Áurea Marília Madureira e Carvalho

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I, the person identified above, declare that I have acted with absolute integrity in the preparation of this work, confirming that in all the work leading to its preparation I have not resorted to any form of falsification of results or to the practice of plagiarism (an act by which an individual, even by omission, assumes the authorship of the intellectual work belonging to another, in its entirety or in parts of it). I also declare that all the sentences I have taken from previous works by other authors have been referenced or reworded, in which case I have cited the bibliographic source

DEDICATORY

I dedicate this work mainly to my mother, who is always by my side. I also dedicate it to my brothers and my best friend Mikael.

" You have to believe. Otherwise, it will never happen."

Neil Gaiman

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SCIENTIFIC PRODUCTION

Communications at international congresses:

Poster presentations

Torres-Portugal M., Couto C., Madureira-Carvalho Á., Ribeiro C. Variation of the physical-chemical parameters of diverse water bodies for study of diatom distribution and composition for forensic investigations. II TOXRUN International Congress, 27 and 28 of May, Porto, Portugal, 2023 (Attachment 1).

Torres-Portugal M., Madureira-Carvalho Á., Couto C., Ribeiro C. Importance of diatoms in the diagnosis of drowning. V Congress of the Portuguese Association of Forensic Sciences/ XIV Scientific Conference on Sciences of the University Institute of Health Sciences/ I TOXRUN International Congress, 7 and 8 of April, Porto, Portugal, 2022 (Attachment 2).

Abstracts in indexed journals

Torres-Portugal M., Couto C., Madureira-Carvalho Á., Ribeiro C. (2023) Variation of the physical-chemical parameters of diverse water bodies for study of diatom distribution and composition for forensic investigations. Scientific Letters, 1 (Sup), 107. https://doi.org/10.48797/sl.2023.107 (Attachment 3).

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ABSTRACT

Diatoms are unicellular microalgae widely distributed in aquatic systems that have proven to be a useful tool in forensic sciences. Different species and communities are characteristic of the ecosystems where they are found, allowing temporal and local associations to be made. In fact, the characteristic diatom composition of an aquatic system, their small size, high resistance to degradation and the fact that they do not occur naturally in the human body can provide relevant information in forensic investigation situations (for example, establishing associative indices between places, individuals and/or objects, and determining the cause of death in drowning cases). However, their composition varies with seasonality and collection site. The aim of this work was to perform a preliminary comparative study of the diatom composition of three aquatic systems with different hydrological and anthropogenic characteristics, trying to perceive the influence of seasonality and physicochemical parameters on the diatom composition of these aquatic systems.

To this end, surface water samples were taken from three types of aquatic systems (a stream, an estuary and two wells) on a seasonal basis (summer, autumn, winter and spring), sampling at two different locations for the stream and the estuary. The samples were processed and analyzed using optical microscopy to identify and quantify the diatoms. The physicochemical parameters of the water samples such as temperature, pH, conductivity, turbidity, dissolved oxygen, and nutrients (nitrates, nitrites, and phosphates) were determined according to established methodologies.

The results showed differences in diatom composition in terms of the types found and abundance in the three different aquatic systems, demonstrating that aquatic systems with different hydrological and anthropogenic characteristics will present different diatom compositions. In addition, there were seasonal and spatial differences. The samples taken from the stream and the estuary showed the greatest diversity and abundance of diatoms, with the estuary samples showing the greatest diversity. In general, for both systems, the greatest abundance of diatoms was found in summer, followed by winter and spring for the stream and estuary, respectively. The water collected from the wells, in two different geographical regions, showed different

compositions. In the well located in the Póvoa de Varzim region, no diatoms were detected in any of the samples taken, while the water collected from the well in the Paredes region showed the occurrence of very small numbers of diatoms. The physicochemical parameters were shown to vary according to seasonality, and it was possible to relate the occurrence of some diatom types to the physicochemical characteristics of the aquatic systems. However, this study is preliminary and to establish a relationship between the occurrence of diatom types and the studied physicochemical parameters, a greater number of sampling points will be required.

This study has mainly demonstrated the difference in the composition of diatoms of different aquatic systems and the relationship with seasonality and collection site. Despite being a pilot study, it will be the starting point for future work to develop a database to support forensic investigation studies.

Keywords: aquatic systems, biodiversity, diatoms, forensic sciences, microalgae.

RESUMO

As diatomáceas são microalgas unicelulares amplamente distribuídas em sistemas aquáticos que têm demonstrado ser uma ferramenta útil nas ciências forenses. Diferentes espécies e comunidades são características dos ecossistemas onde são encontradas permitindo fazer associações temporais e locais. De facto, a composição característica de diatomáceas de um sistema aquático, o seu pequeno tamanho, a alta resistência à degradação e o facto de não ocorrerem naturalmente no corpo humano podem dar informação relevante em situações de investigação forense (por exemplo, estabelecer índices associativos entre lugares, indivíduos e/ou objetos, e determinar a causa da morte em casos de afogamento). No entanto, a sua composição varia com a sazonalidade e local de colheita. Este trabalho teve como objetivo a realização de um estudo preliminar e comparativo sobre a composição de diatomáceas de três sistemas aquáticos com características hidrológicas e antropogénicas diferentes, tentando perceber a influência da sazonalidade e dos parâmetros físico-químicos na composição de diatomáceas desses sistemas aquáticos.

Para isso, foram colhidas sazonalmente (verão, outono, inverno e primavera) amostras de águas de superfície dos três tipos de sistemas aquáticos (uma ribeira, um estuário e dois poços), tendo-se amostrado em dois locais diferentes da ribeira e do estuário. As amostras foram processadas e analisadas por microscopia ótica para identificar e quantificar as diatomáceas. Os parâmetros físico-químicos das amostras de água tais como temperatura, pH, condutividade, turvação, oxigénio dissolvido e nutrientes (nitratos, nitritos e fosfatos) foram determinados de acordo com metodologias já estabelecidas.

Os resultados demonstraram diferenças na composição de diatomáceas relativamente aos tipos encontrados e abundância nos três diferentes sistemas aquáticos demonstrando que sistemas aquáticos com características hidrológicas e antropogénicas diferentes vão apresentar composições de diatomáceas diferentes. Para além disso, verificaram-se diferenças sazonais e espaciais. As amostras colhidas na ribeira e no estuário apresentaram a maior diversidade e abundância de espécies, sendo as amostras do estuário as que apresentaram uma maior diversidade. De forma geral,

para ambos os sistemas, a maior abundância de diatomáceas foi verificada no verão seguida do inverno e da primavera para a ribeira e o estuário, respetivamente. As águas colhidas nos poços, situados em duas regiões geográficas diferentes, mostraram composições diferentes sendo que no poço localizado na região da Póvoa de Varzim, não se detetaram diatomáceas em nenhuma das colheitas realizadas, enquanto que as águas colhidas no poço da região de Paredes demonstraram a ocorrência de um número muito reduzido de diatomáceas. Os parâmetros físico-químicos demonstraram variar de acordo com a sazonalidade e foi possível relacionar a ocorrência de alguns tipos de diatomáceas com as características físico-químicas dos sistemas aquáticos. No entanto, este estudo é preliminar e para estabelecer uma relação da ocorrência entre os tipos de diatomáceas e os parâmetros físico-químicos estudados será necessário um maior número de pontos de colheita.

Este estudo demonstrou, maioritariamente, a diferença na composição de diatomáceas de diferentes sistemas aquáticos e a relação com a sazonalidade e o local de colheita. Apesar de ser um estudo piloto, será o ponto de partida para a realização de trabalhos futuros que permitam o desenvolvimento de uma base de dados para apoiar estudos de investigação forense.

Palavras-Chave: biodiversidade, ciências forenses, diatomáceas, microalgas, sistema aquático.

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LIST OF ABBREVIATIONS AND ACRONYMS

ATP - Adenosine triphosphate

CCME - Canadian Council of Ministers of the Environment

DNA - Deoxyribonucleic Acid

IPMA - *Instituto Português do Mar e da Atmosfera* - Portuguese Institute of Sea and Atmosphere

NTU - Nephelometric Turbidity Units

1. INTRODUCTION

1.1. Algal Diversity

Ecosystems biodiversity is characterized by a number and variety of organisms, from microscopic to macroscopic, that play a key role in ecological foundations. Among them, algae are aquatic organisms naturally abundant in freshwater and marine ecosystems and frequently found in terrestrial habitats with a wide world distribution (Lee, 2008). Due to their key role and prevalence in ecosystems, algae have also been widely explored as environmental trace evidence, offering useful information of forensic interest when reconstructing crime events (Seckbach and Gordon, 2019).

Algae are unicellular or multicellular eukaryotic organisms, mostly photosynthetic, being placed under the Protista kingdom (McLaughlin, 2012). These organisms are recognized with fundamental importance for the functioning of aquatic ecosystems giving the great contribution to carbon fixation and oxygen release into the atmosphere, being the base of the trophic chain as primary producers (McLaughlin, 2012; Falciatore and Mock, 2022). In aquatic environments (lentic or lotic, marine or fresh), algae can remain suspended in the water (planktonic), as well as being fixed to the ground or attached to a surface, depending on their class and/or species. In terrestrial environments, algae generally live in a symbiotic relationship with other organisms (Lee, 2008).

Unlike superior plants, algae are avascular organisms. Thus, they do not have true roots, stems or leaves, although the so-called macroscopic and multicellular algae have a thallus, which can appear as filaments, resembling stems and leaves of plants, such as the *Rhodophyta* (red algae) and *Chlorophyta* (green algae) (Lee, 2008). Microscopic algae are unicellular, although some live-in colonies. Microalgae are commonly used as bioindicators of ecosystem conditions as they are susceptible to ecological variations (Falciatore and Mock, 2022). These variations can be caused by biotic factors (changes in the food chain), or abiotic factors as light (intensity and spectrum) and physicochemical parameters (e.g., changes in pH, temperature, chemical components) resulting in reproductive disturbances affecting the algae population number and consequently leading to variations of ecosystems composition (Kelly, 2000; Lee, 2008; McLaughlin, 2012). Seasonal variation is also an important factor that interferes with microalgae population composition. In fact, seasonal variations cause changes in the

ecosystem abiotic factors that affect microalgae population (Kelly, 2000; Lee, 2008; Smol and Stoermer, 2010).

In freshwater environments, there are two major classes of microalgae, the *Chrysophyceae*, known as golden algae, and the *Bacillariophyceae*, known as diatoms. Diatoms have been more used in ecological and forensic studies (Lee, 2008; McLaughlin, 2012). In fact, seasonal variation of diatom composition in aquatic ecosystems have been shown to give important information in forensic sciences (McLaughlin, 2012; Kaye and Meltzer, 2020; Falciatore and Mock, 2022).

1.2. Diatoms

1.2.1 General Characteristics

Diatoms belong to the phylum Bacillariophyta, with more than 20,000 existing species. Diatoms can be microscopic algae, with a size that can vary from 2.0 to 500 µm. They are composed of a nucleus, cytoplasm, plasma membrane and cell wall. The cell wall of diatoms is their main characteristic, and their size and shape are important features to differentiate species allowing, at the first instance, the classification of diatoms and, afterwards, their identification (Figure 1) (McLaughlin, 2012; Annenkov et al., 2022; Falciatore and Mock, 2022).

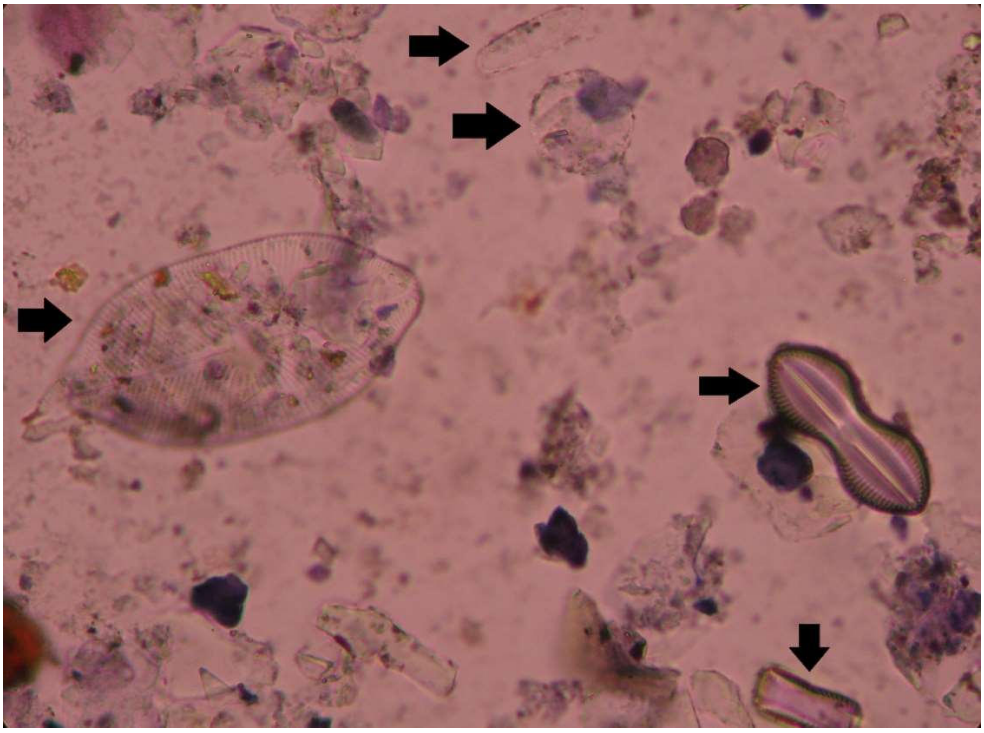

Figure 1. Different shapes and sizes of diatoms (sample collected from the estuary of the Ave River 2, Portugal).

The diatom's cell wall, named frustule, is composed of silica (SiO_2) and provides resistance and hardness, which can be perceived in diatomites, rocks formed by microscopic remains of the cell walls (McLaughlin, 2012; Pereira Júnior et al. 2018; Annenkov et al., 2022).

The structure of the cell wall is divided into two valves, that fit together like a box: the upper valve, known as epivalve, and the lower one called hypovalve (Figure 2). Among them, there is the cingulum, that spans both valves, dividing them into two parts. Cingulum is subdivided into epicingulum (on the epivalve side) and hypocingulum (on the hypovalve side). The set of the epivalve with the epicingulum is called the epitheca and the set of the hypovalve with the hypocingulum is called the hypotheca. The epitheca is larger than the hypotheca since hypotheca is originated from the mother cell during the process of diatom cell division (Figure 2) (Kelly, 2000; McLaughlin, 2012). The structure of diatom cell walls, including the patterns and shapes of these components, is often used for taxonomic classification and identification of different diatom species (Kelly, 2000).

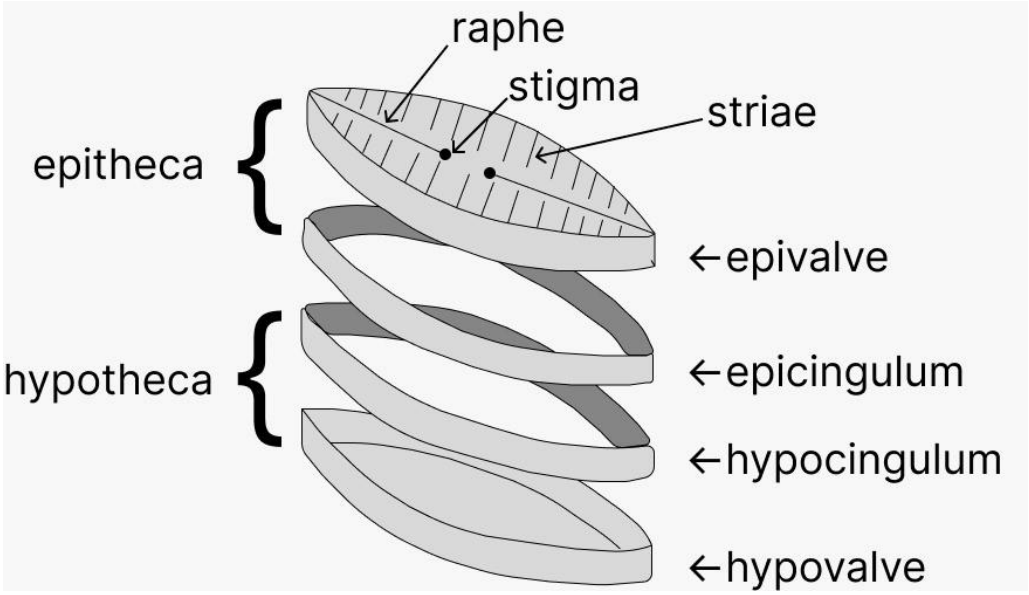

Figure 2. Cell wall structure of diatoms. Adapted from Kelly, 2000.

Based on the shape of the frustules two orders are defined: the Centrales (radial symmetry) and Pennales (elongated). They are classified into three main classes: the Coscinodiscophyceae (centrales), being its morphological structure radial, and the Fragilariophyceae (e.g., *Asterionella* sp., *Fragilaria* sp.) and the Bacillariophyceae (e.g., *Eunotia* sp., *Navicula* sp.), that belong to the pennates and differ from each other by the presence or absence of a structure named raphe (Lee, 2008; Seckbach e Gordon, 2019). Raphe is a slit separating the central area from the apical area (Figure 2 and 3). Raphe can end in different ways in the central and in the apical area (i.e., ending in the central area with the tips facing the same direction or not, and with stigma on the tips or not, like in *Navicula* sp. (Figure 4), ending in the apical area forming a curve or not), being important for diatoms species identification. The raphes are only found on pennates and their main function is locomotion and adhesion (McLaughlin, 2012; Seckbach and Gordon, 2019).

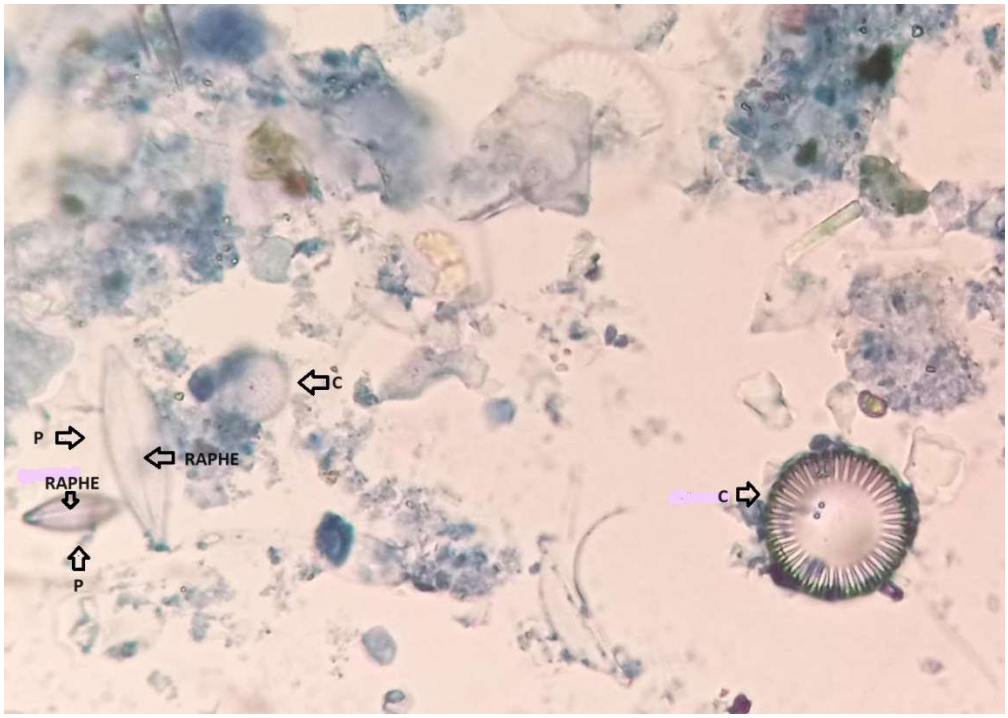

Figure 3. Pennate (P) and centric (C) diatoms, showing the raphe in pennate ones (sample collected from the estuary of the Ave River 2, Portugal).

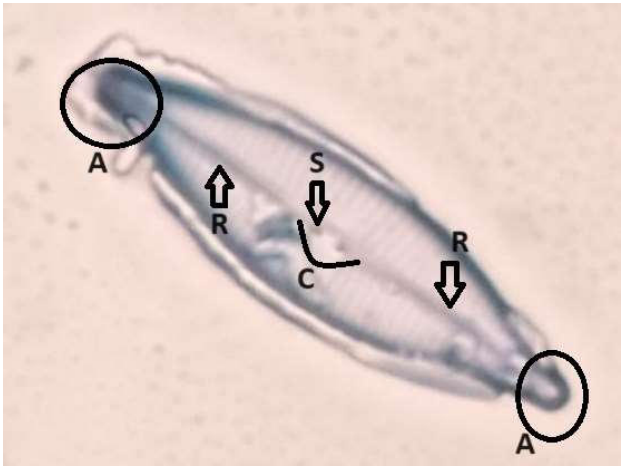

Figure 4. *Navicula* sp. From a sample collected from the estuary of the Ave River 2, Portugal (A = Apical area; R = Raphe; C = Central area; S = Stigma).

Pennate diatoms are usually periphytic, adhering to substrates (algae - epiphytic; sediments - epipelic; rocks - epilithic; animals - epizootic). This is due to their ability to excrete a mucilaginous substance through the pores, which are tubes that cross the face of the valve, the eyespots, the raphe or areolae (in pennates, mainly from the raphe),

which also allows them to move around (Vieira, 2011; McLaughlin, 2012; Pereira Júnior et al. 2018).

Diatoms are not motile, although diatoms with a raphe are able of limited movements by secretion of mucilaginous material whereas central diatoms, which do not have a raphe, follow the movement of the water, and are considered pelagic (Smol and Stoermer, 2010).

1.2.2. Life Cycle

The life cycle of a diatom has an estimated duration of about 6 days, with each population duplicating every 24 hours under favourable conditions. The life cycle is influenced by several factors such as nutrients availability affecting growth, reproduction, and motility (McLaughlin, 2012; Annenkov et al. 2022; Falciatore and Mock, 2022).

Most diatoms are autotrophic, photosynthetic and obtain their nutrients by synthesizing organic compounds, but depending on the environment in which they live, some species can become facultative heterotrophs, and rarely obligate heterotrophs (McLaughlin, 2012). Some nutrients are essential for maintenance of normal growth and frustule formation as vitamin B12, although other organic substances and minerals are also needed. The most important minerals are silica, for reproduction, whereas calcium and iron promote a rapid growth (Pereira Júnior et al. 2018). The growth period varies according to the diatom species, and if it is a single individual, or if it lives in a colony, if it adheres to substrates or if it is pelagic.

When the frustule is formed, the diatom's growth ends although an axial expansion can occur increasing body size. Reproduction also occurs after growth (Annenkov et al. 2022).

1.2.3. Reproduction

Diatoms can reproduce asexually or sexually during their life cycle. Asexual reproduction is more frequent and consists of a simple binary division with each daughter cell receiving one of the parent cell's two frustules (Figure 5). This is used by each daughter cell as the larger frustule, the epitheca, into which a second, small frustule, the hypotheca is constructed (Figure 5). However, binary division causes

reduction of diatom size in one of the daughters since hypotheca of the mother cell is already smaller comparatively to epitheca (Vieira, 2011; Annenkov et al., 2022). As a result, there will come a time when the reduced size results in a critical situation for survival, and it will then be necessary to initiate a sexual reproductive phase, to allow future generations to recover the appropriate size (Smol and Stoermer, 2010; McLaughlin, 2012; Falciatore and Mock, 2022).

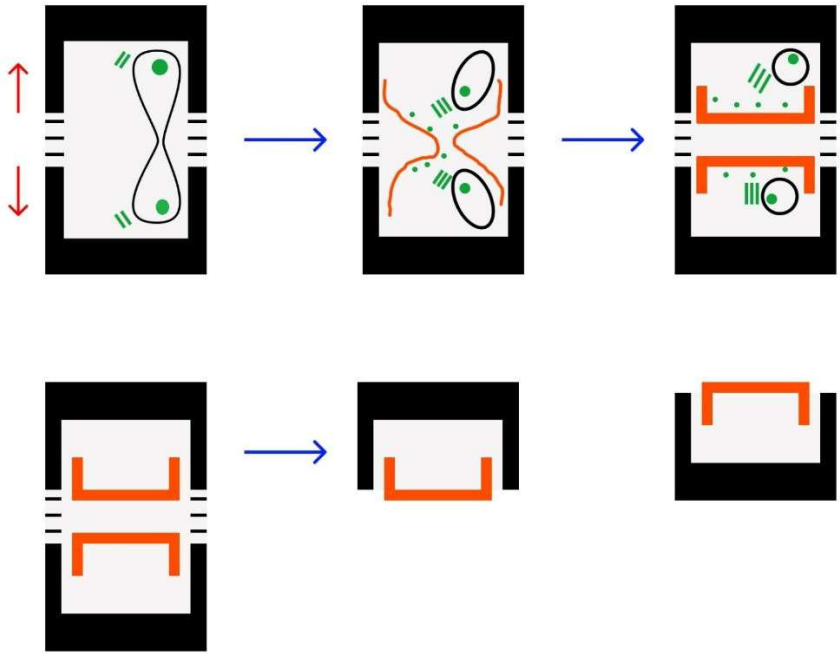

Figure 5. Asexual reproduction of a diatom. The original diploid diatom (mother) undergoes growth binary fission of the protoplasm; this leads to the individuation of the new protoplasm; the formation of hypothecas (orange box) and isolation of the protoplasm; finally forming two new diatoms (daughters). Simple binary division evidencing the difference in size in the daughter cells. Adapted from Vieira, 2011.

Beyond simple binary division, diatoms can also reproduce by parthenogenesis by apomixis, although it is rare. In this case, an unfertilized gamete develops into a haploid individual (Kelly, 2000; Smol and Stoermer, 2010).

Depending on the species of diatom, sexual reproduction can occur by isogamous meiosis (equal, non-flagellated gametes) or anisogamous meiosis (different gametes, one larger and non-flagellated and the other smaller and flagellated) (Lee, 2008; McLaughlin, 2012). Normally, centric diatoms reproduce by isogamous meiosis and pennate diatoms by anisogamous meiosis (Lee, 2008). Sexual reproduction occurs to

allow size species recovery as the auxospores (zygotes) continue developing until they reach the appropriate size of the species and complete the formation of the frustule (Figure 6) (Vieira, 2011).

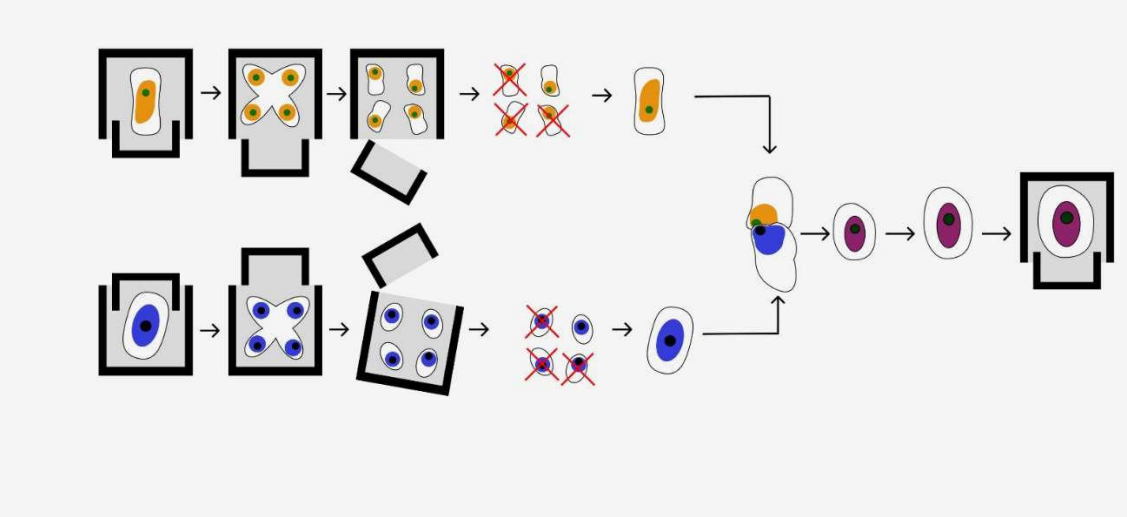

Figure 6. Sexual reproduction, auxospore formation. Adapted from Vieira, 2011.

1.3. Physicochemical parameters that affect the composition of diatom communities in aquatic ecosystems

Some diatoms live everywhere but most are highly sensitive to temperature and physicochemical water characteristics and other environmental conditions that restricts their habitats. Physicochemical water parameters such as temperature, pH, salinity, conductivity, turbidity (light), dissolved oxygen and nutrients availability interfere with diatom species community and number (Kelly, 2000; Pereira et al., 2012; Luis et al., 2016). Due to variability in diatoms composition of a water body, it is possible to infer the characteristics of a waterbody from which an environmental or forensic sample originated, based on the diatom community (composition and number) (Coelho et al., 2016; Kaye and Meltzer, 2020). Additionally, physical and chemical parameters can change due to seasonality or anthropogenic pressures. Centric species are more common in marine waters whereas pennate species are more frequent in freshwaters, although centric diatoms species can also occur in freshwater systems and pennate species in marine waters. In aquatic ecosystems of lentic waters, such as ponds, wells, both types of diatoms may exist (Lee, 2008; Baird et al. 2017).

Temperature is an important parameter that can influence other physicochemical parameters (Baird et al., 2017). Temperature is dependent of seasons and can cause alterations in water bodies physicochemical parameters and consequently on diatom species composition (Lee, 2008; McLaughlin, 2012). Nevertheless, changes in other physicochemical parameters such as pH, salinity, or nutrients may have a greater impact on diatom composition (Santana et al., 2016). Diatoms can be adapted to variations of these parameters to certain limits beyond that it may lead to the interruption of their development or early death (Luis et al., 2016; Baird et al., 2017). According to the Canadian Council of Ministers of the Environment (CCME, 1999), the ideal temperature values for diatoms' aquatic life range from -1.5ºC to 30ºC.

In lotic waters, the pH varies between 6 to 9, and when the temperature increases, it makes the water more alkaline, being favorable for diatom species that are adapted to these conditions (CCME, 1999). Several studies demonstrated that there is an abundance of *Pinnularia* sp. and *Eunotia* sp. at pH between 1.9 and 5.1; whereas *Achnanthidium* sp., *Brachysira* sp., *Eunotiaceae* sp., *Cyclotella* sp., *Ulotrix* sp. are more adapted at pH between 5 and 6.8 and higher conductivity values and *Navicula* sp., *Achnanthidium* sp. and *Nitzschia* sp., at pH between 7 and 8.4 and high concentration of phosphate (Pereira et al., 2012; Rodrigues et al., 2015; Luis et al., 2016; Pereira Júnior et al., 2018). In rainy periods, which occur mainly in the autumn and winter months (and even in early spring), precipitation leads to an increase in water flow that causes the dissolution of chemical components, leading to changes in physicochemical parameters, such as a reduction in pH and an increase in the concentration of metals (metals from rocks, correlated with the salinity of the water), i.e. the movement of water influences the pH level of the water, due to the solubilization of rocks and sediments present in the aquatic environment (Luis et al., 2016; Baird et al. 2017). As these periods continue, the pH concentration rises again, and the concentration of metals decreases, as they are inversely proportional, which consequently leads to an increase in salinity. With this reduction in metal concentration, the presence of teratology in diatom species is reduced (e.g., *Eunotia exigua* - presence of valve defects) and salinity (conductivity) favors the absence of these defects (Luis et al., 2016). The genus *Pinnularia* adapted at low pH and conductivity levels, has its greatest abundance in low water periods, as there

is a greater interaction between sediment and water column (Pereira et al. 2012; Rodrigues et al. 2015).

Conductivity is influenced by salinity and increases towards the ocean. Species as *Entomoneis paludosa* are well adapted to high levels of salinity/conductivity and can be found in water bodies as estuarine waters (Luis et al. 2016). Also, genus *Amphora* can adapt to varying levels of salinity and temperature, contributing to their ecological versatility and can inhabit a wide range of aquatic environments, including both freshwater and marine habitats (Round et al., 1990).

Most diatoms, as photosynthetic organisms, need access to light, which depends on the turbidity of the water (Lee, 2008; Annenkov et al., 2022). The excessive number of suspended particles leads to an increase in turbidity, reducing access to light and, therefore, the diatom population. In rainy seasons, such as autumn, winter and early spring, increased precipitation leads to greater leaching of sediments and organic materials from soils that reach water bodies, increasing turbidity and the hydrodynamics of water bodies. The class *Chlorophyceae* (centrale) is most abundant during periods of lower turbidity, as it remains suspended in the water, requiring access to light (Santana et al., 2016). The CCME, 2002, reports that, for clear flow, the maximum level should be 8 nephelometric turbidity units (NTUs), in a short-term exposure, and in high flow or turbid water it should be between 8-80 NTUs but should not exceed 10% when the value is higher than 80 NTUs.

Dissolved oxygen concentration changes daily and seasonally and is highly influenced by temperature, as it varies along the day, and photosynthesis (a normal diurnal oxygen cycle would be sinusoidal with a maximum concentration late in the day and minimum in early morning) (CCME, 1999; Baird et al., 2017). Concentrations below the threshold (8.0 mg/L) are consistent with high biological activity and in lentic water sites the concentration is higher at the surface than at the bottom (Luis et al., 2016). However, considering the water quality for aquatic life, it can be inferred that in seasons (summer and spring) when the water is warm, dissolved oxygen varies around 6 mg/L. For seasons where the water is considerably cold (autumn and winter), it varies between 9.5 mg/L and 6.5 mg/L. These values are the lowest acceptable dissolved oxygen concentrations, but many species of invertebrates can be adaptable in conditions with an even lower dissolved oxygen concentration (CCME, 1999).

Nitrate and nitrite, an integral part of the nitrogen cycle in the environment, make part of the nutrients necessary for photosynthesis and development of diatoms, with nitrate being preferred, due to its stability (Lee, 2008; Annenkov et al., 2022). Phosphate is essential for the growth of diatoms, as it is an important component of deoxyribonucleic acid (DNA) and adenosine triphosphate (ATP) (Lee, 2008; McLaughlin, 2012; Annenkov et al., 2022). When phosphate is lacking, diatoms can begin to produce organic phosphorus compounds, such as phosphonic acid, to supply their phosphorus needs. However, excess of nutrients such as nitrate and phosphate can lead to an excessive increase in the number of diatoms which can lead to environmental problems such as eutrophication. This occurs when there is an increase in the amount of organic matter and nutrients in the water bodies, leading to a decrease in the amount of available oxygen and a decline in water quality (Baird et al. 2017; Falciatore and Mock, 2022). The presence of low concentration of dissolved oxygen also promotes the transformation of nitrate into nitrite, which is not a favorable compound for the quality of water and aquatic life. According to the CCME, 2004, the adequate values for total phosphorus in freshwater are 10 to 50 µg/L, and these values contribute to the classification of the environment from ultra-oligotrophic (very poor in nutrients) to hypereutrophic (very rich in N and/or P). According to water quality guidelines for the nitrate ion for the protection of aquatic life 550 mg/L is the highest short-term concentration and 13 mg/L is the long term, and regarding the nitrite levels it presents adequate values equivalent to 0.197 mg/L (CCME, 2012; Baird et al. 2017; Falciatore and Mock, 2022). For example, some common genera species in freshwater like *Staurosirella*, *Cyclotella* and *Melosira*, *Synedra* are even more common in eutrophic environments, and adapted in environments with high nitrate levels. Genus like *Caloneis* and *Diploneis* are adapted to variations of these parameters, but genus *Gomphonema* and *Pinnularia* are more common in oligotrophic environments (Round et al., 1990; Killham et al., 1998; Smol and Stoermer, 2010).

Therefore, the main physicochemical parameters of water that affect the composition of diatoms and their population are temperature, pH, conductivity and salinity, turbidity, dissolved oxygen, nitrite and nitrate levels, and phosphate (Santana et al. 2016).

1.4. Application of diatom evidence in forensic investigation

Beyond their key role on ecological function of water bodies, algae can provide significant support evidence in criminal investigation. Application of diatoms in forensic science is most related to diagnosis of death by drowning (Figure 7). However, diatoms are frequently good evidence for association/dissociation of a variety of materials related to crime scenes or suspects to specific geographic areas of water bodies (McLaughlin, 2012; Seckbach and Gordon, 2019). Knowledge about local diatom composition is imperative for comparison of diatoms found in materials, victims, and suspects (Levin et al., 2017; Magni et al., 2020). For instance, the use of diatom test to diagnose drowning is the most widely accepted method for identifying drowning, when other anatomopathological parameters such as cyanosis, foam mushroom, and changes in the skin of the fingertips are not possible to obtain due to advanced decomposition, or aquatic animals that may mask these evidence (they bite/eat the fingers, for example), although it has received some criticism (Seckbach and Gordon, 2019; Zhou et al., 2020; Khurshid et al., 2021). In fact, lungs, kidney, liver and even brain can harbour diatoms accumulated in life (McLaughlin, 2012; Coelho et al., 2016; Seckbach and Gordon, 2019). Confirmation of drowning using the diatom test has been based on the histological analysis of tissues/organs for the detection of diatoms, as they can enter the blood circulation through the pulmonary alveoli or gastrointestinal tract when aspiration or swallowing water containing diatoms occurs (Seckbach and Gordon, 2019). Determination of death by drowning has been ascribed by the presence of diatoms not only in the upper respiratory tract but also in other organs (e.g., spleen, heart, marrow bone) (Figure 7). However, this test is relevant if collection of water samples from the place where the body was found, and at the time of removal of the body is performed. Coelho et al. 2016, carried out a comprehensive study consisting in the characterization of diatoms composition in the Douro River for comparison with the species of diatoms found in the victim's body. Nevertheless, it is important to stress that this test presents some gaps and also, to avoid false positives, it is necessary a careful analysis of the samples during the autopsy procedure, as contamination by diatoms can occur or diatoms can be present in the body by other factors. In fact, Lunetta et al. 2013 showed the presence of diatoms in autopsying non-drowned corpses from dry places. In that work, species of the genera *Nitzschia* sp., *Achnanthes* sp., *Coscinodiscus* sp., *Melosina*

sp. and mainly *Thalassiosira* sp. were found, and authors concluded that organisms could contain for a certain period, diatoms originating from food, mainly fish, crustaceans, and from air inhalation, as there are species that disperse in the air.

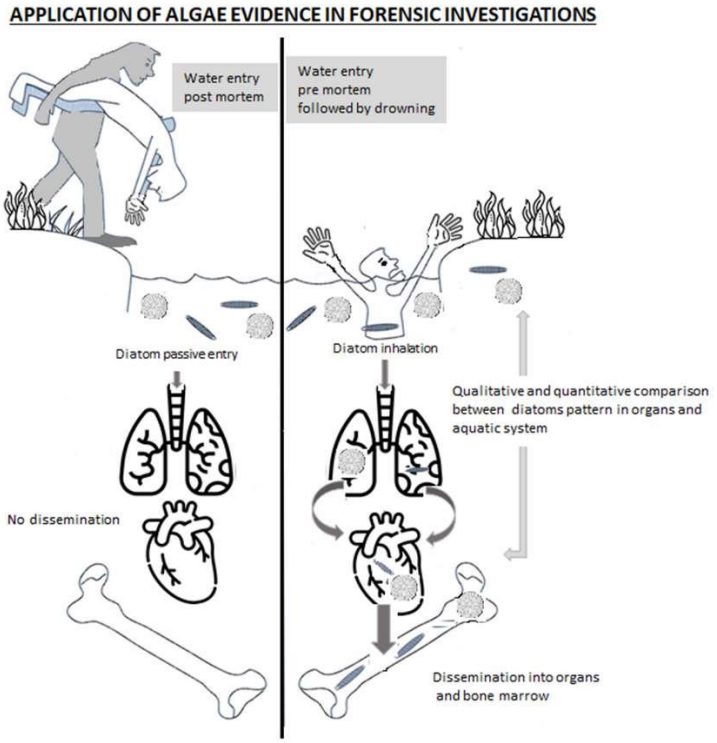

Figure 7. Application of algae evidence in forensic investigations. Adapted from Bernard Knight, Legal Aspects of Medical Practice (Seckbach and Gordon, 2019).

Lin et al. 2014, demonstrated that there is greater evidence of diatom findings in bodies found in fresh waters than in sea waters. One hundred cases of presumed death by drowning and twenty control cases where it was certain that death was due to natural causes were used to check the sphenoid sinus fluid and lung tissue. Of the 100 cases, 94 were positive for drowning, 81 with the presence of diatoms. Of these, 54 had diatoms both in the sphenoid sinus fluid and in the lung tissue, 22 only in the sphenoid sinus fluid, which can be considered according to the exposure time and water intake, and 5 only in lung tissue. The other cases in which the post-mortem diagnosis of drowning was given did not show the presence of diatoms, probably due to the location (swimming pools and bathtubs) where the bodies were found, and thus diatoms may not be present depending on the type of treatment received by the water used. Two of the 6 cases in which death was not due to drowning, evidenced the presence of diatoms in the lung

tissue, which can be questioned whether the cause would be the level of decomposition, open wounds or the hydrostatic pressure that led the diatoms to reach the tissue. The most common diatoms were *Navicula* sp., *Nitzschia* sp., *Cyclotella* sp., *Achnanthes* sp. and *Thalassiosira* sp. commonly found in freshwaters.

According to a study performed by Magrey and Raj, 2014, in bodies found in rivers in India, the diatom test was used for the post-mortem diagnosis of drowning, since the injuries common to drowning were not possible to collect. Collection and analysis of the diatoms present in the places where the bodies were found were carried out together to make a correlation and rule out false positives. All the species found in the bodies and sites belonged to pennate diatom group commonly found in rivers. In that study, it was verified that of the 11 cases studied, only 2 were not due to drowning.

Diatoms can also provide valuable information in other forensic cases. For instance, diatoms are fundamental in terms of trace and geolocation. The Connecticut Case, in 1991, where young suspects were proven guilty of the crimes due to the finding of diatoms and Chrysophytes (golden algae) in their shoes, which were the same species found in the victims' shoes (Siver et al. 1994). *Mallomonas caudata*, a species of Chrysophytes very similar to diatoms, and *Eunotia* sp., a diatom common in freshwaters, were found on shoes (suspects and victims) and at the place where the crime occurred (Siver et al. 1994). Other example of the applicability of diatom identification as a forensic tool to established timeframes is the D.B. Cooper money, reported by Kaye and Meltzer, 2020. This case refers to the skyjacking of an airplane that occurred in November 1971. After receiving the payment, Cooper parachuted from the aircraft and disappeared without a trace. Nine years later, the money was discovered in the Columbia River. The burial site was located 30 km away from the reported jumped area. Initially, it was suspected that the money had been buried in November 1971 during the escape. Nevertheless, diatom found on the surface of bills revealed the presence of *Asterionella formosa* and *Fragilaria s*p. *Asterionella* species are predominant in the summer. Therefore, the assumption that the money was buried in November was not possible. Additionally, this species is planktonic, demonstrating that the money had been immersed in the summer before burial, probably due to entrainment and that Cooper probably died during jump.

Levin et al. 2017, carried out experiments to measure the persistence and transfer of diatoms in different shoe fabrics, and the time when there was a greater and lesser presence of diatoms. It was concluded that regardless of the type of footwear material, after 168 hours of exposure diatoms would be present, and it took at least 4 hours to have a considerable amount of them. Therefore, shoes could be good samples, although in a brief contact limit transfer of diatoms to the fabric may occur and thus, the more time exposed the greater the transfer. Magni et al. 2020, performed an experiment with two species of diatoms in a river in Australia, one of the pennate class (*Navicula* sp.) and the other centrale (*Chaetoceros muelleri*). In that study, it was demonstrated that the type and concentration of diatoms present in the water body affects the transfer and extraction efficiency more than the types of fabrics. It was also verified that 30 minutes of the fabric being submerged would be enough for the presence of diatoms. It also showed that the method of extracting diatoms by hydrogen peroxide is the most effective in obtaining the greatest number of diatoms.

Finally, in relation to geolocation, Coelho et al. 2016, highlighted the need to carry out a database of diatoms, including their seasonal changes and the physicochemical parameters that affect diatom composition, for an accurate determination of the real location of a body, or evidence, since the correlation between diatoms present in the body and objects are not always positive with those of the place found. Diatoms found in the Douro River in Portugal were mainly *Nitzschia* sp., *Aulacoseira* sp., *Gomphonema* sp., *Cyclostephanos* sp., *Achnanthes* sp., *Cyclotella* sp., and *Cocconeis* sp. The same genus may be present at different points of the river, however, depending on the factors mentioned above, their number varies, confirming the need for a database, and spatial distribution for a better use of the test of diatoms in forensic cases.

2. AIMS

This work aimed to provide a comprehensive study about the diatom composition of three different aquatic systems (stream, estuary and well waters) and get insights about the influence of seasonality and physicochemical parameters on the diatom composition of those aquatic systems.

Specifically, this research aimed:

a) to seasonally collect water samples from the three different aquatic systems, with different water hydrological characteristics, located in different areas of the north of Portugal, for comparative studies of diatom composition;

b) to understand the influence of seasonality and physicochemical parameters on diatom community composition by analysing several factors such as temperature, pH, conductivity, turbidity, dissolved oxygen, and nutrients (nitrates, nitrites, phosphates) and at each season and sample site collection.

Data collected will be important for understanding the space-time distribution of freshwater diatoms and for better knowing the factors that can influence the composition of diatom communities. Data will also be available to futurity contribute to the development of a database that can support forensic investigation studies.

3. MATERIALS AND METHODS

3.1. Chemicals, materials and equipments

All chemicals were of analytical reagent grade, unless mentioned otherwise. The hydrogen peroxide 35% (H_2O_2 from Sigma Aldrich) and hydrochloric acid 37 % ACS, (HCl, from Fluka) were acquired from VWR Prolab Chemical (Pennsylvania, USA). The Toluidine Blue O dye was acquired from Sigma-Aldrich (Saint Louis, MO, USA). Entellan® new (refractive index, 1.490 - 1.500) was acquired from Merck (Darmstadt, Germany). Ultrapure water was obtained using a SG Ultra Clear UV plus equipment.

Physicochemical parameters were determined using standard methods and using the following substances: manganese chloride ($MnCl_2$, 99%), purchased from PanReac AppliChem (Barcelona, Spain), potassium iodide (KI) purchased from Riedel, sodium hyposulfite ($Na_2S_2O_3$) from Sigma-Aldrich was purchased from Merck (Darmstadt, Germany), ammonium hydroxide 25% (NH_4OH), was acquired from Sigma-Aldrich (Steinheim, Germany). Sodium nitrite ($NaNO_2$) and nitric acid 65% (HNO_3) were purchase from Merck (Darmstadt, Germany) and Zambelli's reagent was prepared using HCl, sulfanilic acid ($C_6H_7NO_3S$) purchased from Merck (Darmstadt, Germany), crystallized phenol purchased from Sigma-Aldrich (Darmstadt, Germany) and ammonium chloride acquired from PanReac AppliChem (Barcelona, Spain) whereas potassium permanganate ($KMnO_4$) from PanReac AppliChem was acquired from José Manuel Gomes dos Santos (Odivelas, Lisboa) . Sodium nitrate ($NaNO_3$) was acquired from Sigma-Aldrich (Steinheim, Germany). Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}$. 4 H_2O (81-83 %) and antimony potassium tartrate ($K_2Sb_2(C_4H_2O_6)_2$) from Sigma-Aldrich were acquired from Merck (Darmstadt, Germany).

NO_3^- and NO_2^- absorbance was measured using a UV/Vis spectrophotometer (Unicam UV/VIS Spectometer, from ATI Unicam), whereas for the determination of phosphates, a microplate reader BioTek Synergy 2 (Vermont, USA) was used. For measuring turbidity, a Merck Turbiquant 1100T turbidimeter was used, while pH was measured using a Crison pH Meter GLP21, and conductivity was measured using a Crison Conductimeter GLP31. Mercury thermometer to measure the water temperature at each location.

A Thermo Scientific's Heraeus megafuge 1.0R centrifuge and Z 206 A's Hermle, were used for centrifugation of water samples. For analysis and identification of diatoms an

optical microscope (Axiostar plus, from Zeiss) coupled to a digital camera (Canon PowerShot G9) were used to record images.

3.2. Study area and sampling collection

Three types of aquatic ecosystems with different hydrological characteristics and anthropogenic pressures were selected for water sampling, namely a stream Ribeira da Asprela (two sampling points), an estuary - Ave River estuary (two sampling points in the ornithological reserve) and two wells, located in two different geographical zones: Póvoa de Varzim and Paredes (Table 1).

Table 1. Water samples and their sampling location.

WATER TYPE	Sample identification	LOCAL	GPS COORDINATES
Stream	Sample 1 AsR	Asprela stream, Paranhos, Porto	41º10'38"N8º35'51"W
Stream	Sample 2 AsR	Asprela stream, Paranhos, Porto	41º10'36"N8º36'04"W
Estuarine	Sample 1 AvR	Ave River, Ornithological Reserve, Vila do Conde	41º20'28.8”N8º44'26.0”W
Estuarine	Sample 2 AvR	Ave River, Ornithological Reserve, Vila do Conde	41º20'26.1”N8º44'30.5”W
Well	Well PV	Póvoa de Varzim	Private location
Well	Well PD	Paredes	Private location

Surface water samples were collected in Summer (24th July 2022), Autumn (20th November 2022), Winter (03rd March 2023) and Spring (04th June 2023). Samples were collected into glass containers (three replicates for each sampling point) previously cleaned with distilled water. *In situ*, the glass containers were previously passed by local water that were rejected before collection of the samples. Temperature was measured *in situ* using a contact thermometer. All other parameters as pH, conductivity, turbidity, dissolved oxygen, nitrates, nitrites, and phosphates, were determined upon arrival to the laboratory. Two sampling points were selected at each urban freshwater ecosystem to evaluate spatial distribution. Although these sampling points were not representative of the stream/estuary, they were selected to have a preliminary picture of the seasonal and spatial distribution of the diatom composition in both aquatic systems.

Asprela stream is an affluent of the Leça River with most of its length blocked off (by plumbing), and a small area in the open. Its sampling sites cross the university campus, in a recently developed urban park, and underwent a requalification and re-naturalization project in mid-2015.

Two sampling points were also selected at Ave River estuary located in the ornithological reserve area. Although this is a natural reserve, this area is subject to anthropogenic pressures due to agricultural and livestock practices.

Also, surface water samples from two wells located in different geographical areas in the north region of Portugal were collected. The well from Póvoa de Varzim, is in a private sandy soil area, close to a dune cord, whereas the well from Paredes, is also in a private area but near agricultural zones.

3.3. Diatom sample preparation and identification

For identification and quantification of diatoms, 250 mL of water samples were collected at each sampling point in triplicate (Figure 8). Upon arrival to the laboratory, samples were centrifuged using 50 mL centrifuge tubes, at 3000 rpm, for 15 minutes and at 4°C. Then, the supernatant was discarded. A residue of 5 ml per sample was stored at -20ºC until the digestion process.

Figure 8. Sample preparation for further diatom analysis.

A preliminary study was performed to determine the best oxidation procedure for analysing diatoms in water samples. Three different procedures using H_2O_2, $KMnO_4$ and HNO_3were performed according to literature and optimized (McLaughin, 2012; Zhao et al., 2012; Levin et al., 2017; Zhou et al., 2020).

The treatment with H_2O_2 was found to be the most successful regarding execution procedure, extraction, and analysis of diatoms. The procedure was optimized and consisted of the following steps: 5 mL of water sample was homogenized by vortexing, transferred into a beaker and 8 mL of hydrogen peroxide was added. Then, the samples were heated for 15 minutes at 90ºC in a hot plate and, after that, 1 mL of hydrochloric acid 37% was added, allowed to react for 5 minutes at room temperature and then allowed to boil in a hot plate. After that, the beaker was removed from the hot plate and left to stand for 1 hour at room temperature. The content of the beaker was transfer to centrifuge tubes, centrifuged at 3000 rpm and left to stand around 2 hours. After two hours, the supernatant was removed up to 1 mL and distilled water was added to complete the 10 mL being left to stand until the following day. The supernatant was removed again up to 1 mL and transferred to eppendorf tubes and 1 mL of distilled water was added. The samples were centrifuged at 13000 rpm for 10 minutes, the supernatant removed and washed again with distilled water (500 µL). This procedure was repeated 3 times. Finally, the samples were reconstituted in 250 µL and stored at -20ºC until preparation of permanent slides.

For permanent preparation, 50 µL of each sample was put on a coverslip and evaporated on a hot plate and mounted in Entellan® new. For that, a drop of Entellan® was added to a slide placed on a hot plate and covered with the coverslip, the slide was immediately allowed to cool by removing from the hot plate.

Permanent slides were observed using an optical microscope. Nevertheless, using this procedure, some features of diatom frustules were not possible to be observed. Therefore, the procedure was repeated, but before mounting the coverslip in mounting medium, a drop of dye was added. Different dyes were tested as methylene blue, toluidine blue, safranin, eosin-blue, methylene giemsa and fuchsin. The toluidine blue dye showed the best results for observation of diatom characteristics allowing better identification and quantification.

Each replica of each water sample was first visualized under a 10 x 5 objective and a 10 x 100 oil immersion objective following the zigzag movement. Each diatom found was photographed for later identification and the number of diatoms registered. Diatoms

were identified based on literature (Kelly, 2000) and an online tool for diatom identification available at: http://craticula.ncl.ac.uk/EADiatomKey/html/index.html.

3.4. Physicochemical determinations

3.4.1. pH, conductivity and turbidity

The pH and conductivity were determined immediately after arriving at the laboratory (Figure 9).

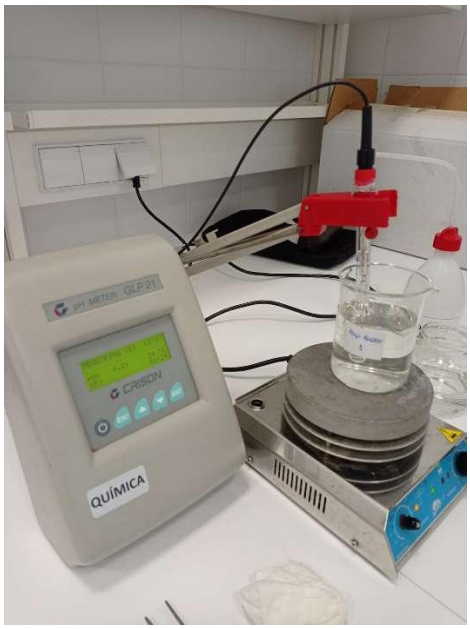

Figure 9. pH meter.

For determination of turbidity, a calibration curve was constructed using a series of calibration standards provided with the equipment that were placed in the device's specific container (Figure 10). The turbidity is measured through the incidence of radiation in the sample and a standard reference of the device is used and the results are presented in NTU (Nephelometric Turbidity Unit).

Figure 10. Turbidimeter.

3.4.2. Dissolved oxygen

For the determination of dissolved oxygen, the Winkler method was used. Manganese ions are added to the sample, oxidizing the dissolved oxygen to form manganese oxides. Next, iodide ions are added, resulting in the release of iodide ions which, in turn, react with the manganese oxide formed, releasing manganese ions and iodine. The iodine released is titrated with a standard solution of sodium hyposulphite. The amount of hyposulphite used in the titration is proportional to the amount of dissolved oxygen in the sample. The results were expressed as mg/L of O_2.

3.4.3. Nitrate, nitrite and phosphates

For measuring nitrate levels, a spectrophotometric method was used. For that, a stock standard solution was prepared using $NaNO_3$, absorbance was read at 220 nm and a calibration curve was constructed ranging from 1.00 to 7.00 mg/L. The results were expressed as mg/L of NO_3^-.

NO_2^- and phosphates were determined using the Zambelli and ascorbic acid methods, respectively. For determination of NO_2^-, a standard solution of $NaNO_2$ was prepared and used for preparation of standards for the calibration curve. The absorbance was read at 435 nm and a calibration curve ranged from 0.02214 to 0.4428 mg/L. The results were expressed in mg/L of NO_2^-. For determining phosphates, absorbance was measured at 880 nm, and a calibration curve was performed from 0.150 to 2.00 µg/mL. The results were expressed as µg/L of phosphates.

3.5. Climate data

Climate data (Table 2) was sourced from the *Instituto Português do Mar e da Atmosfera* - Portuguese Institute of Sea and Atmosphere (IPMA) at https://www.ipma.pt/pt/publicacoes/boletins.jsp?cmbDep=cli&cmbTema=pcl&idDep=cli&idTema=pcl&curAno=-1.

Table 2. Seasonal parameters of Porto's district by IPMA.

PARAMETERS	SEASON			
	SUMMER (JULY 2022)	AUTUMN (NOVEMBER 2022)	WINTER (MARCH 2023)	SPRING (JUNE 2023)
TºC MIN mean	16.6ºC	11.0ºC	9.7ºC	16.1ºC
TºC MAX mean	26.8ºC	17.3ºC	17.8ºC	24.1ºC
TOTAL PRECIPITATION	3.8 mm	295.0 mm	82.3 mm	48.7 mm

4. RESULTS AND DISCUSSION

4.1. Diatom seasonal and spatial distribution

Diatom populations have been demonstrated to be sensitive to environmental changes and seasonal variations as these factors affect hydrological conditions and biogeochemical processes (Kelly, 2000; McLaughlin, 2012; Luís et al., 2016; Annenkov et al., 2022). For instance, precipitation may cause dilution of water systems but also causes surface run-off nearby these systems and possible increasing levels of some chemicals in the receiving waters. For a better understanding of diatom seasonal and spatial distribution, Table 2 shows IPMA climate data at each sampling collection season. Autumn and winter were characterized by high precipitation, with the highest values registered in autumn. In both seasons, temperature was similar with maximum levels reaching ~17˚C. The lower precipitation values were registered in late spring and summer months, although during spring high temperature levels where registered reaching values up to 24˚C.

Most literature reports have shown higher diatom abundance in the autumn and spring seasons, although, some diatom genera have a different seasonal pattern. Moreover, other factors such as physicochemical water parameters, the type of aquatic environment, pollution and climatic variations can interfere with population peak (Lee, 2008; McLaughlin, 2012).

Figures 11 and 12, and Table 3, show the seasonal and spatial distribution diatom composition of Asprela stream. The higher number of diatoms was registered in summer and winter with some types being found only in summer (e.g. *Fragilaria* sp. and *Staurosira* sp.) and others only in winter (e.g. *Luticola* sp. and *Rhoicosphenia abbreviata*). Spring sampling collection coincided with a drought weather that caused an intense reduction of water levels in the stream. In fact, spring of 2023 was the third driest registered in Portugal, which may have affected the diatoms growth population and survival.

In Asprela stream sample 1 AsR, no centric diatoms were found. This can be due to the fact that centric diatoms are more commonly found in estuarine and marine environments. However, contrary to what would be expected, in Asprela riverine sample 2 AsR, a colony of the genus *Melosira*, that inhabits both freshwater and marine habitats, was found.

Other differences in seasonal and spatial distribution were also found. For example, diatoms belonging to the genus *Cocconeis* were only found in summer and winter in sample 1 AsR (Figure 11), whereas the genera *Pinnularia*, *Nitzschia*, and *Eunotia* were only found in sample 2 AsR (Figure 12). Nevertheless, some pennate diatom species from sample 2 AsR were not possible to be identified due to the limitations of optical microscopy. Therefore, although there may be differences in the spatial distribution of diatom species, due to the difficulty in identifying some specimen's interpretation of possible spatial differences should be performed with careful.

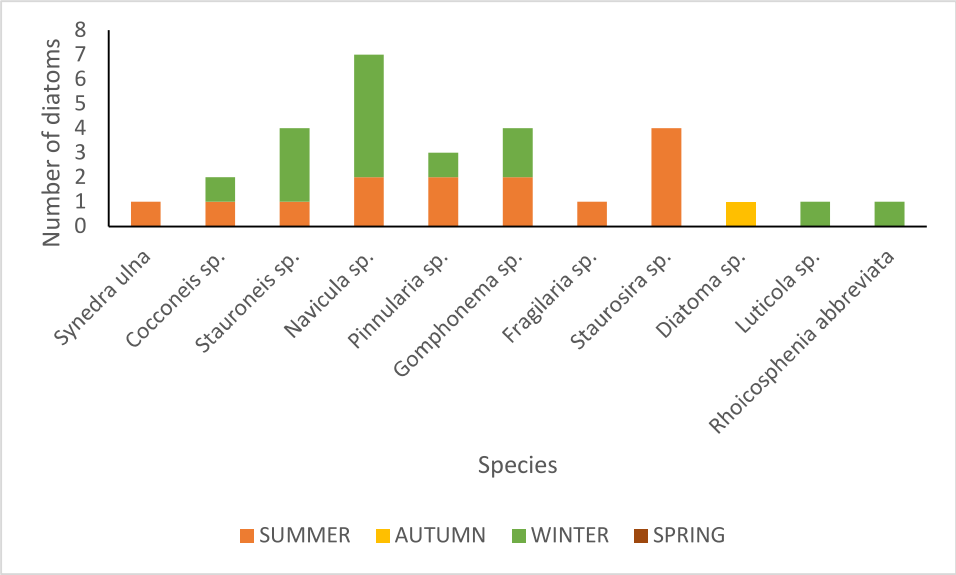

Figure 11. Diatom seasonal variation of Asprela Stream sample 1 AsR.

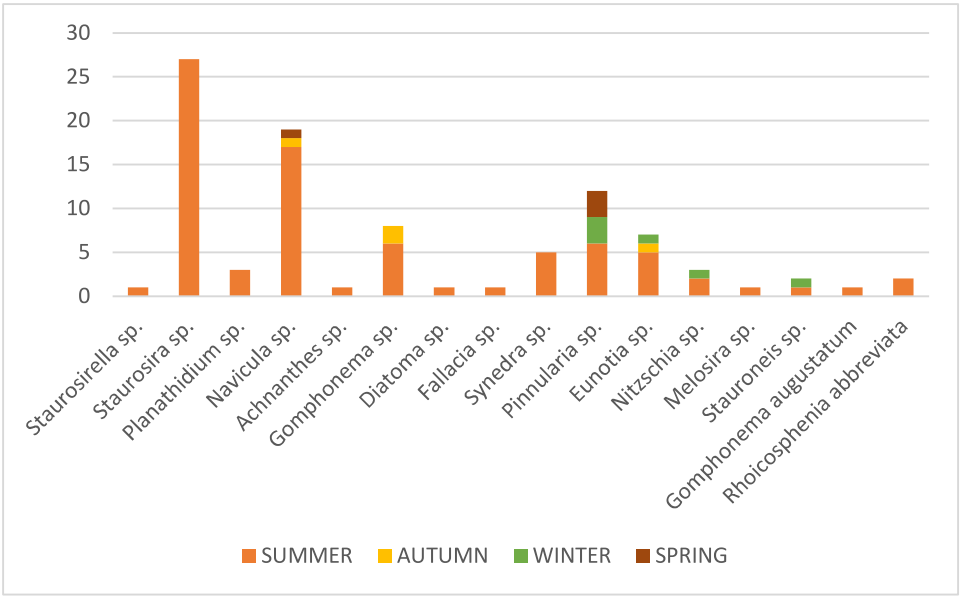

Figure 12. Diatom seasonal variation of Asprela Stream sample 2 AsR.

Table 3. Seasonal and spatial distribution of diatoms in Asprela stream (n.i. = not identifiable).

Sample/collection	Replicate	Summer		Autumn		Winter		Spring	
		Class/ Number	**Identification**	**Class/ Number**	**Identification**	**Class/ Number**	**Identification**	**Class/ Number**	**Identification**
Sample 1 AsR	**1**	**Pennate:** 6 **Centric:** 0	**Pennate:** 1 *Cocconeis* sp. 1 *Stauroneis* sp. 1 *Synedra ulna* 3 n.i.	**Pennate:** 2 **Centric:** 0	**Pennate:** 1 *Diatoma* sp. 1 n.i.	**Pennate:** 28 **Centric:** 0	**Pennate:** 13 Bacillariophyceae 1 *Cocconeis* sp. 1 *Gomphonema* sp. 1 *Luticola* sp. 3 *Navicula* sp. 1 *Rhoicosphenia abbreviata* 3 *Stauroneis* sp. 5 n.i.	**Pennate:** 1 **Centric:** 0	**Pennate:** 1 Bacillariophyceae
	2	**Pennate:** 1 **Centric:** 0	**Pennate:** 1 n.i.	**Pennate:** 1 **Centric:** 0	**Pennate:** 1 Bacillariophyceae	**Pennate:** 4 **Centric:** 0	**Pennate:** 1 *Gomphonema* sp. 2 *Navicula* sp. 1 *Pinnularia* sp.	**Pennate:** 0 **Centric:** 0	
	3	**Pennate:** 25 **Centric:** 0	**Pennate:** 4 Bacillariophyceae 2 *Gomphonema* sp. 1 *Fragilaria* sp.	**Pennate:** 3 **Centric:** 0	**Pennate:** 3 n.i.	**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0	

			2 *Navicula* sp. 2 *Pinnularia* sp. 4 *Staurosira* sp. 10 n.i						
Sample 2 AsR	**1**	**Pennate:** 83 **Centric:** 0	**Pennate:** 14 Bacillariophyceae 1 *Achnanthes* sp. 1 *Diatoma* sp. 1 *Eunotia* sp. 1 *Fallacia* sp. 2 *Gomphonema* sp. 1 *Luticola* sp. 4 *Navicula* sp. 1 *Nitzschia* sp. 2 *Pinnularia* sp. 3 *Planothidium* sp. 1 *Staurosirella* sp. *10 Staurosira* sp. 5 *Synedra* sp. 36 n.i.	**Pennate:** 5 **Centric:** 0	**Pennate:** 2 Bacillariophyceae 1 *Gomphonema* sp. 2 n.i.	**Pennate:** 3 **Centric:** 0	**Pennate:** 3 Bacillariophyceae	**Pennate:** 3 **Centric:** 0	**Pennate:** 1 Bacillariophyceae 1 *Navicula* sp. 1 n.i.

	2	**Pennate:** 53 **Centric:** 1	**Pennate:** 10 Bacillariophyceae 4 *Eunotia* sp. 1 *Gomphonema* sp. 10 *Navicula* sp. 1 *Nitzschia* sp. 4 *Pinnularia* sp. 2 *Stauroneis* sp. 7 *Staurosira* sp. 13 n.i. **Centric:** 1 *Melosira* sp. (colony)	**Pennate:** 3 **Centric:** 0	**Pennate:** 1 Bacillariophyceae 2 n.i.	**Pennate:** 11 **Centric:** 0	**Pennate:** 2 Bacillariophyceae 3 *Pinnularia* sp. 1 *Stauroneis* sp. 5 n.i.	**Pennate:** 3 **Centric:** 0	**Pennate:** 2 Bacillariophyceae 1 *Pinnularia* sp.
	3	**Pennate:** 50 **Centric:** 0	**Pennate:** 20 Bacillariophyceae 1 *Eunotia* sp. 3 *Gomphonema* sp. 1 *Gomphonema augustatum*	**Pennate:** 8 **Centric:** 0	**Pennate:** 1 Bacillariophyceae 1 *Eunotia* sp. 1 *Gomphonema* sp. 1 *Navicula* sp. 4 n.i.	**Pennate:** 5 **Centric:** 0	**Pennate:** 1 Bacillariophyceae 1 *Eunotia* sp. 1 *Nitzschia* sp. 2 n.i.	**Pennate:** 3 **Centric:** 0	**Pennate:**1 Bacillariophyceae 2 *Pinnularia* sp.

			3 *Navicula* sp. 2 *Rhoicosphenia abbreviata* 1 *Stauroneis* sp. 10 *Staurosira* sp. 9 n.i.						

Regarding samples collected from Ave River estuary, differences in seasonal and spatial distribution were also found (Figure 13 and 14, and Table 4). Indeed, similarly, to Asprela stream, samples collected in summer and winter showed the highest content and diversity of diatoms for sampling point 1 AvR, although high variation among the number of diatoms were observed in replicates. Both pennate and centric diatom were found. Sample 1 AvR showed the higher number of centric diatoms in winter compared to the other seasons. It was possible to find diatoms of the genus *Cyclotella* and colonies of different species of the genus *Melosira.* Nevertheless, the majority were centric diatoms or with a diameter of less than 5 µm. These diatoms are possible to belong to the genus *Thalassiosira* but other characteristics beyond size were not possible to be observed and thus confirm identification. Regarding sampling point 2 AvR, winter showed the lower number of diatoms whereas summer, autumn and spring showed similar number of diatoms and low variation in the number among replicates.

Seasonal and spatial distribution was found for genus *Cymbella* (Figure 13) that was only found in the winter and the species *Tabellaria fenestrata* (Figure 14 and 15) in the autumn. Diatoms belonging to the genus *Asterionella* were only found in winter, nevertheless, diatom fragments similar to *Asterionella* sp. were also found in other seasons. In sample 1 AvR, in spring, *Surirella* sp. (Figure 16) and *Cymatopleura* sp. (Figure 17) were found. While it was not possible to identify some diatom species, the shapes of the unidentified diatoms suggested that they may belong to the lanceolate to rhombic *Navicula* sp. or *Achnanthes* sp.

Genera with planktonic, benthic and epiphytic behaviour were found in both sampling points. In fact, even though only surface waters were collected and thus not favouring the occurrence of benthic and epiphytic diatoms, these specimens were found in both sample points. This can be explained by the low deep of the estuary in this area of the natural reserve, being surrounded by local flora and macrophytes.

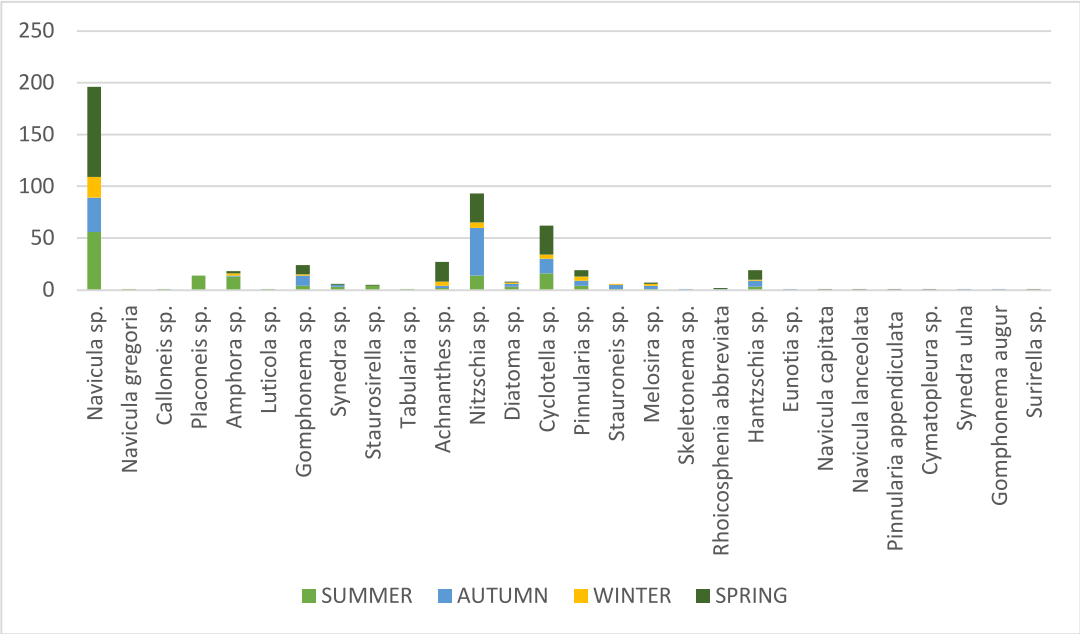

Figure 13. Diatom seasonal variation of Ave River estuary sample 1 AvR.

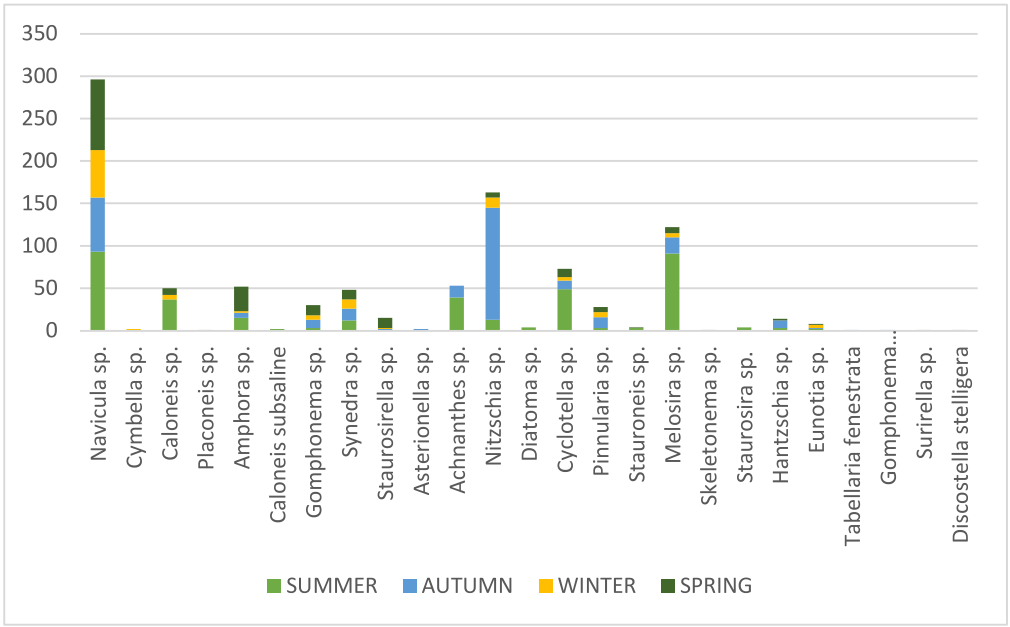

Figure 14. Diatom seasonal variation of Ave River estuary sample 2 AvR.

Table 4. Seasonal and spatial distribution of diatoms in Ave River estuary (n.i. = not identifiable).

Sample/collection	Replicate	Summer		Autumn		Winter		Spring	
		Class/ Number	**Identification**	**Class/ Number**	**Identification**	**Class/ Number**	**Identification**	**Class/ Number**	**Identification**
Sample 1 AvR	**1**	**Pennate:** 183 **Centric:** 17	**Pennate:** 42 Bacillariophyceae 1 *Achnanthes* sp. 4 *Amphora* sp. 1 *Caloneis* sp. 1 *Diatoma* sp. 5 *Gomphonema* sp. 1 *Luticola* sp. 15 *Navicula* sp. 1*Navicula gregoria* 3 *Nitzschia* sp. 10 *Placoneis* sp. 2 *Staurosira* sp. 3 *Staurosirella* sp. 1 *Synedra* sp. 1 *Tabularia* sp. 92 n.i. **Centric:**	**Pennate:** 20 **Centric:** 5	**Pennate:** 1 Bacillariophyceae 1*Gomphonema augur* 1 *Gomphonema* sp. 4 *Navicula* sp. 3 *Nitzschia* sp. 2 *Pinnularia* sp. 1*Rhoicosphenia abbreviata* 7 n.i. **Centric:** 1 *Melosira* sp. (colony) 4 n.i.	**Pennate:** 15 **Centric:** 185	**Pennate:** 2 *Achnanthes* sp. 1 *Hantzschia* sp. 4 *Navicula* sp. 1 *Nitzschia* sp. 1 *Pinnularia* sp. 6 n.i. **Centric:** 1 *Cyclotella* sp. 184 n.i.	**Pennate:** 61 **Centric:** 14	**Pennate:** 7 Bacillariophyceae 6 *Achnanthes* sp. 2 *Amphora* sp. 6 *Gomphonema* sp. 2 *Hantzschia* sp. 13 *Navicula* sp. 6 *Nitzschia* sp. 19 n.i. **Centric:** 3 *Cyclotella* sp. 11 n.i.

			7 *Cyclotella* sp. 10 n.i.						
	2	**Pennate:** 185 **Centric:** 15	**Pennate:** 34 Bacillariophyceae 1 *Achnanthes* sp. 9 *Amphora* sp. 2 *Diatoma* sp. 1 *Gomphonema* sp. 36 *Navicula* sp. 3 *Nitzschia* sp. 3 *Pinnularia* sp. 4 *Placoneis* sp. 1 *Stauroneis* sp. 1*Staurorsirella* sp. 1 *Synedra* sp. 89 n.i. **Centric:** 7 *Cyclotella* sp. 1 *Melosira* sp. (colony) 7 n.i.	**Pennate:** 57 **Centric:** 24	**Pennate:** 5 Bacillariophyceae 2 *Achnanthes* sp. 1 *Amphora* sp. 1 *Diatoma* sp. 1 *Fallacia* sp. 2 *Gomphonema* sp. 2 *Hantzschia* sp. 11 *Navicula* sp. 8 *Nitzschia* sp. 1 *Pinnularia* sp. 1 *Stauroneis* sp. 1 *Synedra* sp. 21 n.i. **Centric:** 6 *Cyclotella* sp. 1 *Melosira* sp. (colony) 1 *Skeletonema* sp. (colony) 16 n.i.	**Pennate:** 22 **Centric:** 178	**Pennate:** 2 *Achnanthes* sp. 2 *Amphora* sp. 1 *Ghomphonema* sp. 11 *Navicula* sp. 3 *Nitzschia* sp. 3 *Pinnularia* sp. **Centric:** 3 *Cyclotella* sp. 2 *Melosira* sp. (colony) 173 n.i.	**Pennate:** 68 **Centric:** 15	**Pennate:** 1 Bacillariophyceae 2 *Achnanthes* sp. 2 *Gomphonema* sp. 5 *Hantzschia* sp. 21 *Navicula* sp. 7 *Nitzschia* sp. 1 *Pinnularia* sp. 1 *Rhoicosphenia abbreviata* 28 n.i. **Centric:** 5 *Cyclotella* sp. 10 n.i.

	3	**Pennate:** 70 **Centric:** 5	**Pennate:** 24 Bacillariophyceae 2 *Gomphonema* sp. 3 *Hantzschia* sp. 5 *Navicula* sp. 8 *Nitzschia* sp. 1 *Pinnularia* sp. 1 *Synedra* sp. 26 n.i. **Centric:** 2 *Cyclotella* sp. 3 n.i.	**Pennate:** 126 **Centric:** 17	**Pennate:** 18 Bacillariophyceae 1 *Eunotia* sp. 2 *Diatoma* sp. 7 *Gomphonema* sp. 4 *Hantzschia* sp. 1 *Luticola* sp. 18 *Navicula* sp. 35 *Nitzschia* sp. 2 *Pinnularia* sp. 3 *Stauroneis* sp. 1 *Synedra* sp. 1 *Synedra ulna* 33 n.i. **Centric:** 8 *Cyclotella* sp. 1 *Melosira* sp. (colony) 8 n.i.	**Pennate:** 13 **Centric:** 187	**Pennate:** 1 *Diatoma* sp. 5 *Navicula* sp. 1 *Nitzschia* sp. 1 *Pinnularia* sp. 1 *Stauroneis* sp. 4 n.i. **Centric:** 187 n.i.	**Pennate:** 159 **Centric:** 41	**Pennate:** 9 Bacillariophyceae 11 *Achnanthes* sp. 1 *Cymatopleura* sp. 1 *Diatoma* sp. 1 *Gomphonema* sp. 2 *Hantzschia* sp. 1 *Navicula capitata* 1 *Navicula lanceolata* 53 *Navicula* sp. 15 *Nitzschia* sp. 1 *Pinnularia appendiculata* 5 *Pinnularia* sp. 1 *Staurosira* sp. 1 *Staurosirella* sp. 1 *Surirella* sp. 1 *Synedra* sp. 54 n.i. **Centric:** 20 *Cyclotella* sp.

									1 *Melosira* sp. (colony) 20 n.i.
Sample 2 AvR	**1**	**Pennate:** 118 **Centric:** 82	**Pennate:** 7 *Achnanthes* sp. 10 *Amphora* sp. 8 *Caloneis* sp. 2 *Caloneis subsaline* 1 *Cocconeis* sp. 2 *Diatoma* sp. 3 *Diploneis* sp. 1 *Eunotia* sp. 4 *Fallacia* sp. 2 *Hantzschia* sp. 41 *Navicula* sp. 5 *Nitzschia* sp. 2 *Pinnularia* sp. 1 *Placoneis* sp. 2 *Stauroneis* sp. 4 *Staurosira* sp. 1 *Staurosirella* sp. 1 *Surirella* sp. 6 *Synedra* sp.	**Pennate:** 167 **Centric:** 33	**Pennate:** 35 Bacillariophyceae 9 *Achnanthes* sp. 2 *Amphora* sp. 2 *Asterionella* sp. 2 *Cocconeis* sp. 1 *Eunotia* sp. 1 *Fallacia* sp. 2 *Gomphonema* sp. 4 *Hantzschia* sp. 26 *Navicula* sp. 52 *Nitzschia* sp. 4 *Pinnularia* sp. 3 *Synedra* sp. 24 n.i. **Centric:** 4 *Cyclotella* sp. 4 *Melosira* sp. (colony) 25 n.i.	**Pennate:** 101 **Centric:** 16	**Pennate:** 30 Bacillariophyceae 2 *Cocconeis* sp. 2 *Fallacia* sp. 4 *Gomphonema* sp. 14 *Navicula* sp. 5 *Nitzschia* sp. 2 *Pinnularia* sp. 1 *Staurosirella* sp. 5 *Synedra* sp. 36 n.i. **Centric:** 1 *Cyclotella* sp. 2 *Melosira* sp. (colony) 13 n.i.	**Pennate:** 168 **Centric:** 17	**Pennate:** 20 Bacillariophyceae 2 *Amphora* sp. 4 *Caloneis* sp. 11 *Diploneis* sp. 1 *Eunotia* sp. 39 *Fallacia* sp. 21 *Navicula* sp. 1 *Nitzschia* sp. 2 *Pinnularia* sp. 4 *Synedra* sp. 48 n.i. **Centric:** 1 *Melosira* sp. (colony) 16 n.i.

			15 n.i. **Centric:** 30 *Cyclotella* sp. 37 *Melosira* sp. (colony) 15 n.i.						
	2	**Pennate:** 119 **Centric:** 81	**Pennate:** 15 Bacillariophyceae 3 Fragillariophyceae 9 *Achnanthes* sp. 2 *Amphora* sp. 25 *Caloneis* sp. 1 *Cocconeis* sp. 2 *Diatoma* sp. 11 *Diploneis* sp. 1 *Eunotia* sp. 4 *Fallacia* sp. 16 *Navicula* sp. 6 *Nitzchia* sp. 1 *Pinnularia* sp. 3 *Synedra* sp. 20 n.i.	**Pennate:** 158 **Centric:** 42	**Pennate:** 22 Bacillariophyceae 5 *Achnanthes* sp. 2 *Amphora* sp. 2 *Cocconeis* sp. 2 *Fallacia* sp. 6 *Gomphonema* sp. 1 *Gomphonema acuminatum* 1 *Hantzschia* sp. 23 *Navicula* sp. 43 *Nitzschia* sp. 6 *Pinnularia* sp. 9 *Synedra* sp. 36 n.i. **Centric:** 2 *Cyclotella* sp.	**Pennate:** 105 **Centric:** 23	**Pennate:** 13 Bacillariophyceae 1 *Amphora* sp. 4 *Caloneis* sp. 5 *Diploneis* sp. 2 *Eunotia* sp. 1 *Gomphonema* sp. 24 *Navicula* sp. 3 *Nitzschia* sp. 5 *Synedra* sp. 47 n.i. **Centric:** 2 *Cyclotella* sp. 21 n.i.	**Pennate:** 170 **Centric:** 30	**Pennate:** 24 Bacillariophyceae 19 *Amphora* sp. 3 *Caloneis* sp. 5 *Diploneis* sp. 4 *Fallacia* sp. 5 *Gomphonema* sp. 2 *Hantzschia* sp. 30 *Navicula* sp. 3 *Nitzschia* sp. 2 *Pinnularia* sp. 7 *Staurosirella* sp. 5 *Synedra* sp. 61 n.i. **Centric:** 2 *Cyclotella* sp.

			Centric: 11 *Cyclotella* sp. 36 *Melosira* sp. (colony) 34 n.i.		12 *Melosira* sp. (colony) 28 n.i.				3 *Melosira* sp. (colony) 1 *Skeletonema* sp. (colony) 24 n.i.
	3	**Pennate:** 148 **Centric:** 52	**Pennate:** 33 Bacillariophyceae 23 *Achnanthes* sp. 3 *Amphora* sp. 4 *Caloneis* sp. 1 *Cocconeis* sp. 4 *Diploneis* sp. 9 *Fallacia* sp. *3 Gomphonema* sp. 36 *Navicula* sp. 2 *Nitzschia* sp. 1 *Stauroneis* sp. 3 *Synedra* sp. 26 n.i. **Centric:** 8 *Cyclotella* sp.	**Pennate:** 128 **Centric:** 21	**Pennate:** 33 Bacillariophyceae 2 *Amphora* sp. 1 *Diploneis* sp. 2 *Gomphonema* sp. 5 *Hantzschia* sp. 15 *Navicula* sp. 37 *Nitzschia* sp. 3 *Pinnularia* sp. 2 *Synedra* sp. 1*Tabellaria fenestrata* 27 n.i. **Centric:** 4 *Cyclotella* sp. 1 *Discostella stelligera*	**Pennate:** 119 **Centric:** 29	**Pennate:** 26 Bacillariophyceae 1 *Amphora* sp. 1 *Caloneis* sp. 1 *Cocconeis* sp. 2 *Cymbella* sp. 3 *Diploneis* sp. 2 *Eunotia* sp. 3 *Gomphonema* sp. 18 *Navicula* sp. 4 *Nitzschia* sp. 1 *Pinnularia* sp. 1 *Synedra* sp. 56 n.i. **Centric:** 3 *Melosira* sp. (colony) 26 n.i.	**Pennate:** 169 **Centric:** 31	**Pennate:** 24 Bacillariophyceae 8 *Amphora* sp. 1 *Caloneis* sp. 1 *Cocconeis* sp. 10 *Diploneis* sp. 3 *Fallacia* sp. 2 *Gomphonema* sp. 32 *Navicula* sp. 2 *Nitzschia* sp. 1 *Stauroneis* sp. 15 *Staurosirella* sp. 4 *Synedra* sp. 66 n.i. **Centric:** 8 *Cyclotella* sp.

			18 *Melosira* sp. (colony) 26 n.i.		3 *Melosira* sp. (colony) 13 n.i.				3 *Melosira* sp. (colony) 20 n.i.

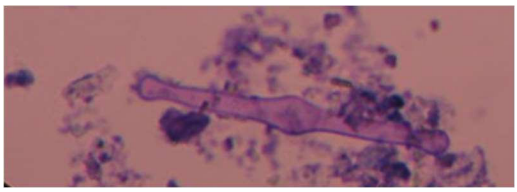

Figure 15. *Tabellaria fenestrata* diatom from Ave River estuary sample 2 AvR (autumn sample collection).

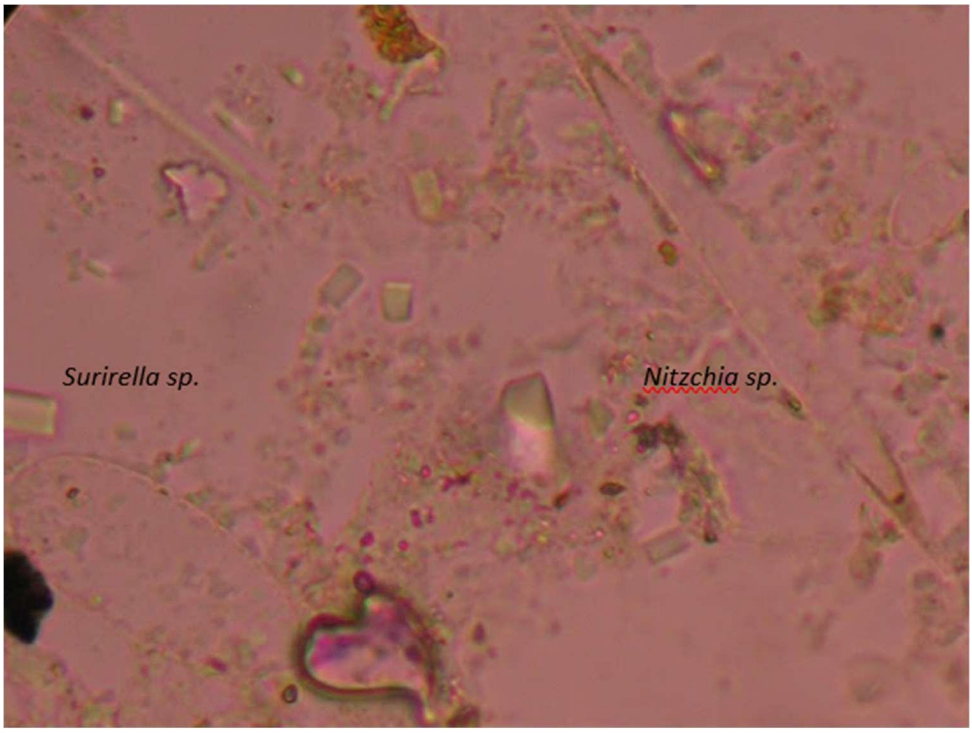

Figure 16. Genera *Surirella* and *Nitzschia* diatoms from Ave River estuary sample 1 AvR (winter sample collection).

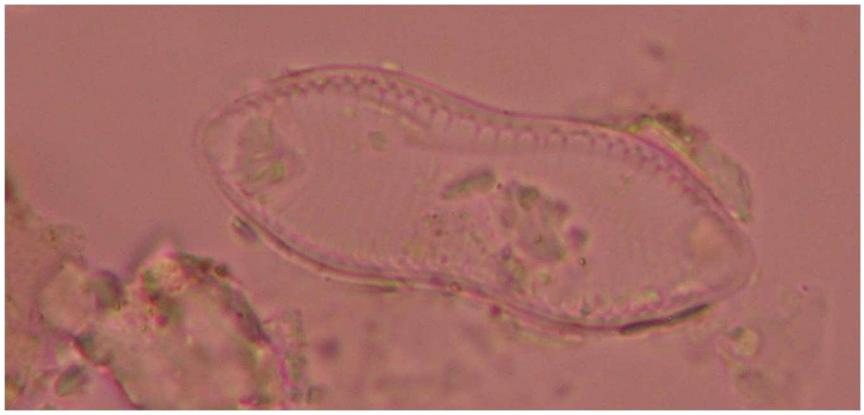

Figure 17. *Cymatopleura sp.* diatom from Ave River estuary sample 1 AvR (winter sample collection).

Seasonal and spatial distribution of diatoms from water samples collected from the wells are shown in Table 5. In both samples, the presence of diatoms was almost inexistent. In well PV, i.e., collected in Póvoa de Varzim, only two diatoms of the Bacillariophyceae class were found in one replicate, possible due to contamination of glass flasks used for collection or during sample digestion.

Regarding sample from PD well, i.e., collected in Paredes, although the number of diatoms was very low, the presence of diatoms was found in three seasons (summer, winter, and spring), and despite its low number, the possible contamination during storage or digestion was disregarded. This low population density may be due to leaching, particularly in winter, as the pennate diatoms may float since the genera found have benthic living behaviour.

The low abundance of diatoms in wells can be due to diverse factors. Indeed, planktonic diatom are denser than water thus, in non-hydrodynamic aquatic systems such as wells, diatoms will tend to sink and be deposited at the bottom. Also, the lack of light for photosynthesis and growth is an important factor that may contribute to the lower abundance of diatoms in wells or ground waters.

Table 5. Seasonal and spatial distribution of diatoms from Póvoa de Varzim (PV) and Paredes (PD) wells (n.i. = not identifiable).

Sample/collection	Replicate	Summer		Autumn		Winter		Spring	
		Class/ Number	Identification	Class/ Number	Identification	Class/ Number	Identification	Class/ Number	Identification
Well PV	1	**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0	
	2	**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0	
	3	**Pennate:** 2 **Centric:** 0	**Pennate:** 2 Bacillariophyceae	**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0	
Well PD	1	**Pennate:** 1 **Centric:** 0	1 n.i.	**Pennate:** 0 **Centric:** 0		**Pennate:** 3 **Centric:** 0	**Pennate:** 3 Bacillariophyceae	**Pennate:** 2 **Centric:** 0	**Pennate:** 2 *Caloneis* sp.
	2	**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0		**Pennate:** 1 **Centric:** 0	**Pennate:** 1 *Navicula* sp.	**Pennate:** 1 **Centric:** 0	n.i.
	3	**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0		**Pennate:** 0 **Centric:** 0	

4.2. Physicochemical parameters

The physicochemical parameters of water samples are shown in Table 6. Temperature showed a seasonal variation ranging between 11 °C in winter (sample 1 AvR and sample 2 AvR) to 21.5 °C in summer (both wells). The lowest pH value (5.26) was determined in spring in water samples collected from PD well whereas the highest value was determined in winter in Asprela stream, sample 1 AsR (7.80) indicating an overall weak acid to weak alkaline nature. Estuarine water samples showed the higher conductivity values in all sampling collections, as expected due to proximity to the sea. Portugal is mainly characterized by a warm temperate climate, with higher pluviosity during the winter and early spring and dry weather in summer, therefore, temperature, pH and conductivity corroborate a seasonal expected variation. Values are also similar to others reported in similar water systems (Rodrigues et al., 2015; Coelho et al., 2016; Luis et al., 2016; Santana et al., 2019).

Beyond the previous parameters, rainy and dry seasons also interferes with other physicochemical characteristics such as turbidity causing changes in light intensity that may affect photosynthesis taxa and growth. Indeed, with the increase in precipitation there is a greater movement of water, turning over the sediments present at the bottom and increasing turbidity. The higher values were always found in Ave River estuary and the highest values were found in autumn followed by winter in both sampling points. In fact, compared to other seasons, in the autumn of 2022, the total rainfall was 295 mm in the Porto district in November (Table 2). This may explain the high value of turbidity determined in the estuarine samples, with values > 80 NTU.

High pluviosity also enhances leaching of agricultural fields increasing values of nutrients as nitrogen and phosphate.

Considering a short/acute exposure, all samples are within acceptable nitrate values for aquatic life quality. However, considering chronic exposure, values determined in spring season were above 13 mg/L for all samples except for PV Well.

Regarding nitrite levels, considering long-term exposure, only water samples collected from both wells were within the acceptable range. Water samples collected from both Asprela stream and Ave River estuary were above 0.197 mg/L (except sample AsR from summer).

Dissolved oxygen levels vary with water temperature, being in cold water (autumn and winter) 6.5 - 9.5 mg/L and in warm waters (spring and summer) 5.5 – 6.0 mg/L (CCME, 1999). Values below those limits can interfere with aquatic life. Values below established limits were determined in water sample 2 AsR in autumn, both wells in summer and water samples AvR also collected in summer corroborating with the high temperature registered in this period (Table 2).

Phosphate levels are highly important indicators for determining nutrient levels in the aquatic environment, whether the environment is oligotrophic or eutrophic, i.e. poor or rich in nutrients, respectively. As expected, the wells are considered ultraoligotrophic, since the values were not detectable. The same occurred with the stream samples. For the estuary samples, water samples are considered hypereutrophic, as expected due to the high intensity of agricultural practices nearby, and the values exceed 100 µg/L as can be seen in the Table 6. In autumn, it was not possible to determine the phosphate content in water samples.

Table 6. Physicochemical parameters of the selected water bodies: Asprela stream (1 AsR; 2 AsR), Ave River estuary (1 AvR; 2 AvR) and two wells (PV = Póvoa de Varzim; PD = Paredes).

Physical-chemical parameters	**Summer**	**Autumn**	**Winter**	**Spring**
Sample 1 AsR				
T(ºC)	20.5	15	13	18
pH	7.43	7.05	7.80	7.08
Conductivity (µs/cm)	424	145	336	295
Turbidity (NTU)	0.07	13.3	2.18	1.2
D.O (mg/L)	6.04	8.91	8.10	6.45
Nitrate (mg/L NO_3^-)	1.99	0.432	3.24	**59.6**
Nitrite (mg/L NO_2^-)	0.133	**0.226**	**0.212**	**0.587**
Phosphate (µg/L)	ND	ND	ND	ND
Sample 2 AsR				
T(ºC)	19	15	13	18

pH	7.04	7.10	7.43	6.74
Conductivity (µs/cm)	393	193	244	272
Turbidity (NTU)	0.36	4.77	1.98	0.53
D.O (mg/L)	6.02	**6.33**	7.54	6.35
Nitrate (mg/L NO_3^-)	0.049	0.626	2.35	**42.3**
Nitrite (mg/L NO_2^-)	ND	**0.276**	**0.400**	**0.282**
Phosphate (µg/L)	ND	ND	ND	ND
Sample 1 AvR				
T(ºC)	19.5	16.8	11	19
pH	7.09	7.22	6.68	7.27
Conductivity (µs/cm)	13100	333	310	4300
Turbidity (NTU)	1.7	236	2.56	2.57
D.O (mg/L)	**4.11**	11.4	8.94	6.95
Nitrate (mg/L NO_3^-)	1.16	1.56	3.55	**45.4**
Nitrite (mg/L NO_2^-)	0.79	**4.99**	**1.41**	**0.289**
Phosphate (µg/L)	117	-	ND	ND
Sample 2 AvR				
T(ºC)	19.5	17.5	11	19
pH	7.68	7.14	6.54	7.79
Conductivity (µs/cm)	29300	2920	6880	15260
Turbidity (NTU)	2.84	590	41.4	81.5
D.O (mg/L)	5.69	7.66	10.2	6.60
Nitrate (mg/L NO_3^-)	0.589	0.643	1.06	**18.9**
Nitrite (mg/L NO_2^-)	**1.12**	**0.650**	**0.636**	**1.45**
Phosphate (µg/L)	247	-	0.448	188
Sample Well PV				
T(ºC)	21.5	15.5	12	18
pH	6.43	7.00	6.41	6.6
Conductivity (µs/cm)	509	631	308	388

Turbidity (NTU)	0.03	3.71	2.12	52.9
D.O (mg/L)	**3.55**	8.01	7.93	5.96
Nitrate (mg/L NO_3^-)	2.61	0.157	0.053	2.74
Nitrite (mg/L NO_2^-)	ND	0.123	0.111	0.096
Phosphate (µg/L)	ND	-	ND	ND
Sample Well PD				
T(ºC)	21.5	16	12	21
pH	5.33	6.31	6.84	5.26
Conductivity (µs/cm)	118	37.1	255	72.6
Turbidity (NTU)	0	0.48	0.01	0.01
D.O (mg/L)	**4.67**	8.53	7.24	6.23
Nitrate (mg/L NO_3^-)	0.93	0.056	0.64	**19.3**
Nitrite (mg/L NO_2^-)	ND	0.111	0.088	0.077
Phosphate (µg/L)	ND	-	ND	ND

4.3. Diatom composition and relationship with physicochemical parameters

The physical and chemical parameters such as temperature, pH, conductivity/salinity, and nutrients content such as nitrate, nitrite, phosphates affect diversity and population abundance of diatoms (Rodrigues et al., 2015; Luis et al., 2016; Vajravelu et al., 2018). On the other hand, physicochemical parameters show seasonal fluctuation due to rainfall, tidal inflow, among others. Besides, anthropogenic activities may enhance nutrient contents.

Phosphate is of great importance for the development of most diatom species. Genera such as *Nitzschia*, *Cyclotella* and *Melosira*, bloom in eutrophic environments with high levels of nitrate and phosphate, contributing to an increase in diatom biomass. On the other hand, genus *Gomphonema*, known for its preference for oligotrophic conditions, dominates in clear, well-oxygenated waters with lower nutrient concentrations. High abundance of *Nitzschia* sp., *Cyclotella* sp. and *Melosira* sp. diatoms were found in the estuary samples (Table 4) in comparison to *Gomphonema* sp. corroborating with the high levels of these nutrients in the estuary. Regarding Asprela

stream, high abundance of *Nitzschia* and *Navicula* diatoms were also found. The species belonging to these genera show high adaptability to polluted environments, with high nitrite levels, and oligotrophic characteristics.

Similarly, dissolved oxygen levels in the water affect diatom metabolism, with species such as most *Nitzschia* responding to well-oxygenated conditions.

In addition, the adaptability of the genus *Amphora* to diverse environments, both freshwater and marine, highlights the versatility of certain diatom genera. This genus was found in the estuary water samples and prefers high salinity conductivity levels corroborating the determined conductivity levels of water samples collected in this aquatic system.

With regard to the applicability of these identifications in forensic science, we can conclude that although we have knowledge about the behavior of diatoms in each region, the different aquatic environments, as well as the changes in physicochemical parameters caused by global warming, affect their behavior. This tells us which diatoms we can find in each location and why.

When analyzing the results presented in tables 3 to 6, there is some doubt as to whether these diatoms contribute exclusively to forensic investigations. However, quantitative analysis of the specific genus is useful. For example, diatoms such as *Navicula* sp., known for its high tolerance to pollution, and *Nitzschia* sp., which can exhibit benthic, epiphytic, and sometimes planktonic behaviour, depending on the species, provide valuable information.

As a result, although the work had its limitations, there is enough material to obtain a useful tool of what diatoms can be found in these chosen regions and a comparative study regarding communities found in different aquatic systems with difference hydrological and anthropogenic pressures.

5. CONCLUSIONS

The study revealed the complex interaction of biotic and abiotic factors that influence the composition and abundance of diatom populations. By collecting and analyzing water samples from different environments, such as streams, estuaries and wells, it was possible to learn more about the dynamic nature of diatom communities and their response to different environments, seasonal variation, and physical and chemical factors. It became clear that the population density and species assemblage of diatom in water bodies are influenced by various factors, including hydrological conditions, nutrient availability, and anthropogenic activities.

We found that the hydrological and anthropogenic factors of the selected aquatic systems had a significant impact on diatom composition and physicochemical parameters. For example, in the estuary of the Ave River, we found a higher concentration of *Navicula* sp. diatoms, which are known to have a high tolerance to pollution. In contrast, the Asprela stream zone had a higher concentration of *Nitzschia* sp. diatoms, which can have benthic, epiphytic, and sometimes planktonic behavior, depending on the species.

Several important differences were identified in the composition and distribution of diatoms between the estuarine zones of the Ave River and the Asprela stream. For example, in the estuary of the Ave River, a higher number of diatoms that prefer high salinity/ conductivity levels were found, which corroborates the conductivity levels determined from the water samples collected in this aquatic system. In contrast, the Asprela stream side area had a higher number of diatoms that prefer lower salinity levels.

In addition to our findings on the stream and estuarine zones, it is important to note that our research also included an analysis of diatom distribution in well water samples. We observed that the presence of diatoms in the well samples was almost non-existent, with only a few diatoms of the Bacillariophyceae class found in one replicate of the Póvoa de Varzim well sample. This minimal presence of diatoms in the well water samples may be attributed to various factors, including possible contamination during collection or digestion, as well as the non-hydrodynamic nature of well water, which may lead to diatoms settling at the bottom due to their density.

Furthermore, the low abundance of diatoms in the well water samples may also be influenced by factors such as limited light for photosynthesis and the benthic living behavior of certain diatom genera. These factors contribute to the lower abundance of diatoms in well or ground waters compared to surface water bodies.

While our initial focus was on the stream and estuarine zones, the inclusion of well water samples in our research provides valuable insights into the distribution and abundance of diatoms in non-surface water environments. This aspect of our study underscores the importance of considering diverse aquatic systems when investigating diatom populations and their ecological dynamics.

Overall, our research has provided valuable insights into the seasonal and spatial distribution of diatoms in different aquatic systems. While there are still limitations to our understanding of diatom behavior and their usefulness in forensic investigations, we believe that our findings can serve as a useful tool for future research in this field.

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7. ATTACHMENTS

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7.1. Attachment 1. Poster Communication at the V Congress of the Portuguese Association of Forensic Sciences/ XIV Scientific Conference on Sciences of the University Institute of Health Sciences/ I TOXRUN International Congress.

Variation of the physical-chemical parameters of diverse water bodies for study of diatom distribution and composition for forensic investigations

M. Portugal[1], C. Couto[1,2*], A. Madureira-Carvalho[1,3], C. Ribeiro[1]

[1]TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, Gandra, Portugal.
[2]LAQV/REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal.
[3]LAQV/REQUIMTE, Laboratory of Pharmacognosy, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal

* E-mail:cristina.couto@iucs.cespu.pt

BACKGROUND

Diatoms are microalgae of fundamental ecological role in aquatic ecosystems; however, these microorganisms have been shown to provide valuable information for forensic investigations. Due to the characteristic diatom distribution in a water body, the presence of diatoms in objects may allow to correlate to a specific aquatic system and/or a suspect (Figure 1). Additionally, in cases of suspicious of death by drowning, the presence of diatoms in some organs and/or bones may give important information to support confirmation (Figure 2) [1-3].

Fig.1. Objects (t-shirt and knife) that may link to suspects.

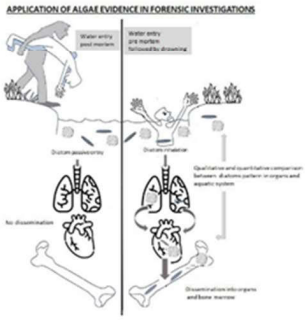

Fig. 2. In death by drowning, diatoms can be found in differents organs. Unlike of dead for other causes, if diatoms are found, they will be located only in the upper respiratory tract (even if the person has been previously exposed to diatoms).

The occurrence of diatoms, concerning diversity and frequency in a water body, depends on spatial, temporal and physical-chemical water parameters [3,4].

OBJECTIVE

The aim of this study was to determine the seasonal and spatial variation of the physical-chemical parameters of different water for further correlation with the presence and geo-temporal variation of diatom composition.

METHODS

Six sampling points were selected in different regions of the Porto District:

- two estuarine waters, in the natural reserve area of the Ave River (Figure 3);
- two stream waters, on the Asprela streams (Figure 4);
- two well waters (Póvoa de Varzim and Paredes).

Samples were collected seasonally (Summer, Autumn and Winter) and temperature, conductivity, turbidity, pH, dissolved oxygen, nitrate, nitrite and phosphate were measured.
Water samples aliquots were separated for further analysis for diatom composition.

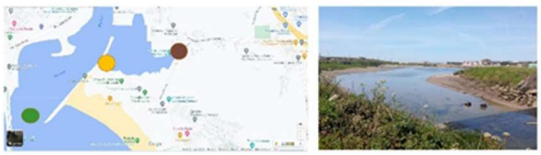

Fig. 3. a) Location of the sampling points from Ave River (brown = Ave river bridge; yellow = Ave river reserve; green = the end of the estuary). b) view from the Ave river bridge.

Fig. 4. a) Location of the sampling points from Asprela streams. b) Asprela stream.

RESULTS AND DISCUSSION

Results are presented in tables 1-3.

PHYSICO-CHEMICAL PARAMETERS	AVE RIVER RESERVE	AVE RIVER BRIDGE	ASPRELA FEUP	ASPRELA FEP*	WELL PÓVOA	WELL PAREDES
T (°C)	19.5	19.5	20.5	19.0	21.5	21.5
pH	7.68	7.09	7.43	7.04	6.43	5.33
Conductivity (µs/cm)	29300	13100	424	393	509	118
DO (mg/L)	5.69	4.11	6.04	6.02	3.56	4.87
Nitrate (mg/L)	0.59	1.16	1.89	0.049	2.61	0.93
Nitrite (mg/L)	1.12	0.79	0.13	nd	nd	nd
Turbidity (NTU)	2.84	1.70	0.07	0.36	0.03	0.00

Table 1. Summer sampling.
*FEUP = Faculdade Engenharia do Porto; **FEP = Faculdade Economia do Porto; ***nd = not detected

PHYSICO-CHEMICAL PARAMETERS	AVE RIVER RESERVE	AVE RIVER BRIDGE	ASPRELA FEUP	ASPRELA FEP*	WELL PÓVOA	WELL PAREDES
T (°C)	17.5	16.8	16.0	15.0	15.5	16.0
pH	7.14	7.22	7.05	7.10	7.00	6.31
Conductivity (µs/cm)	2920	333	149	193	531	37
DO (mg/L)	7.66	11.40	8.91	6.33	8.01	8.83
Nitrate (mg/L)	0.64	1.56	0.43	0.63	0.16	0.06
Nitrite (mg/L)	0.65	4.99	0.23	0.28	0.12	0.11
Turbidity (NTU)	590	226	13.3	4.77	3.71	0.48

Table 2. Autumn sampling.
*FEUP = Faculdade Engenharia do Porto; **FEP = Faculdade Economia do Porto

PHYSICO-CHEMICAL PARAMETERS	AVE RIVER RESERVE	AVE RIVER BRIDGE	ASPRELA FEUP	ASPRELA FEP*	WELL PÓVOA	WELL PAREDES
T (°C)	11	11	13	13	12	12
pH	6.54	6.68	7.8	7.43	6.41	6.84
Conductivity (µs/cm)	6880	310	336	244	306	255
DO (mg/L)	10.2	8.94	8.09	7.54	7.90	7.24
Nitrate (mg/L)	1.06	3.65	3.24	2.35	0.0533	0.643
Nitrite (mg/L)	***	-	-	-	-	-
Turbidity (NTU)	41.4	2.56	2.18	1.98	2.12	0.01

Table 3. Winter sampling.
*FEUP = Faculdade Engenharia do Porto; **FEP = Faculdade Economia do Porto; ***- = not determined

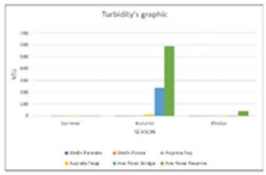

Figure 5. Parameters of turbidity of the three seasons.

Physical-chemical parameters showed seasonal variation. In Summer, water temperature ranged from 19 to 21.5ºC whereas in Winter from 11 to 13ºC. Also, due to intense rain periods, in Autumn and Winter, high levels of turbidity were noted, especially when compared to Summer (Figure 5). Spatial differences were also found. The highest turbidity value was 590 NTU, at Ave nature reserve, which may affect the light availability. During the rainy season, the water flow was higher, with a higher exchange of material from the soil surface and water, leading to a greater availability of nutrients (nitrate and nitrite) and a greater concentration of dissolved oxygen (Table 1-3, in bold). According to aquatic life quality criteria, the levels found for dissolved oxygen, nitrate, nitrite, and pH were within acceptable limits for this type of ecosystems. Overall, the well waters showed the lower nutrients content except in summer (Table 1-3, underlined). Physical-chemical parameters may affect diatom composition. Data about diatom composition is ongoing, but preliminary studies showed a low occurrence of diatoms in well water samples.

CONCLUSIONS

These results suggest that, for these well water bodies, the presence of suspended diatom may be low which may difficult the use of these organisms for forensic investigation. Nevertheless, further experiments are ongoing to correlate seasonal and spatial variation of the physical-chemical parameters with composition of other water bodies.

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7.2. Attachment 2. Poster Communication at the II TOXRUN International Congress.

IMPORTANCE OF DIATOMS IN THE DIAGNOSIS OF DROWNING

Mariana Torres-Portugal[1*], Áurea Madureira-Carvalho[1,2], Cristina Couto[1,3], Cláudia Ribeiro[1,4]

[1]TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, Advanced Polytechnic and University Cooperative (CESPU), CRL, 4585-116 Gandra, Portugal; [2]REQUIMTE/LAQV, Laboratory of Pharmacognosy, Department of Chemistry, Faculty of Pharmacy, University of Porto, 4200-319 Porto, Portugal; [3]LAQV/REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal. [4] Interdisciplinary Center of Marine and Environmental Research (CIIMAR), University of Porto, Edifício do Terminal de Cruzeiros do Porto de Leixões, Matosinhos, Portugal.
*Email: mtpramirez@gmail.com

INTRODUCTION

Diatoms are photosynthetic unicellular microalgae found in the majority of the aquatic habitats, where they play an important role as primary producers (Figure 1) [1,2].

Fig. 1. Different aquatic ecosystems provide different species of diatoms.

Diatoms characteristic is the frustule, a cell wall composed of silica (SiO_2), which provides resistance and different shapes, sizes, and colours, according to the species (Figure 2A) [3]. The morphology of the frustule is the main characteristic for identifying these organisms. Figure 2B shows the basic external structure of a diatom.

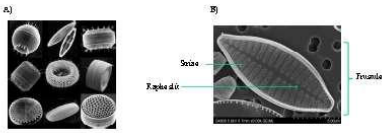

Fig. 2. A) The diatom cell wall provides different shapes and sizes allowing their identification; B) Basic external structure of a diatom.

Species and communities are diverse and environmentally specific due to their sensitivity to physicochemical properties of aquatic systems such as: pH, temperature, light, type of aquatic ecosystem and seasonal changes (Figure 3) [4,5].

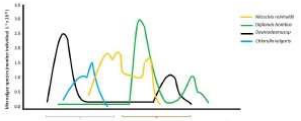

Fig. 3. Communities are specific of an aquatic habitat and seasonal changes.

Diatoms have been of great importance in forensic investigation providing a useful tool for different purposes including the diagnosis of drowning. In fact, objects of a crime, or persons linked to an accident or crime scene related with water, will have algae in/on them [1,2,4]. Their persistence due to the silica cell wall makes possible to detect them also in drowning victims, even in advanced states of decomposition.

Therefore, the purpose of this study is focused on diatoms and their traditional use in forensic investigations for the diagnosis of drowning death cases.

MATERIALS AND METHODS

Search was based on ScienceDirect and PubMed database considering the following keywords alone or in combination: diatoms, forensic science, and drowning in both humans and animals.

RESULTS AND DISCUSSION

A death by drowning may be either accidental, suicidal or homicidal. Diagnosis of death by drowning is based on drowning signs, however, these signs are absent when the body is decomposed or skeletonized.

Therefore, diatoms have a relevant importance in assisting post-mortem diagnosis of an individual found in an aquatic environment. It is possible to distinguish whether death occurred by drowning or by other cause, through the identification of diatoms present in lungs, brain, heart, kidney, liver and bone marrow (e.g., sternum and femur) or only in the upper respiratory tract (Figure 4) [2,3,4].

Diatoms possess two characteristics that makes them an important tool in forensic investigation:

- They are resistant to putrefaction
- They do not occur naturally in the body

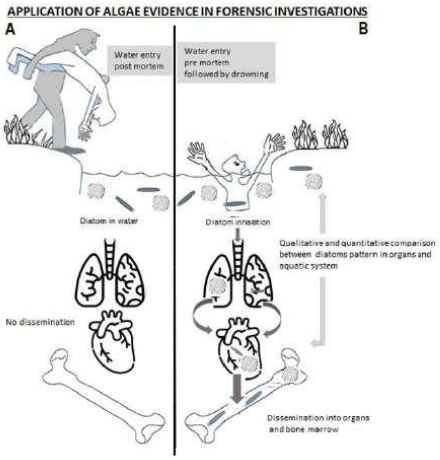

Fig. 4. Unlike dead for other causes (A), in death by drowning, diatoms can be found in differents organs (B).

Distinctive diatom species and pattern also help in the geolocation as algal communities are distinctive of certain aquatic ecosystems or water quality.

In fact, some studies have shown a correlation between organs and diatom pattern and drowning sites.

Additionally, their adherence to surfaces also allows the use of diatoms in the establishment of associative indexes between water submerged/ in contact objects and persons, aquatic systems and even seasons [3,5].

CONCLUSIONS

The identification and pattern of diatoms in a specific aquatic environment can therefore be a useful tool in forensic investigations.

The importance of diatoms should not be underestimated, and their role in Forensic Sciences should be valued.

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7.3. Attachment 3. Abstract at Scientific Letters, 1 (Sup), 107.

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Poster 65

Variation of the physical-chemical parameters of diverse water bodies for study of diatom distribution and composition for forensic investigations

M. Portugal [1], C. Couto [1,2,*], A. Madureira-Carvalho [1,3] and C. Ribeiro [1]

[1] TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, Gandra, Portugal
[2] LAQV/REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal
[3] LAQV/REQUIMTE, Laboratory of Pharmacognosy, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal

* Correspondence: cristina.couto@iucs.cespu.pt

Abstract

Background: Diatoms are microalgae of fundamental ecological role in aquatic ecosystems; however, these microorganisms have been shown to provide valuable information for forensic investigations. Due to the characteristic diatom distribution in a water body, the presence of diatoms in objects may allow to correlate to a specific aquatic system and/or a suspect. Additionally, in cases of suspicious of death by drowning, the presence of diatoms in some organs and/or bones may give important information to support confirmation [1-3]. The occurrence of diatoms, concerning diversity and frequency in a water body, depends on temporal and physical-chemical water parameters [3,4]. **Objective:** The aim of this study was to determine the seasonal and spatial variation of the physical-chemical parameters of different water for further correlation with the presence and geo-temporal variation of diatom composition. **Methods:** Six sampling points were selected in different regions of the Porto District: two wells (Póvoa de Varzim and Paredes); two on the Asprela streams; two in the natural reserve area of the Ave River. Samples were collected seasonally (Summer, Autumn and Winter) and temperature, conductivity, turbidity, pH, dissolved oxygen, nitrate, nitrite and phosphate were measured. Water samples aliquots were separated for further analysis for diatom composition. **Results:** The results showed a seasonal and spatial variation of physical-chemical water parameters. High levels of turbidity were found in autumn, in all water bodies and the sampling point located in natural reserve showed the highest value (589.6 NTU), and the highest content of nutrient which may affect diatom composition. Preliminary studies showed a low occurrence of diatom in well water samples. **Conclusions:** These results suggest that, for these water bodies, the presence of suspended diatom may be low which may difficult the use of these organisms for forensic investigation. Nevertheless, further experiments are ongoing to correlate physical-chemical parameters with composition of other water bodies.

Keywords: diatoms; physical-chemical parameters; well; river; streams

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7.4. Attachment 4. Abstract at RevSALUS - Revista Científica Internacional da Rede Académica Das Ciências Da Saúde Da Lusofonia, 4 (Sup), 147.

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Importance of diatoms in the diagnosis of drowning

Mariana Torres-Portugal[1*], Áurea Carvalho[1,2], Cristina Couto[1,3], Cláudia Ribeiro[1,4]

[1]TOXRUN – Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL, 4585-116 Gandra, Portugal.

[2]LAQV/REQUIMTE, Pharmacognosy Laboratory, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, 4200-319 Porto, Portugal.

[3]LAQV/REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal.

[4]Interdisciplinary Center of Marine and Environmental Research (CIIMAR), University of Porto, Edifício do Terminal de Cruzeiros do Porto de Leixões, Matosinhos, Portugal.

*✉ mtpramirez@gmail.com

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Resumo

Introduction: Diatoms are unicellular microalgae ubiquitous in lotic or lentic aquatic ecosystems, though they can also be found on humid land surfaces, even if less frequently [1,2]. Individual species and communities are diverse and environmentally specific due to their sensitivity to pH, temperature, and type of aquatic ecosystem [3,4]. Diatom cell wall is composed of silica (SiO_2), which provides resistance and different shapes, size, and colours according to the species [5]. Diatoms have been of great importance in forensic investigation providing a useful tool for the diagnosis of drowning. In fact, objects of a crime or persons linked to an accident or crime scene that takes place in water will have algae in or on them [1,2,3]. **Objectives:** This work focuses on the significance of diatoms in the diagnosis of drowning death cases. **Methods:** Search was based on ScienceDirect and PubMed database and considering the following keywords alone or in combination: diatoms, forensic science, and drowning. **Results:** Diatoms have a relevant importance in assisting post-mortem diagnosis of an individual found in an aquatic environment. It is possible to distinguish whether death occurred by drowning or by other cause, through the identification of diatoms present in bone marrow, kidney, liver, or only in the upper respiratory tract [2,3,5]. Distinctive diatoms species and pattern also help in the geolocation as algal communities are distinctive of certain aquatic ecosystems or water quality. In fact, some studies have been shown the correlation between tissue and diatom pattern and drowning sites. Additionally, their adherence to surfaces also allows to establish a link between submerged objects, persons, and aquatic system and even seasons [4,5].**Conclusions:** The identification and pattern of diatoms in a specific aquatic environment can therefore be a useful tool in forensic investigation. The importance of diatoms should not be underestimated, and their role in Forensic Sciences should be evaluated.

Keywords: diatoms; drowning; aquatic ecosystem; microalgae; forensic science

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