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Departamento de Biologia

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Impact of wildfire ashes on aquatic systems: diatoms.

Impacto de cinzas de incêndios florestais em sistemas aquáticos: diatomáceas.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Salomé Fernandes Pinheiro de Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, e co-orientação do Doutor Nelson José Cabaços Abrantes, investigador assistente no Departamento do Ambiente e Ordenamento e no CESAM da Universidade de Aveiro, e da Doutora Isabel Maria Alves Natividade Campos, investigadora júnior do Departamento do Ambiente e Ordenamento da Universidade de Aveiro.

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palavras-chave

Incêndios florestais, cinzas, metais, hidrocarbonetos aromáticos policíclicos, extratos aquosos de cinzas, diatomáceas, teratologias.

resumo

Os incêndios florestais constituem um grande problema ambiental em países do sul da Europa, sendo um fenómeno recorrente e com diversos impactos. Um deles é o seu papel na produção, mobilização e distribuição de contaminantes, em particular metais e hidrocarbonetos aromáticos policíclicos (PAHs). Ambos são contaminantes de grande preocupação, elevada persistência ambiental, toxicidade e tendência a bioacumular e biomagnificar. Os incêndios florestais também têm a capacidade de aumentar a escorrência superficial, sendo que o consequente transporte de sedimentos e cinzas com PAHs e metais fixados pode acabar em habitats aquáticos. Desta forma, o *input* de escorrência superficial com diferentes contaminantes pode culminar em efeitos tóxicos em espécies aquáticas, entre as quais diatomáceas.
Tendo em conta que estas microalgas são um bom indicador ecológico, testes ecotoxicológicos foram feitos em *Nitzschia palea* (NPAL) e *Achnanthidium minutissimum* (ADMI) com dois extratos aquosos de cinzas (AEA), feitos com cinzas de baixa e alta severidade (LS e HS), derivadas de incêndios de baixa/média severidade (primeira campanha, em novembro) e alta severidade (segunda campanha, em março), respetivamente. Considerando que as diatomáceas reagem ao ambiente envolvente, foi observada uma resposta diferente aos dois AEA. Um aumento significativo do crescimento foi observado nos testes de toxicidade das duas espécies em AEA de alta severidade, enquanto em LS ocorreu o oposto, com NPAL a apresentar um EC_{50} de 104,98% e ADMI, mais sensível a apresentar um EC_{50} de 84,78%. As diferenças na toxicidade dos AEA podem ser devidas a diferenças na sua química. Ambas as espécies demonstraram respostas similares em todas as concentrações em ambos os AEA considerando anomalias da frústula (cerca de 50% de valvas teratológicas).
As teratologias também ocorreram nas comunidades de diatomáceas do rio Pequeno nomeadamente em *Karayevia oblongella* (KOBG), mais abundante na primeira campanha e *Achnanthidium minutissimum* (ADMI) que foi mais abundante na segunda. Este resultado pode ser derivado dos diferentes momentos de amostragem (Outono e Primavera, respetivamente).
Resumindo, o presente estudo demonstra os potenciais impactos de incêndios florestais em meios lóticos, comprometendo as condições químicas e ecológicas. Em adição demonstra que futuras pesquisas devem ser feitas, com o intuito de compreender melhor e avaliar os impactos reais dos incêndios florestais nos ecossistemas.

keywords Wildfires, ash, metals, polycyclic aromatic hydrocarbons, aqueous extracts of ash, diatoms, teratologies.

Abstract

Forest fires are a major environmental problem in countries of southern – Europe, being a recurrent phenomenon, with distant impacts. One of them is their role on the production, mobilization and distribution of contaminants, in particular metals and polycyclic aromatic hydrocarbons (PAHs). Both of them are contaminants of major concern, due to their high environmental persistence, toxicity and tendency to bioaccumulate and biomagnify. Wildfires also have the ability to increase runoff and the consequent transport of sediments and ash with PAHs and metals attached can end up in downstream aquatic habitats. Therefore, the input of surface runoff with different contaminants, could culminate in toxic effects on aquatic species, amidst diatoms.

Since these microalgae are a good ecological indicator, ecotoxicological tests were performed on *Nitzschia palea* (NPAL) and *Achnanthidium minutissimum* (ADMI) with two aqueous extracts of ash (AEA) made with ashes of low and high severity (LS and HS), from wildfires of low/moderate severity (fist campaign in november), and high severity (second campaign in march), respectively. Whereas diatoms react to the surrounding environment, they responded differently to the different AEA. A significant increment on the growth in the toxicity tests of both species was observed in high severity AEA, while in the LS the opposite occurred, with NPAL presenting an EC_{50} of 104.98% and ADMI, the most sensitive presented an EC_{50} of 84.78%. The differences in AEA toxicity may be due to differences in its chemistry. The two species demonstrated similar responses in every concentration in both AEA concerning abnormal cell wall (around 50% of teratological valves).

Teratologies also occurred in diatom communities of river Pequeno mainly in *Karayevia oblongella* (KOBG), more abundant in the first campaign and in *Achnanthidium minutissimum* (ADMI) more abundant in the second. This may be due to the different sampling season (autumn and spring respectively).

All in all, the present study highlights the potential impacts of wildfires on water bodies, compromising the chemical and ecological conditions. In addition, it demonstrates that further research should be done in order to better understand and evaluate the real impact of forest fires in the ecosystems.

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1. Introduction

Wildfires are a recurrent and natural phenomenon of most forest ecosystems in Mediterranean countries of southern - Europe, especially in dry years (Pausas, 2004; Pereira *et al.*, 2015). Nowadays, fire regime is strongly connected to land abandonment, that combined with the extensive planting of highly flammable tree species results in an increment of fuel load (Lloret, 2004; Moreira *et al.,* 2009; Carmo *et al.,* 2011; Shakesby, 2011). During the last decade a decreasing number of forest fires in Portugal (minus 3.6% of occurrences) was observed, whilst an increment of 428% of Burnt area occurred, with 442.418 ha of forest area affected in a single year (2017) (DGAPPF, 2017). The natural regime of wildfires is changing (IPCC, 2013; Moreira *et al.*, 2011; Mermoz, Kitzberger, & Veblen, 2005), and in the future these events will probably suffer a rise in severity and frequency in Portugal due to human activities, climate change, bad management of the forest areas, fire suppression and the character of the country's forestry activities (Fischlin *et al.* 2007; Pereira *et al.*, 2006, 2014).

Forest fires induce direct and/or indirect impacts and the post-fire induced overall changes can lead to short-, medium-, and long- term variations, such as changes in hydrological and geomorphological processes (DeBano *et al.*, 2005; Shakesby, 2011; Shakesby & Doerr, 2006). They can have large environmental, social and economic consequences, including: economic losses, impacts on terrestrial, aquatic and atmospheric compartments, adverse effects on biodiversity and land-use sustainability, and risk for public health and safety. In addition, forest fires also provide severe and instant impacts on the function and structure of ecosystems and their different compartments, while affecting the structure and composition of vegetation, shaping the landscape, and impacting biogeochemical cycles (Bodí *et al.*, 2014; Flannigan, Stocks, & Wotton, 2000; Neary, Ryan, & DeBano, 2005; Smith *et al.*, 2011).

An important consequence of wildfires with ecological, hydrological and geomorphological implications is the deposition of a layer of ash on the topsoil. According to Bodí *et al.* (2014), ash is the "particulate residue remaining, or deposited on the ground, from the burning of wildland fuels and consisting of mineral materials and charred organic components", and it's composed of small-sized granulates that can be assimilated into the soil profile after its deposition. It is naturally a very heterogeneous material, conditioned by: type and plant species, part Burnt (needles, bark, timber), soil type, combustion process, extensiveness and temperature (fire severity), meteorological conditions before,

after and during fire, the mixture of different flammable materials, and the time of exposure to elevated temperatures, which influences the chemical and physical characteristics of the ashes and its leachates as well as its spatial distribution (Abrantes *et al.*, 2017; Balfour & Woods, 2013; Bodí *et al.*, 2014; Demeyer *et al.*, 2001; Gabet & Bookter 2011; Goforth *et al.* 2005). It is alkaline and primarily constituted by carbonates, oxides and hydroxides, being the major inorganic components magnesium (Mg), calcium (Ca), silicon (Si) and potassium (K) (Demeyer *et al.*, 2001; Etiégni & Campbell, 1991; Gabet & Bookter, 2011; Pandey & Singh, 2010; Plumlee *et al.*, 2007) and in smaller quantities sodium (Na), sulphur (S) and phosphorous (P) (Bodí *et al.*, 2014). However, it also includes major and trace elements, like mercury (Hg), zinc (Zn), copper (Cu), chromium (Cr), aluminum (Al), iron (Fe), lead (Pb), nickel (Ni), vanadium (V), cobalt (Co), cadmium (Cd) and manganese (Mn). Some of these components are essential for living organisms such as zinc (Zn), iron (Fe) and copper (Cu), for example for gene regulation, protein structure stability and electron transfer reactions. Conversely others like mercury (Hg), cadmium (Cd) and lead (Pb) may replace or displace essential metals, which causes toxic effects, interfering with the correct functioning of enzymes and associated cofactors (Gifford *et al.*, 2004), being lead one of the primary inorganic contaminants in humans, animals and plants (Mu *et al.*, 2018). Polycyclic aromatic hydrocarbons (PAHs), produced during the combustion process, should also be taken into consideration due to their carcinogenic and mutagenic properties, wide range of ecotoxicological effects, environmental persistence, high toxicity, and predisposition to bioaccumulate and bioamplify in the food chain, which makes them dangerous contaminants (ATSDR, 1995; IARC, 1991, 2010).

Ashes can be mobilized or redistributed from its original site by means of water erosion, surface runoff or wind, into lakes, footslopes, reservoirs, surface depressions, streams and rivers, having impacts outside the Burnt area and affecting water quality and aquatic organisms. Hence, wildfires and the subsequent rainfall are key factors in the distribution of trace, minor, and major chemical elements associated to ashes in the terrestrial as well as aquatic compartments (Abdel-Shafy, 2015; Blandon *et al.*, 2014; Bodí *et al.*, 2014; Santín *et al.*, 2015; Smith *et al.*, 2011). These compounds may have impacts on surface and groundwater chemical composition, throughout their release by leaching from ash-soil interactions into the soil profile, combustion of vegetation, soil organic matter mineralization, or being eroded by surface runoff. Therefore, the mobilization of these compounds makes these processes important inputs of contaminants into rivers and estuaries, either in dissolved or particulate form. They are persistent and potentially toxic,

tending to accumulate over time and possessing a long lasting effect after elimination of the major sources, holding depositional features, and being a threat to aquatic systems (Campos *et al.*, 2012, 2016; Liu *et al.*, 2014; Nunes *et al.*, 2017; Silva *et al.*, 2015, 2016; Smith *et al.*, 2011; Verma & Jayakumar, 2012), bringing cumbersome consequences for aquatic biodiversity and water quality (Bodí *et al.*, 2014). Therefore, aquatic communities (*e.g.* fishes, macro-invertebrates, periphyton, phytoplankton and amphibians) may suffer injurious effects, due to accumulation of ash or some of its components in aquatic deposits (Bodí *et al.*, 2014), even one year after the fire event (Silva *et al.*, 2015). Additionally, post-fire inputs of particles can also contribute to water degradation (alter color, taste, odor), increment of the quantity of nutrients beyond the recommended limits, decreasing life-span of reservoirs and deterioration of water treatment processes (Bladon *et al.*, 2014). As a consequence, it leads to problems in potable water supplies (Bodí *et al.*, 2014), thus causing possible costs for water managers (Abrantes *et al.*, 2017).

The different geochemical forms of the various elements possess the ability to change when ash is transferred from the production site into anoxic/suboxic sediments in rivers and reservoirs (Santín *et al.*, 2015). It's important to underline that the water-sediment interaction plays a crucial part on regulating metals' transport processes (Thouzeau *et al.*, 2007). Regarding the PAHs in surface water, and depending on their physiochemical properties, they are able to oxidize, volatilize, photodegrade, bind to particulates, and in the river sediments PAHs can be biodegraded or accumulated in the aquatic organisms (Abdel-Shafy & Mansour, 2015). PAHs have high affinity for organic carbon and low solubility, which allows them in aquatic systems, to be found sorbed to particles instead of suspended in the water column or settled in the bottom (Abrantes *et al.*, 2017).

Regarding the potential toxicity of ash loaded runoff, Campos *et al.* (2012) and Silva *et al.* (2015) pointed-out that compounds mobilized or released by wildfires caused toxicity to standard aquatic organisms, like the macrophyte *Lemna minor* and the microalgae *Raphidocelis subcapitata* decreasing their growth, and the bioluminescent bacterium *Vibrio fischeri,* causing inhibitions in the luminescence of the bacteria (Abrantes *et al.*, 2017). Nunes *et al.* (2017) using a biomarker-based approach, found early-warning signals in the fish *Gambusia holbrooki* due to toxicity triggered by aqueous runoff and stream water samples collected from a forest Burnt area (Abrantes *et al.*, 2017). Hence, these studies point out that the production and mobilization of toxic chemicals by wildfires are harmful to aquatic species.

Besides the toxic effects, some studies have already focused on the consequences of wildfires on freshwater communities, including macroinvertebrates (Earl & Blinn, 2003; Mellon, Wipfli, & Li, 2008; Minshall, 2003; Silva *et al.*, 2016; Verkaik *et al.*, 2013), periphyton (Cowell, Matthews, & Lind, 2006; Earl & Blinn, 2003; Verkaik *et al.*, 2013) and fish (Rinne & Neary, 1996; Verkaik, *et al.*, 2013). They all testified shifts in communities' composition and reductions on abundance after-fire, although, after a certain time, communities returned to pre - fire conditions. Some of the changes suffered by the ecosystems occur during burning, like the alteration of the quality and quantity of organic matter content. It can vary from its nearly destruction to increased contents, mostly due to external inputs that includes forest necromass, partly charred litter and leaves, as well as residual ash (Certini, 2005; González-Pérez *et al.*, 2004; Knicker *et al.*, 2005; Mataix-Solera *et al.*, 2011). When that ash reaches the surface of a river, not only micronutrients are injected into the water but also macronutrients that have the ability to cause an increase in phytoplankton, especially in nutrient-limited areas (Zhang *et al.*, 2017).

Photoautotrophic organisms, such as macrophytes, and microalgae are essential to aquatic systems, being producers and the basis of the entire food chain (da Silva *et al.*, 2009; Luís *et al.*, 2011). Among distinct algae groups, diatoms play a crucial role in aquatic systems. They are eukaryotic, unicellular or colonial algae, that can be divided morphologically into centric or pennate showing radial or bilateral symmetry of the siliceous frustule ornamentation, respectively (Round *et al.* 1990). The cell wall of diatoms, the siliceous frustule, is the basis for the morphological identification due to its intricately and diversified ornamentation. Diatoms are geographically and ecologically very widely distributed and its estimated that there are about 100000 taxa (Mann & Vanormelingen, 2013; Ross & Mann, 1986), which makes it possible to be found in almost every ecosystem on the planet where nutrients and light exist in satisfactory quantities (Round *et al.*, 1990; Vieira, 2014). The production and storage of lipids and carbohydrates by microalgae is controlled by the availability of nutrients such as phosphorus (P), silica (Si) and nitrogen (N) (Vieira, 2014).

Freshwater diatoms are widely used as indicators of water quality, including the evaluation of trophic status and organic pollution (van Dam *et al.*, 1994; da Silva *et al.*, 2009; Feio *et al.*, 2009; Luís *et al.*, 2011; Um *et al.*, 2018), due to their clear response to variations in nutrient levels (Vilmi & Karjalainen, 2015) and organic content (van Dam *et al.* 1994) at the species level. Additionally, diatom assemblages are very diverse, with

different environmental preferences and tolerances, a quick response to chemical alterations of the adjacent medium, and short life cycles, being among the biological quality elements specified in the European Water Framework Directive for management of water resources (van Dam *et al.*, 1994; da Silva *et al.*, 2009; Feio *et al.*, 2009; Luís *et al.*, 2011; Mu *et al.*, 2018). They belong to one of the biggest groups of silicified organisms that possess a siliceous frustule. Thus, Si is a core macronutrient for their growth (Jiang *et al.*, 2014[a], 2014[b]).

Intraspecific variation allows each one to adapt to changes in the environment and even under the same conditions, microalgae demonstrate differences at biochemical and physiological levels (Blomberg & Garland, 2002; Esteves *et al.*, 2018; Keck *et al.,* 2016). It is also known that phenotypic characters have the ability to evolve in an independent way from the genotypic ones, which results in sensitivity differences not measurable at the genetic level (Esteves *et al.*, 2018). Diatoms occur, not only in the water column as planktonic organisms, but may also develop extensive benthic communities as they are capable of attaching to all types of substrates, responding in a faster way to environmental alterations than higher level organisms (van Dam *et al.*, 1994).

Trace elements can accumulate in aquatic organisms by means of uptake from suspended particles and sediment, directly from water, or by the consumption of lower trophic level organisms (Wang, 1987). In addition environmental concentrations tend to result in more sub-lethal responses. Thus, assessing the degree of stress that populations are laid on could help enhance the monitoring process, as well as ecological management (Esteves *et al.*, 2018). Diatoms can develop teratologies of the siliceous cell wall with alterations in the shape and ornamentation. These teratologies are sub-lethal responses to environmental stressors, but studies haven't been successful in demonstrating a relationship between the gradient of exposure and the proportion of teratologies, only being able to compare contaminated sites with non-contaminated. Observed deformities may occur in the striation pattern, the raphe/sternum, the general shape of the valve and other structures, or can be a combination of diverse modifications. The frequency of teratologies has been reported as a good biomarker for metal and organic contamination, since different contaminants have different toxic modes of action. It should be taken into account that the tolerance to teratologies is environment and species - dependent, making optimal conditions mitigate their occurrence and suboptimal conditions worsen (Falasco *et al.*, 2009; Lavoie *et al.*, 2017).

It should also be taken into consideration that microalgae in their natural habitat will experience environmental oscillations, from variations on sunlight supply (throughout the day as well as dissimilarities on cloud cover) to undulations on temperature (daily and in seasonal cycles) (Jiang *et al.*, 2014[b]), and that diatoms respond to chemical, hydromorphologic and physical changes in the environment (Elias *et al.*, 2015). Prior studies also demonstrate that growth and photosynthetic rates of phytoplankton have a tendency to increase succeeding nutrient addition or ash enrichment, although the responses may change among different species (Zhang *et al.*, 2017).

Understanding the impacts of wildfires is indispensable for risk assessment of environmental contamination, ecosystem management and sustainability, and different biological components must be considered in order to evaluate the status of the affected habitats (Abrantes *et al.*, 2017; Campos *et al.*, 2016; Nunes *et al.*, 2017; Silva *et al.*, 2015).

Despite the attempts to establish cause-effect relationships, the consequences of wildfires along the aquatic food chain are still being ignored. In particular, the study of the effects of contaminated sediments from Burnt areas in benthic organisms exposed for long periods, as diatoms, assumes high relevance, as these will impact the entire ecosystem throughout bottom-up relationships. Hence, this work aimed to study the impact of post-fire contamination in freshwater diatoms, either in terms of community composition or specific toxicity. To address this main goal, two specific objectives were defined: (i) study the effects of wildfires on diatom community structure and (ii) assess in the laboratory the response of two diatom species with distinct tolerances (*Nitzschia palea* (Kützing) W. Smith and *Achnanthidium minutissimum* (Kützing) Czarnecki) to aqueous extracts of ashes collected from low to moderate and high severity wildfires affecting eucalyptus forests.

2. Materials and Methods

2.1. Study area

The study area was located near the Nespereira de Cima village, Oliveira de Azeméis municipality, Aveiro district, north-central Portugal (Figure 1). At catchment scale, the study took place in River Pequeno (Figure 2), considered a Northern river of small drainage area (N1 < 100 km^2) concerning its national typology (INAG, IP, 2008). At River Pequeno, 3 study sites were defined for the collection of water, sediments and phytobenthos: upstream (Up - 40°47'16.3"N 8°25'38.0"W), within (Burnt - 40°46'50.4"N 8°26'06.2"W) and downstream (Down - 40°46'03.2"N 8°26'39.8"W) the Burnt area (Figure 2). A low/moderate severity fire affected the area on October 3rd 2018, and a second one of high severity on March 27th 2019. The wildfires consumed an area of around 66.394 ha and 320.39 ha, respectively, predominantly covered by eucalyptus (Figure 2). The mean annual temperature varies within 10 to 20 ºC. The area is characterized by a temperate climate, with wet winters and dry mild summers (IPMA, 2019). Over the past 20 years, the annual rainfall ranged from 2896.7 mm to 1204.76 mm (SNIRH, 2019).

Figure 1 – Geographical localization of the study area Nespereira de Cima village.

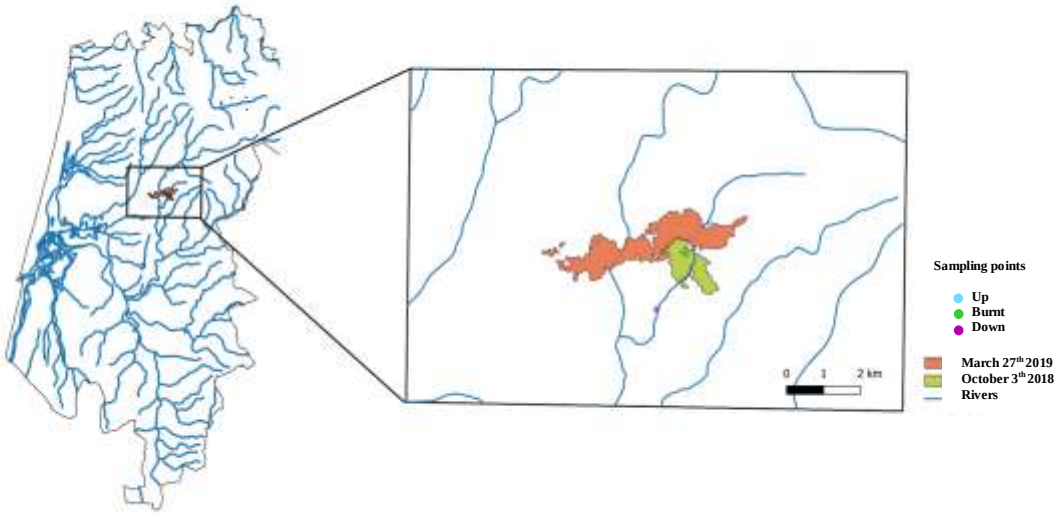

Figure 2 - Catchment, sampling points in River Pequeno and wildfires' area.

Fire severity was assessed according to the methodology described in Shakesby and Doerr (2006) and Keeley (2009). Hence, fire severity assessment was based on the degree of consumption of the canopies of 10 randomly selected trees as well as of the litter layer (partial vs. total) and on ash color (black vs. grey), which was classified by visual inspection. In the first event, the fire consumed the tree canopies only partially but the litter layer entirely and produced black ashes. In the second fire, it totally consumed both the tree canopies and the litter layer, and produced grey ashes. On the basis of these observations, fire severity was classified as low to moderate in the first fire and high severity in the second one.

2.2. Sampling Design

2.2.1. Ash

One Burnt eucalyptus slope was selected in the catchment area for each ash sampling campaign (one after the first fire and another after the second fire event). On each slope, one transect was laid out across the full length of the slope section. Five equidistant points were established from the top to the bottom of each slope to take into account ash spatial heterogeneity. At each of the five points of sampling transect, a grid was laid out and a plot of 50 x 60 cm was sampled for ash. At each sampling plot the entire ash layer was collected with a brush and a spoon, in order to avoid mixture with soil, and after that, the ash samples were sieved separately through a 2 mm mesh and

transported to the laboratory in plastic bags, under dark conditions. In the laboratory, the sieved ash samples were air-dried and then mixed in a container to produce a single composite sample which was subsequently stored at - 20ºC in dark plastic bags (to reduce microbial activity and to prevent photolysis of the PAHs) until the preparation of the aqueous extracts (Campos *et al.*, 2015, 2016).

2.2.2. Water and Sediments

Sampling of water and sediments in River Pequeno took place after the two fire events previously referred in each sampling site (upstream, Burnt and downstream the Burnt area). The first sampling occurred on the 15th November, 2018 (registered as 1) and the second on the 5th June, 2019 (registered as 2). The first sampling moment occurred after heavy rainfall, which could enable runoff into the water stream. At each sampling site and campaign, pH, electric conductivity (EC), dissolved oxygen (DO), temperature (TºC) and total dissolved solids (TDS) of water were measured in the field using a multiparameter probe (Hanna Instruments, HI 9829), as well as the current velocity (FP111 Global Water Probe). At the same time two surface water samples were collected *per site* into polyethylene terephthalate bottle for metal and nutrient analysis and into amber glass bottle for PAHs and carbon analysis. Two sediment samples were also collected at each sampling site and campaign. Samples were transported to the laboratory in cool boxes. In the laboratory, water samples were preserved and filtered, accordingly to the different chemical determination and stored at - 20ºC till further processing. Also the filters were stored at - 20ºC prior to the analysis of their contents (metals, PAHs and carbon). Sediments were air-dried and sieved through a 2 mm mesh and ground. Samples were stored in a black plastic bag and aluminum bags at - 20°C until further analysis.

2.2.3. Phytobenthos

After the two fire events, in the stream at the three established sampling sites, and avoiding shades and pools of stagnant water, epilithic samples were collected by scraping approximately 100 cm^2 of submerged stones with a toothbrush. These sampling processes followed the Water Framework Directive (WFD) protocol for *phytobenthos* (INAG, 2008; WFD, 2000). The samples were then divided in two: one of the sub-samples was preserved with formalin (ca. 8% final concentration), while the other was kept alive.

2.2.3.1. Sample processing

From the live sub-sample, upon arrival at the laboratory, a small part was cleaned and oxidized using potassium dichromate ($K_2Cr_2O_7$) and concentrated nitric acid (HNO_3) for 24h at room temperature. After this period, oxidation by-products were removed by, three centrifugations (1500rpm) (Hettich, Universal 16A). Permanent slides were mounted using Naphrax® (INAG, 2008). In each sample, the diatoms were identified to the lowest taxonomic category possible using available identification literature (Krammer 2000, 2001, 2009; Krammer and LangeBertalot, 1986, 1988, 1991[a], 1991[b]; Prygiel and Coste, 2000), and at least 400 valves were counted. Valve teratologies were also analyzed and counted (Annex I). The study of diatoms took place under the light microscope (Leitz Biomed 20 EB) equipped with a 100x objective with a numerical aperture of 1.32.

2.4. Preparation of aqueous extracts of ash (AEA) and analytical procedures

The aqueous extracts of ash (AEA) embodies the contaminant matrix that reaches aquatic systems after a wildfire. Aqueous extracts of ash from the low/moderate and high severity wildfires, previously referred, were prepared for posterior chemical analysis and for toxicity testing using unialgal diatom cultures. The resulting ashes of the low/moderate severity fire event are of low severity (LS), and the ashes originated on the high severity wildfire are of high severity (HS).

Five grams of ashes were mixed in 1L of the culture medium used for the tested species in ecotoxicological assessments. These mixtures were then placed in flasks wrapped in aluminum foil, in order to be protected from light, and were subjected to a period of 8h stirring in an orbital shaker at 220rpm (SCANSI SK-O330-Pro), being stored at 4ºC for a maximum period of 24h before its use in the ecotoxicological tests (Silva *et al.*, 2015).

It must be noted that the amount of ashes used to obtain the aqueous extracts of ashes (AEA) may have resulted in an ash concentration with larger proportions than that expected in natural runoff. On the account of the variability present in the environmental concentration, it is also extremely difficult to know the exact proportions of ash and solvent (in this case culture medium DVII) that should be used to make the AEA. For that reason, in this study several concentrations were tested in order to cover the approximate concentration present in the runoff.

2.4.1. Analytical procedures

2.4.1.1. Sediments samples

Approximately 500 mg of dry sediments (40°C) were digested with *aqua regia* (3 HCl:1 HNO_3) in covered Teflon beakers. The mixture was heated on a DigiPrep HotBlock at 95°C - 100°C until dryness. After this, 10ml of HNO_3 (4M) were added to the Teflon vessels and the solution was filtered through 0.45µm Whatman® Nucleopore™ filters to remove all ash particles and transferred into polypropylene volumetric tubes. After cooling, the solution was diluted to 50ml with Milli-Q® water (USEPA 3050B, 1996). Concentration of metals: vanadium (V), chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd) and lead (Pb) were determined by inductively coupled plasma mass spectrometry (ICP-MS - Thermo Elemental, X-Series). Quality control of the analytical procedures was ensured by the analysis of Certified Reference Material (CRM) and by testing every 10^{th} sample in duplicate. Blanks were prepared following the same analytical procedure and run in parallel with the CRM and samples.

The analysis of the PAH contents of sediments were restricted to the 16 priority PAHs defined by the United States Environmental Protection Agency (USEPA, 1995): acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benzo(a)anthracene (BaA), chrysene (CHR), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), dibenzo(a,h)anthracene (DBA), indeno(1,2,3-cd)pyrene (IND) and benzo(g,h,i)perylene (BGP). The PAH contents of the ash samples were analyzed following the procedure described in Martins *et al.* (2012). Prior to the actual analysis, however, the unfrozen samples were dried at 40ºC to minimize the loss of the volatile PAHs (Berset *et al.*, 1999). Pressurized liquid extraction (PLE) was performed using an ASE (Accelerated Solvent Extraction) 200 system (Dionex, USA) equipped with 24 stainless steel extraction cells. The extraction cells were lined with cellulose filter paper, packed (from bottom to top) with 5g of sample mixed with diatomaceous earth, spiked with 1ml surrogate standards obtained from Supelco (Bellefonte, PA, USA) containing acenaphthene-d^{10} (0.408µg ml^{-1}), phenanthrene- d^{10} (0.397µg ml^{-1}), chrysene-d^{12} (0.397µg ml^{-1}) and perylene-d12 (0.433µg ml^{-1}) and topped with cellulose filter paper. Extractions were performed with a mixture of hexane:acetone (1:1, v:v) at 100ºC and 1500psi for 5 min, followed by a static extraction step, with one cycle (5 min) and a flush volume of 60% of the extraction cell volume. Organic extracts were then concentrated by a rotator evaporator rinsed with hexane. The extracts were evaporated under a gentle stream of N2 to 2ml and fractionated with silica:alumina (1:1) and sodium sulphate glass column. The first fraction, corresponding to aliphatic hydrocarbons, was eluted with 20ml of n-hexane and not analyzed. The second fraction, containing the PAHs compounds, was

eluted with 30ml of a hexane of n-hexane/dichloromethane (9:1, v:v) and 40ml n-hexane/ dichloromethane (4:1, v:v), evaporated by a rotator evaporator and then concentrated to 0.5ml under a gentle stream of N2 for prior analysis. With each set of samples to be analyzed, a solvent blank, a standard mixture and a procedural blank were run in sequence to check for contamination, peak identification and quantification. Analyses of PAHs were performed on a gas chromatography-mass spectrometry (GC-MS).

2.4.1.2 AEA and Water samples

Water and AEA samples were filtered through 0.45µm Whatman® Nucleopore™ filters for the metals' contents. The suspended particulate matter retained in the filter (dried at 40°C) was totally digested with an acid mixture (*aqua regia* and HF) following the procedure described by Caetano *et al.* (2007). After the acid digestion phase metals were analyzed by ICP - MS, as described before. The aqueous filtered samples, which correspond to the dissolved metal fraction, were preserved with double - distilled NHO_3 to pH < 2 and were analyzed directly by ICP-MS, as previously described. Quality control of the analytical procedures was ensured by the analysis of Certified Reference Material (CRM) and by testing every 10^{th} sample in duplicate. Blanks were prepared following the same analytical procedure and run in parallel with the CRM and samples. Total concentrations for each individual metal represent the sum of the dissolved and particulate phase.

The AEA and water samples for the determination of PAHs were filtered (1000ml) using Whatman® microfiber filters GF/F (0.7µm) to analyze the dissolved and particulate phase. The particulate PAHs levels were analyzed using the same procedure as described for the ash samples. The dissolved PAHs levels of the filtered samples were analyzed using solid phase extraction (SPE) which was conducted in an SPE vacuum six-port SPE manifold using Bakerbond speedisk H_2O-Phobic DV B extraction disk from J. T. Baker (Avantor Performance Materials, USA), according to the established procedures (Munch *et al.,* 2012). The PAHs extracts were analyzed by using a gas chromatography-mass spectrometry (GC–MS) system (Thermo® DSQ) following the conditions described by Martins *et al.* (2008). Total PAHs concentrations represent the sum of the dissolved and the particulate PAHs for each sample.

The AEA and water samples were also analyzed with respect to total nitrogen (TN), nitrite (NO_2-N), nitrate (NO_3-N), phosphate (PO_4^{3-}) and total, organic and inorganic carbon. For the TN the samples were subjected to an oxidative digestion followed by quantification by molecular absorption spectrometry (MAS) (APHA, method 4500-N C, 2017). As for the

nitrite and nitrate contents, the samples were analyzed using the chromotropic acid method (West & Lyles, 1960) and N-(1-Naphthyl)-ethylendiamine method (ISO15923-1, 2013), respectively. The concentration of ammonium and phosphate was determined by the phenate method (APHA, method 4500-NH3 F, 2017) and by the ascorbic acid method (ISO15923-1, 2013). The carbon contents in both matrices were determined based on the high-temperature combustion method (APHA, method 5310 B, 2017) and by first filtering the samples through 0.7µm Whatman® microfiber filters GF/F. The filtrate (dissolved fraction) and filters (particulate fraction) were measured directly using the total organic carbon analyzer (multi N/C® 3100 and the accessory TOC solid module HT 1300, respectively).

2.5. Diatom culture conditions

This study was performed with two freshwater diatom species, quite frequent in rivers and streams, *Nitzschia palea* (Kützing) W. Smith (NPAL) and *Achnanthidium minutissimum* (Kützing) Czarnecki (ADMI) that show different ecological profiles concerning nutrients and organic matter. NPAL is more tolerant to alterations in the surrounding environment (Esteves *et al.*, 2017). To select the best culture conditions for diatom growth the two diatom species were grown in two culture media Chu10 (Annex II) and DVII (Annex III) – (Isabelle, 2011; Stein, 1973; Hughes & Lund, 1962), and subjected to two photoperiods (light:dark - 12:12h and 16:8h) in culture chambers (SANYO, Versatile Environmental Test Chamber) at 20º ± 2ºC temperature and with a light intensity of about 72.973 $\mu E \cdot m^{-2} \cdot s^{-1}$ (TES-1332 – Luxmeter).

2.6. Growth curves

Growth curves were determined for both species in both media and photoperiods previously reported, which resulted in the decision of using DVII medium, with 16:8h photoperiod in toxicity tests. For each species and set of tested conditions, three Erlenmeyer flasks were homogenized in an ultrasonic bath for about 30s (Bandelin, Sonorex Super, RK 102 H), and 1mL counting chambers were filled, with 1mL sample preserved with a few drops of Lugol solution and allowed to settle before counting about 200 cells. Lugol solution increases the weight of the cells and therefore, promotes sedimentation, besides killing (immobilizing) the diatoms for easier cell counting (Esteves *et al.*, 2018). Cell growth was estimated by daily cell counting using 1mL counting chambers under the inverted light microscope (Leitz Labovert SF). The growth rate (GR, day^{-1}) was then calculated according to equation 1:

$$GR = (lnxf - lnxi)/t \quad \text{(Equation 1)}$$

where (*ln xf*) is the natural logarithm of cell density at the end of the assay, (*ln xi*) is the natural logarithm of cell density at the beginning of the assay and (*t*) is the duration period of the assay in days. Daily cell counting was interrupted when the decline/death phase of the growth curve was attained for each diatom species.

2.7. Ecotoxicological tests

In order to assess the effects of AEA on the growth rates of NPAL and ADMI, ecotoxicological assays were performed following the OECD 2011 guideline (OECD, 2011). An initial inoculum in exponential growth phase of approximately 10^4 cells mL^{-1} were exposed to AEA in 100mL Erlenmeyer flasks, with 50mL final volume. The Erlenmeyers were corked to minimize accidental contamination and evaporation, but still allowing air exchange. For a period of 96h, each species was exposed to different AEA concentrations: 0 (control solution, only composed by DVII culture medium), 12.5, 25, 50, 75 and 100% (only composed by AEA), with three replicates being established for every concentration (Table 1). The tests took place at 20º ± 2ºC, with a 16:8 h photoperiod and with a light intensity of 72.973 $\mu E \cdot m^{-2} \cdot s^{-1}$ (TES-1332 - Luxmeter). Every Erlenmeyer was manually stirred once a day until the deposit disappeared, to avoid cell agglutination and to promote active gas exchange (Silva *et al.*, 2015). Only the ecotoxicological tests performed in *Nitzschia palea* fulfilled the respective validity requirements (OECD, 2011). According to OECD (2011), the test is only valid if the biomass in the control cultures increases exponentially by a factor of at least 16 within a 72 – hour test period, which was not observed in any test on *Achnanthidium minutissimum* (control values on a 96h – test period: 33112.5, 30252.1, 23769.5 cells mL^{-1}). Regardless of this outcome, the tests were conducted in order to evaluate different responses between groups. For greater truthfulness and credibility of results, the experience should be replicated using controls to ensure research principles.

Table 1 – Final concentrations of DVII medium and AEA per concentration tested (mL).

	Control	**12.50%**	**25%**	**50%**	**75%**	**100%**
Medium	50	43.75	37.5	25	12.5	0
AEA	0	6.25	12.5	25	37.5	50

After the exposure period, cell density was determined by direct counting 400 cells under the inverted light microscope (Leitz Labovert SF) and the growth rate was calculated according to equation 1, previously referred in 2.6, to determine the influence of AEA on diatom growth.

2.8. Diatom valve teratologies

In order to study the effects of AEA on diatom cell wall (siliceous frustule, composed of two valves) morphology, permanent slides of a random replica per AEA concentration were mounted as referred in 2.3. Three countings of 100 valves in each slide were made and types and number of teratologies were registered. Deformed valves were photographed under the light microscope Zeiss Axioplan 2 Imaging (100x objective and 1.40 numerical aperture) equipped with an Olympus DP70 digital camera.

2.9. Data analyses

2.9.1. Diatom communities' structure

The statistical analyses of the diatom communities were performed using the software Primer 6 (Primer – E Ltd, Plymouth, UK). A multidimensional scaling analysis (MDS) was made, based on a Bray-Curtis similarity test (data transformed by double square root; Primer 6). The diatom communities in the sampling locations were compared by means of a distance–based permutational multivariate analysis of variance, PERMANOVA test. Furthermore, in order to determine the most representative taxa (those contributing the most to the similarity within groups) of the statistically different groups, a SIMPER analysis (double square route, Bray–Curtis similarity; Primer 6) was performed.

Diatom indices are designed to compile all the information given by the autoecological preferences of the diatom community. The IPS (Indice de Polluosensibilité Spécifique) index was calculated for each sampling site, to evaluate the ashes effect on the ecological status of water. Computation of this index was done with OMNIDIA software (version 5.2) (Lecointe *et al.*, 1993). The ecological status class assessment of the sampled locations, was made resourcing the Ecological Quality Ratio (EQR). The EQR values, which express the ratio of the observed IPS value to the reference IPS value for the type of water bodies in study (EQR = observed IPS / reference IPS), were determined based on an IPS reference value of 19, due to the fact that the river in study is in northern Portugal with a catchment area <100 km^2 (INAG, 2009).

2.9.2. Ecotoxicological test

Statistical analyses of the ecotoxicological tests were made featuring the software IBM SPSS statistics 24. The verification of the normality and variance was performed by means of a Shapiro–Wilk test and an Equal Variance test. One–way analysis of variance was used to test statistically significant differences between AEA concentrations. A Dunnett's test was then performed in order to highlight the statistically significant differences with the controls. Differences were considered significant at a $p < 0.05$. EC_x values and corresponding 95% confidence levels were determined by means of a nonlinear regression analysis, using a logistic equation fitted to the data through the least squares method.

The statistical analyses of the teratologies' percentage occurring during the ecotoxicological tests were made on Primer 6 (Primer – E Ltd, Plymouth, UK). Bray-Curtis similarity test and PERMANOVAS were made, followed by Pair Wise Tests in order to compare the percentage of teratologies present in the different concentrations.

3. Results

3.1. Environmental Parameters

3.1.1 Stream Sediments

In an overall view, metals' concentration in the stream sediments were similar between the sampling campaigns, with cadmium (Cd) always below quantification limit, and manganese (Mn) and zinc (Zn) showing the highest concentrations (Table 2).

All the 16 PAHs analyzed were below the quantification limit (0.05 mg kg^{-1}).

Total carbon (TC) was higher in the sampling site affected by the wildfire (Burnt), in both sampling moments (Table 3).

Table 2 - Metal concentrations in stream sediments (mg kg^{-1}) on both campaigns.

Campaign date	Sample	V	Cr	Mn	Co	Ni	Cu	Zn	As	Cd	Pb
15/nov/18	Up	33	26	239	8	26	22	111	40	< 0.2	17
	Burnt	33	26	240	8	24	21	107	40	< 0.2	16
	Down	33	25	263	9	26	22	110	43	< 0.2	16
05/jun/19	Up	33	27	206	7	24	22	114	39	< 0.2	18
	Burnt	31	25	232	7	22	19	111	31	< 0.2	16
	Down	32	25	191	9	26	22	103	32	< 0.2	16

Table 3 - Total carbon (TC) present in stream sediments (g kg-1) in both campaigns.

Campaign date	Sample	TC
15/nov/18	Up	2.9
	Burnt	7.5
	Down	5.5
05/jun/19	Up	2.8
	Burnt	8.2
	Down	4.6

3.1.2 Stream water

With the measurements made on the field it was possible to verify a minimum general decrease on the dissolved oxygen from the first campaign (15/nov/18) to the second (5/jun/19), while a small increment on the temperature, electric conductivity, total dissolved solids and current velocity occurred. The pH was found on the range of 6.53 and 7.34, being always in the neutral interval (Table 4).

Table 4 - Temperature (TºC), pH, electric conductivity (EC), total dissolved solids (TDS), dissolved oxygen (O_2) and current velocity measured on the field on both campaigns.

Campaign date		T ºC	pH	EC (µS cm^{-2})	TDS (mg l^{-1})	O_2 %	O_2 mg L^{-1}	$V_{current}$
15/nov/18	Up	14.3	6.66	50	24	111	11.1	40-42
		14.3	6.53	51	24	108	10.8	
	Burnt	14.1	6.68	51	25	111	11.3	30-54
		14.1	6.69	50	25	106	10.9	
	Down	13.6	6.67	48	25	110	11.3	23-26
		13.6	6.71	54	35	118	11.1	
5/jun/19	Up	14.9	7.12	88	43	91	10.9	20-30
		14.9	6.93	85	60	88	9.9	
	Burnt	14.7	7.34	71	42	89	9.1	30-60
		14.6	7.32	87	51	85	8.8	
	Down	15.1	7.15	60	32	87	9.7	30-90
		15.1	7.22	71	41	84	9.0	

Contrary to stream sediment samples, in water samples, metals were less noticed and those detected were present at lower concentrations. Vanadium and Cu remained relatively stable through the different samples and campaigns. On the other hand, Mn and As showed higher concentration at the Burnt and Down sites of both campaigns. Cobalt, Ni, Zn, Cd and Pb were below the limit of quantification. Likewise, Cr in the Down site in november and in the Burnt site in june was also below the limit of quantification (Table 5).

Table 5 - Metal concentrations in stream water samples (µg L^{-1}) collected in both campaigns.

Campaign date	Sample	V	Cr	Mn	Co	Ni	Cu	Zn	As	Cd	Pb
15/nov/18	Up	2.2	1.3	23	< 0.20	< 2.00	2.8	< 5.00	0.48	< 0.20	< 0.50
	Burnt	2.0	1.5	27	< 0.20	< 2.00	2.7	< 5.00	0.68	< 0.20	< 0.50
	Down	2.0	< 0.50	32	< 0.20	< 2.00	2.4	< 5.00	0.88	< 0.20	< 0.50
05/jun/19	Up	2.0	1.2	15	< 0.20	< 2.00	2.2	< 5.00	0.38	< 0.20	< 0.50
	Burnt	2.2	< 0.50	30	< 0.20	< 2.00	2.6	< 5.00	0.69	< 0.20	< 0.50
	Down	2.1	1.3	28	< 0.20	< 2.00	2.2	< 5.00	0.55	< 0.20	< 0.50

PAHs were all below the limit of quantification, except NAP in the second campaign, FLU and ANT in the first campaign, and PHE in both campaigns, all in the Down sampling site (Table 6).

Table 6 – Quantification of detected PAHs in the stream water samples (µg L^{-1}) collected in both campaigns.

PAH		15/nov/18			5/jun/19		
		Up	Burnt	Down	Up	Burnt	Down
Naphtalene	NAP	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.01
Fluorene	FLU	< 0.01	< 0.01	0.03	< 0.01	< 0.01	< 0.01
Phenanthrene	PHE	< 0.01	< 0.01	0.05	< 0.01	< 0.01	0.01
Anthracene	ANT	< 0.01	< 0.01	0.06	< 0.01	< 0.01	< 0.01

Relatively to nutrients, the water samples from the first campaign were similar considering inorganic carbon (IC) and total particulate carbon (TCp). Nitrates and nitrites

were above limit of quantification only in Down and Burnt sampling sites of the second campaign. Likewise, TCp showed the highest concentration in the second sampling moment, particularly in the Burnt and Down sites. In relation to total nitrogen, total organic carbon (TOC) and total dissolved carbon (TCd), a oscillation between sampling sites was observed, especially in the first campaign (Table 7).

Table 7 - Quantification of nutrients in stream water samples collected in both campaigns. Total Organic Carbon (TOC), Inorganic Carbon (IC), Total dissolved Carbon (TCd) and Total particulate Carbon (TCp).

Campaign date	Sample	Total Nitrogen (mg N l^{-1})	Nitrate (mg NO_3^--N l^{-1})	Nitrite (mg NO_2^--N l^{-1})	Phosphate (mg PO_4^{3-} l^{-1})	TOC (mg C l^{-1})	IC (mg C l^{-1})	TCd (mg C l^{-1})	TCp (mg C l^{-1})
15/nov/18	Up	15.3	< 1	<0.01	0.76	2.2	1.1	3.2	7
	Burnt	< 0.5	< 1	<0.01	0.20	5.6	1.3	6.9	7
	Down	1.1	< 1	<0.01	0.61	7.2	1.7	8.9	7
5/jun/19	Up	20.4	< 1	<0.01	0.47	6	1.9	8.1	14
	Burnt	8.4	2.9	0.03	0.41	11	2.2	13	28
	Down	10.7	3.4	0.01	0.42	6.6	2.1	8.7	29

3.1.3 Aqueous Extracts of Ash (AEA)

The pH values measured in both AEA were within the acceptable range values for diatom growth, with 7.63 for the low severity AEA and 7.94 for the high severity AEA.

The metal concentrations on both AEA are similar, with exception of Mn, Co, Cu, Zn and As. Mn, Cu and Zn can be found in higher concentration on the low severity AEA, while Co and As on the high severity AEA (Table 8).

Table 8 - Metal concentrations in AEA (µg L^{-1}) used in the ecotoxicological tests (low severity - LS and high severity – HS).

AEA	V	Cr	Mn	Co	Ni	Cu	Zn	As	Cd	Pb
LS	25	20	897	6.8	19	60	226	4.2	2.7	19
HS	27	22	728	11	18	51	180	13	2.7	18

Relatively to the presence of PAHs (Table 9), only Naphtalene (NAP) and Phenanthrene (PHE) were detected in both AEA. Acenaphthylene (ACE), Anthracene (ANT), Fluoranthene (FLT) and Pyrene (PYR) were only observed in high severity AEA.

Table 9 – Quantification of detected PAHs in the AEA (µg L^{-1}) used in the ecotoxicological tests (low severity - LS and high severity - HS).

PAH		LS	HS
Naphtalene	NAP	0.67	1.5
Acenaphthene	ACE	< 0.01	0.02
Phenanthrene	PHE	0.05	0.14
Anthracene	ANT	< 0.01	0.01
Fluoranthene	FLT	< 0.01	0.03
Pyrene	PYR	< 0.01	0.01

According to Table 10, the low severity AEA possesses a higher concentration of nitrates and carbon in all forms, while the high severity AEA showed a higher concentration of total nitrogen and phosphate.

Table 10 - Quantification of nutrients in AEA used in the ecotoxicological tests (low severity - LS and high severity - HS). Total Organic Carbon (TOC), Inorganic Carbon (IC), Total dissolved Carbon (TCd) and Total particulate Carbon (TCp).

AEA	Total Nitrogen (mg N l^{-1})	Nitrate (mg NO_3^--N l^{-1})	Nitrite (mg NO_2^--N l^{-1})	Phosphate (mg PO_4^{3-} l^{-1})	TOC (mg C l^{-1})	IC (dissolved) (mg C l^{-1})	TCd (mg C l^{-1})	TCp (mg C l^{-1})
LS	51	11.5	< 0.01	51.2	22	6.8	29	55
HS	57	8.5	< 0.01	65.4	13	5.9	19	39

3.2. Analysis of diatom communities

According to the MDS 2D plot, there is a clear separation between the first (autumn) and second (spring) sampling events shown by the diatom community structure (Figure 3). The PERMANOVA global test further demonstrated that these differences are

statistically significant (*t* = 1.9887, *P(MC)* = 0.038), which shows that the structure of diatom communities changed considerably between the two sampling moments. During each season the three sites appear separated in Figure 3, which means that the diatom communities are different between sites.

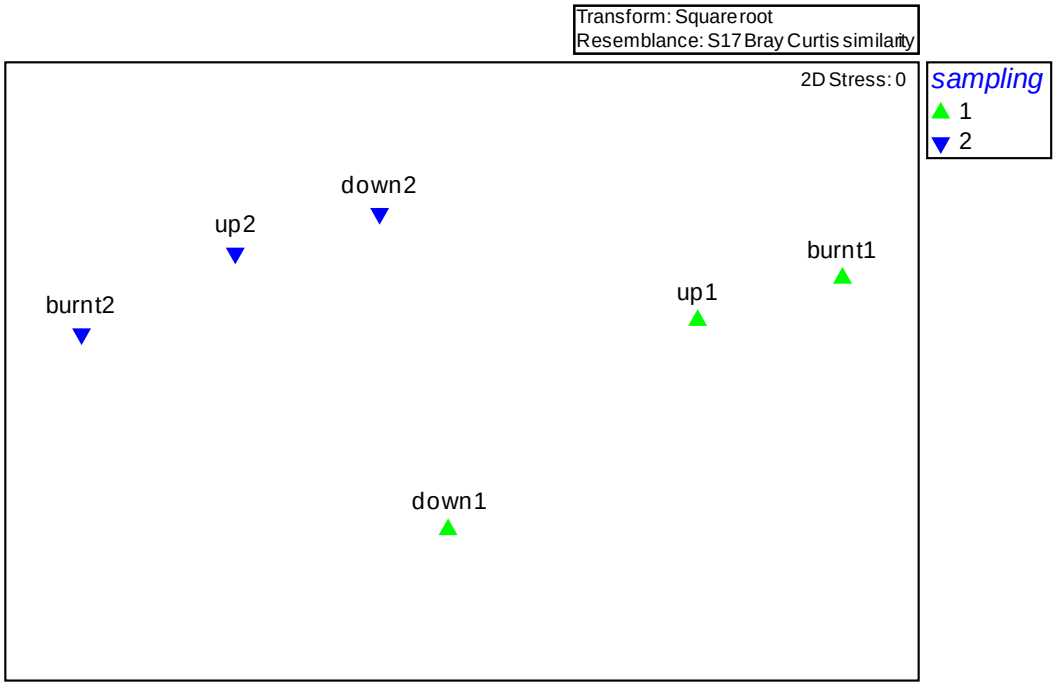

Figure 3 - Multidimensional scaling analysis ordination of diatoms of the first sampling moment – 15/nov/18 (1) and the second – 5/jun/19 (2).

SIMPER analysis revealed a similarity of 57.19% within samples collected in autumn, and a similarity of 62.87% within samples collected in spring. *Karayevia oblongella* (Østrup) Aboal (KOBG) contributed with about 10% to the dissimilarity between these two groups (58.03%), which was more abundant in autumn compared to sampling in spring (Table 11 and Annex IV). During the first sampling campaign (15/nov/18) KOBG showed higher percentage of abnormal valves and was the dominant taxon, in the Up and Burnt sampling locations (Table 11 and Table 112). In Downstream, *Navicula notha* J.H. Wallace dominated and ADMI demonstrated more teratological valves (Table 11 and Table 12). In the spring it's possible to observe the dominance of ADMI in the Up and Down locations, while *Fragilaria rumpens (*Kützing*) G.W.F.Carlson* dominates Burnt (Table 11).

Table 11 – Number of valves of the dominant species in the three sampling sites of the autumn (15/nov/18) and spring campaigns (5/jun/19). Shaded cells represent the dominant taxa in each location.

Specie		15/nov/18			5/jun/19		
		Up	Burnt	Down	Up	Burnt	Down
*Achnanthidium minutissimum (*Kützing*) Czarnecki*	ADMI	58	48	97	191	84	78
Karayevia oblongella (Østrup) Aboal	KOBG	278	321	40	57	44	76
Karayevia oblongella (Østrup) Aboal anormale	KOTG	13	19	6	11	0	14
Navicula notha J.H. Wallace	NNOT	13	0	116	5	9	2
*Fragilaria rumpens (*Kützing*) G.W.F.Carlson*	FRUM	0	0	0	22	105	6

The percentage of abnormal valves of *Karayevia oblongella* (KOTG) was also higher in autumn 2018 than in spring 2019 (Table 12). In autumn and site Up the percentage of abnormal valves (KOTG) was 5.6% of all *Karayevia oblongella* (KOBG) counted, while in the Burnt it was 13% and in the Down sampling site of 4.5% total KOBG. In the spring campaign on Up site KOTG had a percentage of 13% of all *Karayevia oblongella* counted, in the Burnt area of 16.2% and in the downstream location of 15.6%.

A few taxa which were only present in spring also contributed to the dissimilarity between the two sampling moments (e.g.: *Fragilaria rumpens* (FRUM), *Eolimna minima* (EOMI), *Fragilaria perminuta* (FPEM) and *Achnanthidium minutissimum* abnormal form (ADMT)). Despite the presence of ADMI in autumn samples, abnormal valves were only counted on Down sampling point, with the higher percentage (Table 12). It represents 14% of the total counted taxa, and approximately 67% of all ADMI. In spring samples ADMT was counted on the three sampling sites, and of all *Achnanthidium minutissimum* (ADMI) counted 15% showed abnormal morphology (teratologies) (ADMT) on the Up site, 4.5% on Burnt and 39.1% on the Down sampling site. *Frustulia saxonica* (FSAX) was only counted in autumn despite its observation in spring.

Table 12 - Percentage of counted valves of *Achnanthidium minutissimum* normal valves (ADMI) and teratological valves (ADMT) and of *Karayevia oblongella* normal valves (KOBG) and teratological valves (KOTG) in the two sampling moments considering the entire community (100%). (For the other counted taxa see Annex I).

Specie	15/nov/18			5/jun/19		
	Up	Burnt	Down	Up	Burnt	Down
ADMI	14.8	11.9	20.9	43.8	20.6	21.5
ADMT	0.0	0.0	14.0	7.8	1.0	13.8
KOBG	70.7	79.7	8.6	13.1	10.8	21.0
KOTG	3.3	4.7	1.3	2.5	0.0	3.9

The Ecological Quality Ratio (EQR) values observed in the studied stream classified the water as good quality status ([0.73 – 0.97[) despite the differences (Table 13). There was no clear pattern in changes of water quality regardless of the sampling site (Burnt site, before and after the influence of the fire event) or between wildfire events.

Table 13 - EQR values of the different sampling locations and respective water quality class.

Campaign date	Sample	EQR	Water quality class
15/nov/18	Up	0.83	Good
	Burnt	0.85	Good
	Down	0.86	Good
5/jun/19	Up	0.79	Good
	Burnt	0.85	Good
	Down	0.77	Good

3.3. Ecotoxicological Tests

3.3.1. Growth curves

Growth curves were determined for *Nitzschia palea* (NPAL) and *Achnanthidium minutissimum* (ADMI) in two photoperiods (light:dark - 12:12h and 16:8h) and in the culture mediums DVII (Figure 4) and Chu10 modified (Isabelle, 2011; Stein, 1973; Hughes & Lund, 1962) (Figure 5). The composition of both media can be found in Annex II and Annex III.

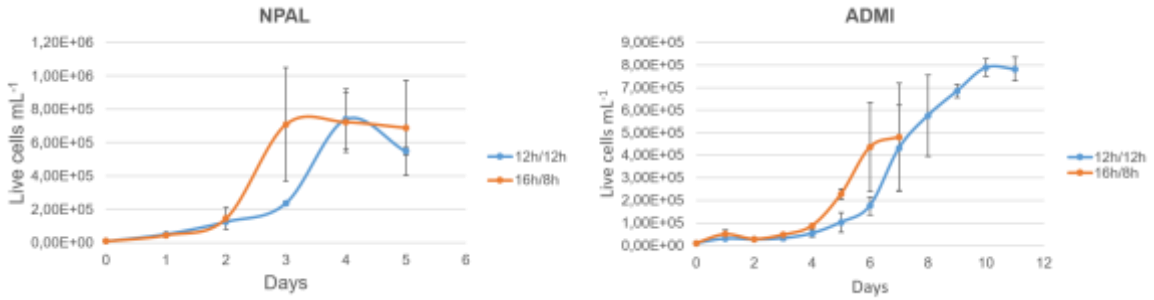

Figure 4 - Growth curves for *Nitzschia palea* (NPAL) and *Achnanthidium minutissimum* (ADMI) in both photoperiods (light:dark - 12:12h and 16:8h), in DVII culture medium.

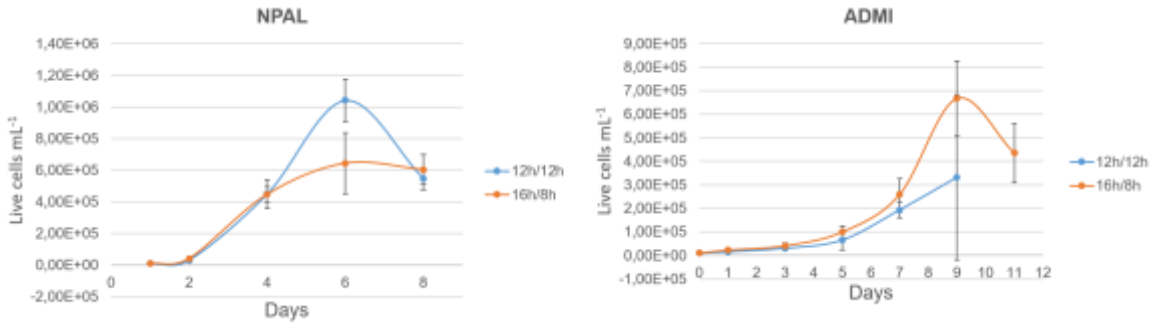

Figure 5 - Growth curves for *Nitzschia palea* (NPAL) and *Achnanthidium minutissimum* (ADMI) in both photoperiods (light:dark - 12:12h and 16:8h), in Chu10 modified (Isabelle, 2011; Stein, 1973; Hughes & Lund, 1962) culture medium.

The best growth for both species was attained with the DVII medium. The exponential growth phase was reached at day 6 (436009 cells ml^{-1}) and day 3 (708378 cells ml^{-1}) for *Achnanthidium minutissimum* and *Nitzschia palea* respectively (Figure 4).

3.3.2. Inhibition test

The verification of the normality and variance displayed that every test showed statistically significant differences (Table 14). *Achnanthidium minutissimum* (ADMI) tested with AEA of low severity demonstrated statistically significant differences in the concentrations of 12.5% and 25%, and in every concentration on AEA of high severity (Figure 6). On the other hand, only in the 100% concentration of AEA of low severity were revealed statistical significant differences for *Nitzschia palea* (NPAL) while in the high severity the concentrations of 12.5% and 25% were the ones showing significant statistical differences (Figure 7).

Table 14 - One-way analysis of variance (ANOVA) results for the growth rates of *Achnanthidium minutissimum* (ADMI) and *Nitzschia palea* (NPAL), exposed to High Severity (HS) and Low Severity (LS) AEA.

Specie / AEA	***p***	***df***	**F**
ADMI HS	<0.001	5	120.513
ADMI LS	<0.001	5	20.020
NPAL HS	<0.001	5	11.231
NPAL LS	0.005	5	6.214

df — degrees of freedom; F — F statistic; *p* — p value (probability level for significant effects: $p < 0.05$).

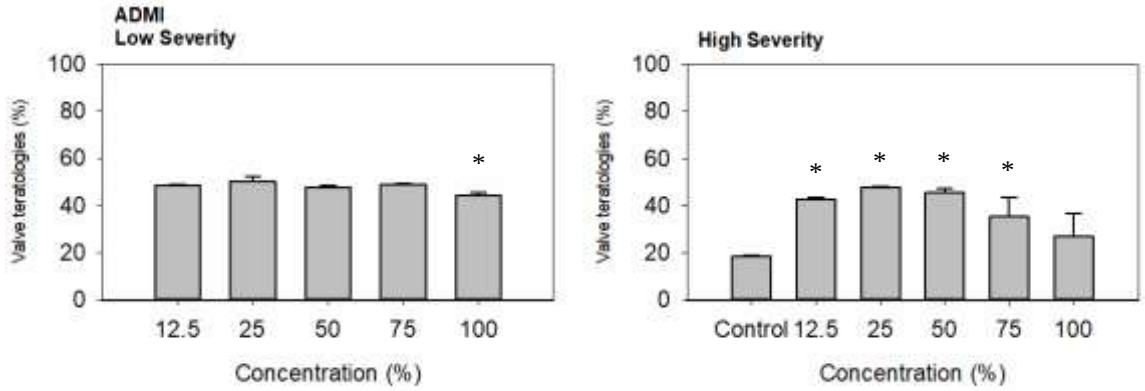

Figure 6 - Growth rate of ADMI after 96h in different concentrations of AEA originated from ashes of low and high severity, with the respective standard deviation. Statistically significant differences ($p < 0.005$) between every concentration (low severity graph) and between the controls and remaining concentrations (high severity graph) are indicated with an asterisk (*).

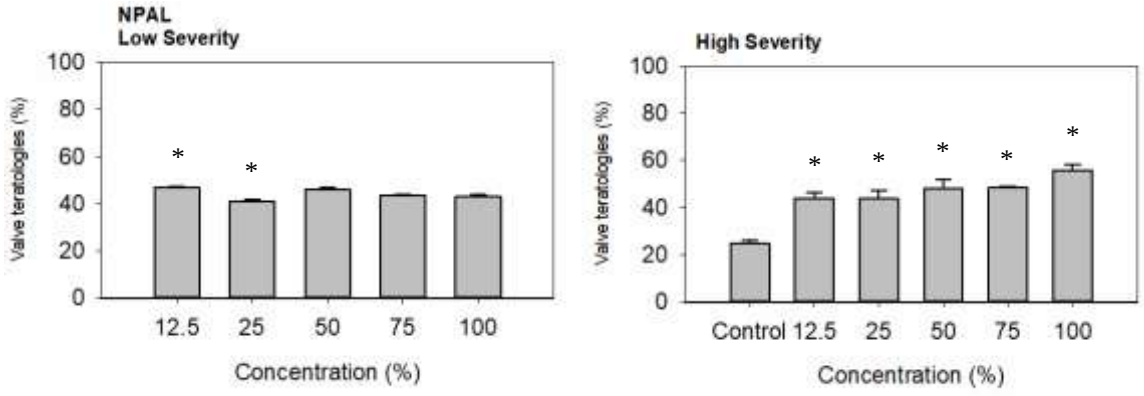

Figure 7 - Growth rate of NPAL after 96h in different concentration of AEA originated from ashes ashes of low and high severity, with the respective standard deviation. Statistically significant differences ($p < 0.005$) between every concentration (low severity graph) and between the controls and remaining concentrations (high severity graph) are indicated with an asterisk (*).

The values of EC_{50} and EC_{10} were estimated and a toxic effect was only observed in the AEA from low severity (LS) ashes. NPAL had the higher values, being EC_{50} of 104.98% and the EC_{10} 62.36%, while on ADMI were of 84.78% and 45.07%, respectively (Table 15).

Table 15 - EC_{10} and EC_{50} values (% AEA concentration) and respective 95 %-confidence limits for growth rates of *Achnanthidium minutissimum* (ADMI) and *Nitzschia palea* (NPAL), exposed to High Severity (HS) and Low Severity (LS) AEA for 96h. 'NT' stands for – no toxicity.

Specie / AEA	**EC_{10} (%)**	**EC_{50} (%)**
ADMI HS	NT	NT
ADMI LS	45.066 (-0.193 – 90.599)	84.778 (55.768 – 113.787)
NPAL HS	NT	NT
NPAL LS	62.359 (40.013 – 84.760)	104.977 (88.789 – 121.166)

3.3.3. Teratologies

The teratologies observed, present in Figure 8 and Figure 9 for ADMI and NPAL respectively, were: irregular valve outline/abnormal shape, such as the width of the central or longitudinal areas (Figure 8 – b, c, d, e, f, g, j, k, l, m, n, o, p, u and v; Figure 9 – d and o), kidney shape (Figure 8 – s, t, w, x, y and z; Figure 9 - f, g, h, i, j, k and l), changes in cell length (Figure 8 – g and u; Figure – d), and mixed deformities (Figure 8 – h, i, q and r; Figure 9 – c and e).

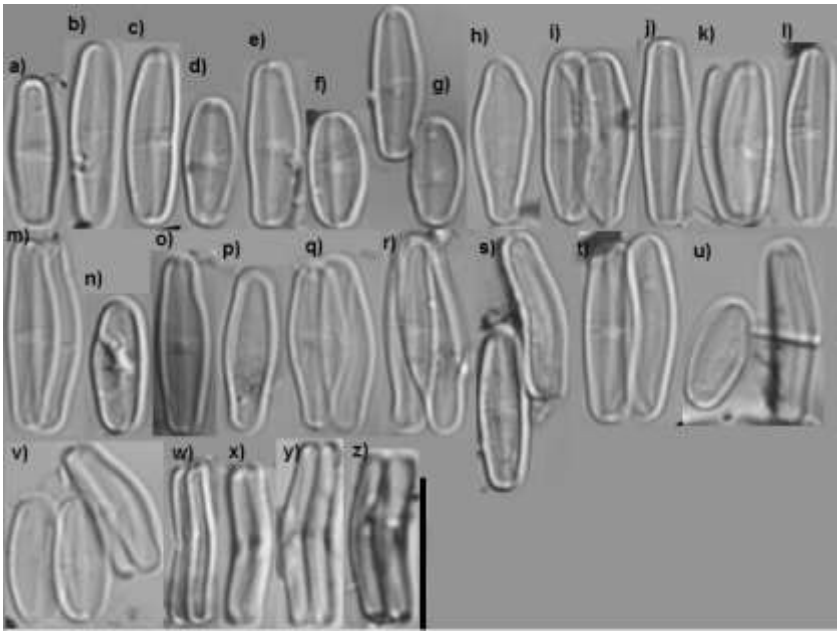

Figure 8 - Teratologies observed in ADMI during the ecotoxicological test with ashes of low severity. It can be observed: normal cells (a), different types and degrees of valve abnormal shape (b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q and r), and mixed deformities (h, i, q and r). Scale bar = 10.0 µm.

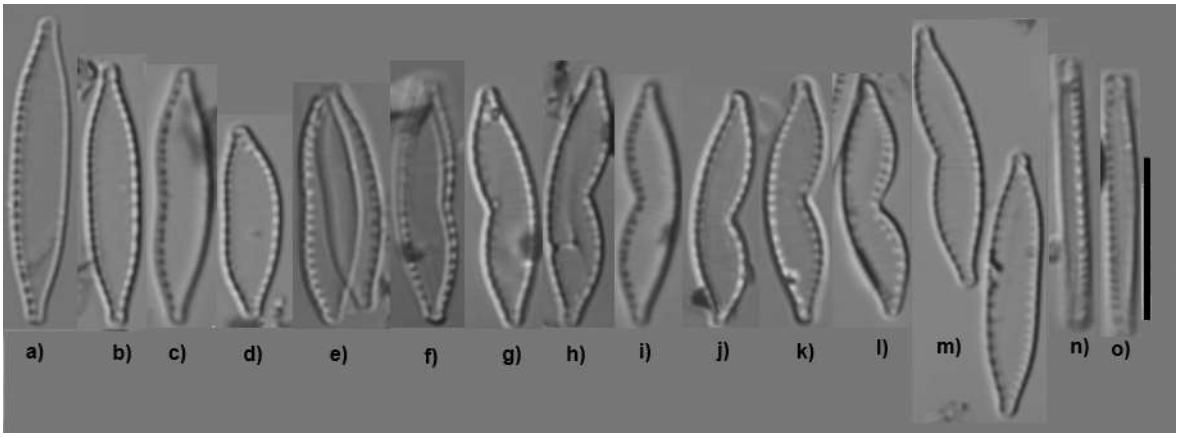

Figure 9 - Teratologies observed in NPAL during the ecotoxicological test with ashes of low severity. It can be observed: normal cells (a, b and n), slight valve abnormality (c, d, e, m and o) and kidney shape (f, g, h, I, j, k, l and m). Scale bar = 10.0 µm.

Teratologies were detected in both species as previously referred, so in order to check for statistically significant differences of percentages of teratologies between AEA concentrations a PERMANOVA analysis was performed (Table 16). The percentage of teratological valves in *Achnanthidium minutissimum* (ADMI) on AEA of low severity is significantly lower in the 100% concentration comparing with the remaining ones (Figure 10). In *Nitzschia palea* (NPAL) statistically significant differences were detected in the concentrations of 12.5% and 25%, while 12.5% was statistically different from 25, 75 and 100%; 25% was statistically different from 50 and 75%; and 50% was statistically different from 75 and 100% (Figure 11). On the other hand, in the high severity AEA, ADMI revealed statistically significant differences between the control and the concentrations of 12.5%, 25%, 50% and 75% (Figure 10), whereas NPAL presented significant differences with all the concentrations (Figure 11).

Table 16 – PERMANOVA main tests results for the teratologies percentage of *Nitzschia palea* (NPAL) and *Achnanthidium minutissimum* (ADMI), exposed to Low Severity (LS) and High Severity (HS) AEA.

Specie / AEA	***p***	***df***	**Pseudo - F**
NPAL LS	0.001	4	18.505
ADMI LS	0.003	4	7.347
NPAL HS	0.001	5	48.327
ADMI HS	0.002	5	8.752

df — degrees of freedom; F — F statistic; *p* — p value (probability level for significant effects).

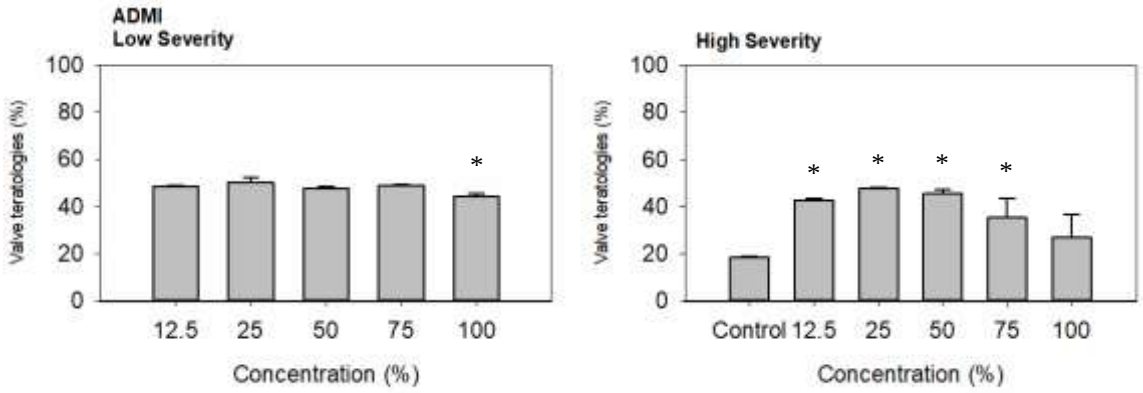

Figure 10 - *Achnanthidium minutissimum* (ADMI) percentage of valve teratologies after 96h in different concentrations of low and high severity AEA, with standard deviation. Statistically significant differences ($p < 0.005$) between every concentration (low severity graph) and between the controls and remaining concentrations (high severity graph) are indicated with an asterisk (*).

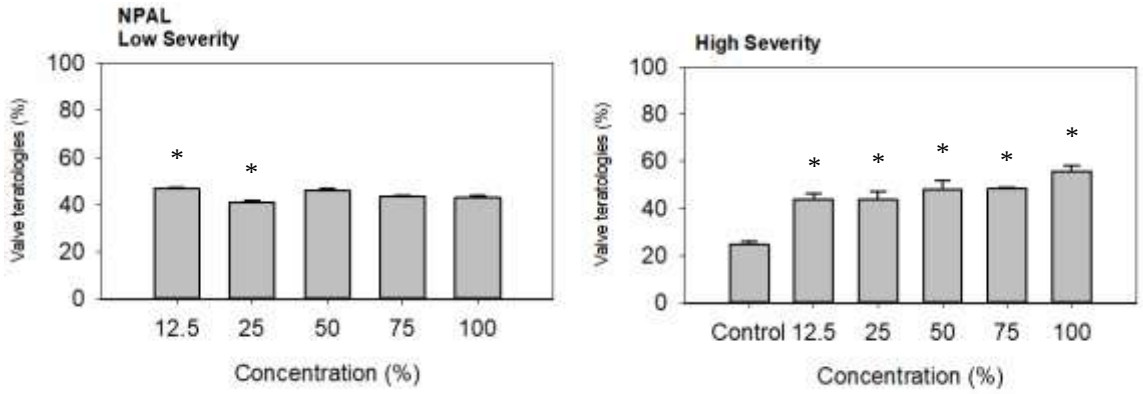

Figure 11 - *Nitzschia palea* (NPAL) percentage of valve teratologies after 96h in different concentrations of low and high severity AEA, with the respective standard deviation. Statistically significant differences ($p < 0.005$) between every concentration (low severity graph) and between the controls and remaining concentrations (high severity graph) are indicated with an asterisk (*).

4. Discussion

4.1 Environmental parameters

In general, the stream sediments collected possessed similar concentrations of metals between sampling periods. Also, by comparing the sampling sites, no noticeable differences were found between locations. In what concerns the PAHs in the sediments, all of them were found below the limit of quantification, even in the sites with the influence of the wildfire (Burnt and Down). In opposite, the TC on the stream sediments was higher

in the Burnt sampling location in both sampling moments, followed by Down and finally by Up site.

Regarding the metal concentration in the stream water samples, just Mn and As suffered a rise from the Up site to the Down. Most PAHs were below or close to the limit of quantification. These results were contradictory to what was found by Olivella *et al.* (2006) that testified a higher presence of PAHs as a result of a wildfire. Total carbon in stream waters revealed a similar pattern as it was found in sediments, with higher values found in the sites potentially affected by the post-fire contamination. Total nitrogen and phosphate were always higher in the Up site compared with the Burnt and with the Down sampling locations.

Hence, based on the chemical results, the present study revealed that in both campaigns ashes hadn't reached the sampled stream. In fact, during the sampling just slight evidences of ash presence were found in sediments, and the stream water was transparent, with total dissolved solids ranging from 24 to 35 mg L^{-1} on the first campaign and between 32 and 60 mg L^{-1} on the second . According to Earl & Blinn (2003), the water quality in streams reacts immediately to the entry of ash following a wildfire. Nonetheless, if the sampling moments don't overlap the ash overland flow into the water system, the water chemistry may pass undetected, in particular with limited ash flow (Earl & Blinn, 2003), which was the case of the present study. In addition, metal concentrations are generally low in surface water, since they are adsorbed onto particles suspended in the water column and then accumulated in the sediments (Miao *et al.*, 2006).

Concerning the metal concentrations in the aqueous extracts of ashes (AEA), similar concentrations were found between the Low Severity (LS) and the High Severity (HS). With exception of Mn and As, that were found at higher concentration compared to sediment, all the other analyzed metals were at similar concentrations on both AEA and in sediment. It's extremely important to highlight that the medium used for the diatom culture could have influence in the presence of some chemicals on the aqueous extracts of ash. In this study no chemical analysis was performed on the culture medium used, but as shown in Annex III, a trace elements solution was added. It included Mn, Zn, V, Cd, Co, Cu, Ni and Cr, which could contribute to the concentrations observed. In addition, the medium also contains vitamins, phosphate and nitrates that could influence the nutrients' concentration. Thus, the alterations registered through the different AEA concentrations, could have been influenced by its dilution with DVII medium. Silva *et al.* (2015) studied the chemical composition of AEA, and when both matrices (ashes and AEA) are compared, it's evident that the recovery/remobilization of chemical elements from ashes into AEA

fluctuates within the different compounds, which results from the affinity of the chemical elements towards the available organic material (Koc) and water solubility. Consequently, elements with lower Koc and higher solubility may potentially occur in higher concentrations in aqueous extracts due to their particle size, chemical specification, mineralogy of the ash and the pH of the solution (Silva *et al.*, 2015). In the present study, the high values of Mn, Zn and Cu in the AEA may have resulted from the ashes (GSI, 2012; Silva *et al.*, 2015; Campos *et al.*, 2016). In terms of PAHs in the AEA, Naphtalene (NAP) and Phenanthrene (PHE) showed the highest concentrations, particularly in the high severity AEA. The dominance of NAP was also reported by Silva *et al.* (2015) and Vila-Escalé *et al.* (2007). In these cases, as suggested by Manoli & Samara (1999), there is an inverse relationship of water solubility and molecular weight of PAH, since they have a hydrophobic nature and reduced solubility. With regard to nutrients, an increment in carbon (in all of its forms), total nitrogen, nitrate and phosphate was observed in both AEA as also seen by Belillas & Roda (1993), Beschta (1990), Schindler *et al.* (1980) and Tiedemann *et al.* (1978). Total nitrogen (TN) as well as total carbon (TC) were more significant in the AEA made with low severity ashes (LS), which indicates that a decrease of these two nutrients with fire severity occurs as confirmed by several other studies (Murphy *et al.*, 2006; Khanna *et al.*, 1994; Goforth *et al.*, 2005; Pereira *et al.*, 2012). The decay of TN with fire severity is proportional to the quantity of organic matter consumed by the fire, which is a good predictor of fire severity (DeBano & Conrad, 1978; Gray & Dighton, 2006; Murphy *et al.*, 2006; Neary *et al.*, 2005; Ponder *et al.*, 2009; Qian *et al.*, 2009). Nonetheless, carbon was also found in the high severity AEA, suggesting the entrapment of carbon in ashes of medium – high severity wildfires, as also observed by Bodi *et al.* (2011), Goforth *et al.* (2005) and Raison & McGarity (1980).

4.2 Diatom communities

Previous studies on metal polluted rivers have demonstrated that various diatoms possess different ways of responding to environmental degradation (e.g. Hellawell, 1978): at the individual level with alterations in frustule morphology (eg. Falasco *et al.*, 2009; Pandey & Bergey, 2016; Pandey *et al.*, 2014), as showed in the present study; at a community compositional level, changing the diversity patterns as well as dominant taxa, as seen in 4.2 and in Annex I (Falasco *et al.*, 2009; Shi *et al.*, 2015); a reduction in biomass, and alterations in the community structure (Falasco *et al.*, 2009).

Several studies have already stated that environmental conditions can alter the valve morphology of diatoms (Cox, 1995; Geissler, 1970[a], 1970[b], 1982, 1986; Jahn, 1986; McBride & Edgar, 1998; Schultz, 1971; Schmid, 1976; Wendker & Geissler, 1988), but the mechanisms that can induce them are not well understood. Its hypothesized that cellular processes engaged in wall formation and cell division suffer variations on account of chemical or physical stressors (Lavoie *et al.*, 2017). Teratologies can be observed in natural diatom assemblages but with low frequency, yet the presence of multiple stressors increases their occurrence (Lavoie *et al.*, 2017), such as external contaminants. In the present study, in the first sampling moment, 3.82% of the total diatoms counted in the Up site were teratological, while in the Burnt were 4.71% and in the Down 15.3%. In the second campaign 10.3%, 1% and 17.63% were observed in the Up, Burnt and Down sampling sites, respectively. Dziengo–Czaja *et al.* (2008) reported a much higher percentage of teratological cells (around 25%), but in marine diatoms, due to contaminated marine sediments. Pandey & Bergey (2016) and Pandey *et al.* (2015) also stated deformities under Cu and Zn treatment on periphytic diatom communities.

As previously referred, ashes had no or a minimum impact in stream water as well as sediments, and therefore water class provided by diatom assemblages was the same (good water quality) for all three sites and both campaigns. In addition, and also as mentioned before, metals suffered an increment in the first campaign in the Down site, and on the second sampling moment on the Burnt. Relatively to the diatom community in those places, it's possible to observe a change of the dominant taxa, being in the Down sampling site of the first campaign *Navicula notha* (NNOT) and *Achnanthidium minutissimum* (ADMI), and on the Burnt site of the spring campaign *Fragilaria rumpens* (FRUM) and ADMI as well. In the remaining locations of both campaigns the dominant taxa were always ADMI and KOBG. As shown by Luís *et al.* (2009, 2011) and da Silva *et al.* (2009), metal contamination could have the ability to entail the algae communities' successions into more pollution tolerant species (Gustavson & Wängberg, 1995), which culminates in an increased tolerance of communities (Blanck *et al.*, 1988), and in a possible reduction of species diversity and richness (Leland & Carter, 1984; Medley & Clements, 1998), as well as morphological modifications. Since the observed changes are minor, the alterations verified on the metals' concentrations are not reflected on diatom communities.

Previous studies (e.g. Cetin, 2008; Dela–Cruz *et al.*, 2006; Sabater & Roca, 1990) testified the influence of temperature on diatom communities, predominantly on

community diversity and composition. Elias *et al.* (2012) stated the influence of temperature and time of year on diatom communities, reporting differences between two groups of seasons: spring/summer and autumn/winter. They verified the occurrence of 17 species in spring/summer that were absent in the autumn/winter. Some of these taxa were also found in our study only in spring/summer (*Nitzschia dissipata* - NDIS), or were more abundant (*Encyonema minutum* - ENMI, *Nitzschia recta* - NREC, *Eunotia minor* - EMIN, *Navicula rhynchocephala* – NRHY, *Gomphonema parvulum* – GPAR and *Eolimna minima* - EOMI). *Achnanthidium minutissimum* (ADMI) and *Gomphonema pumilum* (GPUM) were present in both studies with higher abundance in spring/summer. Like in the present study, Elias *et al.* (2012) stated that *Karayevia oblongella* (KOBG) was the dominant taxon through all year, which suggests low sensitivity to season changing.

Despite the observed structural differences on diatom communities, the EQR (IPS) values classified all sites in both campaigns as Good ecological status, because shifts in community structure replaced *taxa* with similar autoecology, the basis for the index used (IPS) (Kelly & Whitton, 1995).

4.3 Ecotoxicological Tests

The high severity AEA provoked a growth rate stimulation with increasing concentration of AEA in both species, which culminated in the observation of no toxicity. Contrarily, the low severity AEA induced toxicity to *Achnanthidium minutissimum* and *Nitzschia palea*, leading to a gradual decrease of growth with increasing AEA concentration. Zn and Cu have the ability to be absorbed by diatoms for growth, construction of membranes and cell walls, respiration processes and photosynthesis, but the optimal ranges for these processes are usually narrow (De Filippis & Pallaghy, 1994; George, 1990; Martin & Coughtrey, 1982). These metals are present in higher concentration in low severity AEA which, according to the decreasing growth rate curves, suggests that they overcome the previously referred narrow optimal range, or the noxious effects of other AEA chemicals outgrowth its benefits. ADMI was the most affected species with an EC_{50} of 84.78%. Silva *et al.* (2015) and Campos *et al.* (2012) tested runoff with ashes from a medium – high severity wildfire in mixed stands of pine and eucalyptus and stated that they were toxic for the microalga *Pseudokirchneriella subcapitata* with an EC_{50} of 35% and of circa 70% to 100%, respectively.

Among the factors that can constrain the growth of the aquatic producers, the total suspended solids assume a singular importance by reducing light penetration, the photosynthesis rate, and consequently contributing to lower growth rates (Moreira-Santos *et al.*, 2004). Notwithstanding, studies by Kieskamp (2014) revealed that uncontaminated solids at similar turbidity values to ashes do not affect the growth of the green algae *Raphidocelis subcapitata*, while the species responded negatively to the presence of wildfire ash rich in toxic substances such as polycyclic aromatic hydrocarbons (PAHs) and metals. The differences in toxicity found between the Low severity and High severity AEA may be due to differences in its chemistry including the water-extractable nutrients (Pereira, Úbeda, & Martin, 2012). Both AEA presented similar concentrations of V, Cr, Ni and Cd, but the Low severity AEA possessed more nitrates and carbon in all of its forms, as well as higher concentration of Mn, Cu, Zn and Pb. Hence, diatoms could be responding to a higher toxicity in the Low severity AEA, since some metals exert effects that are substantial even in low concentrations, due to the fact that they can cause damage on the metabolic processes of the essential elements (Gifford *et al.*, 2004; Morin, 2003), e.g. Cd (or Co) can replace Zn requirement (Price & Morel, 1990). The tolerance mechanisms define the responses to heavy metals, and if high levels are not tolerated by diatoms, algal growth is suppressed and cells will die (Gledhill *et al.*, 1997; Payne & Price, 1999); but if present at non-lethal concentrations, tolerance mechanisms may allow metal accumulation that can be transferred along the food chain (Davies, 1978).

On the other hand, Mu *et al.* (2018) showed that exposure to Cd causes cell deformities and Pb induces an increase in width on diatoms as well as deformed frustules, also shown by Pandey *et al.* (2014) and seen in the present study. High severity AEA presented a range between 27.1% and 48% of teratological valves (100% and 25% concentration, respectively) for ADMI, and from 43.7% to 55.7% for NPAL at concentrations of 25% and 100%, respectively. Low severity AEA caused about 50% of teratologies in the total counted cells of ADMI in every concentration, being the lower value of 44.52% found on the maximum concentration, and the higher 50.3% at 25% concentration; it has slightly lower percentages on NPAL, being the lowest of 41% in the 25% concentration and the highest of 46.7% on 12.5% concentration. Taking this in consideration, ADMI presented more teratologies in the low severity AEA, while NPAL on the high severity, but no influence of the AEA concentration was observed. Falasco *et al.* (2009) suggest that teratological forms may be considered an indicator of ecosystem health, being their severity and frequency related to the stress magnitude. The proportion of abnormal organisms offers a basis for assessment of metal pollution (da Silva *et al.*,

2009; Falasco *et al.*, 2009; Morin *et al.*, 2012), and in the present study it is possible to verify the presence of contaminants, but all the percentage of teratologies are similar, not being possible to take any further conclusions.

Valve deformities may be a response to nutrient–rich conditions (Falasco *et al.*, 2009), and the nutrient uptake in algae can also be influenced, among others, by light, temperature, nutritional status and pH (Shi *et al.*, 2015). Light, temperature and pH were defined and controlled in the laboratory in order to avoid their influence on the toxicological tests performed. Teratologies can also be caused by low pH, organic compounds, drought conditions, salinity levels, light intensity, UV, other toxic compounds such as cyanide, polycyclic aromatic hydrocarbons (PAH) and pesticides, as well as metal contamination, which makes teratologies a response to chemicals when in highly contaminated sites (da Silva *et al.*, 2009; Falasco *et al.*, 2009; Lavoie *et al.*, 2017). Luis *et al.* (2009, 2011), da Silva *et al.* (2009) and Morin *et al.* (2008) also noted that with high metal concentrations diatoms suffer morphological changes. However, it's extremely difficult to distinguish the effect of a single metal on diatom communities, being the use of artificial microcosms a possible way to determine cause–effect relationships (Falasco *et al.*, 2009). In the present study we selected two species with contrasting auto-ecology concerning saprobity and trophy: *Achnanthidium minutissimum* (ADMI) is considered a sensitive taxon, preferring high dissolved oxygen media; *Nitzschia palea* tolerates nutrient and organic contamination. Considering other issues of environmental contamination for example, Falasco *et al.* (2009) defined ADMI as a good indicator of heavy metal contamination.

When size reduction occurs and the frustule deforms (e.g. outline of the valves becomes oversimplified), the differentiation of normal and teratological cells becomes very difficult (Falasco *et al.*, 2009). In fact, with the decrease of the cell size, significant alterations in the number, pattern of striae and areolae, as well as shape occurs (Ross & Mann, 1986), as it was possible to verify in the present study. Morel *et al.* (1978) and Thomas *et al.* (1980) testified the swelling of the cells, as was also observed in this study in some ADMI valves. Frustule deformities were also described in previous studies mainly when diatoms were exposed to Cd and Zn (e.g. Adshead-Simonsen *et al.*, 1981; Barber & Carter, 1981; Cattaneo *et al.*, 2004; Dickman, 1998; Foster, 1982; Gold *et al.*, 2003; Harding & Whitton, 1976; Kelly & Whitton, 1989; McFarland *et al.* 1997; Thomas *et al.*, 1980; Yang & Duthie, 1993).

Since the chemical analyses *per si* ignores the interaction between contaminants as well as their bioavailability, the kind of ecotoxicological test performed in this study provides a more integrative assessment of the risks posed by wildfires to aquatic ecosystems (Smolders *et al.*, 2003). Additionally, there is not much literature concerning the toxic effects of runoff from recently Burnt areas on aquatic biota (e.g. Campos *et al.*, 2012; Nunes *et al.*, 2017; Silva *et al.*, 2015, 2016). This narrows the direct comparison of our results with the literature, as well as detailed analysis of the mechanisms of toxicity involved. In contrast, this sort of baseline studies could unleash further developments and encourage the investigation of the effects of wildfires on the aquatic biota.

5. Final considerations

Wildfires are environmentally unsettling due to loss of forest area and biodiversity, drastic and immediate impacts on structure and functioning of ecosystems, as well as environmental contamination through toxic elements (Silva *et al.*, 2015). They often threaten water quality (both chemical and ecological), through the transfer of eroded soil as well as ash into aquatic environments, which can induce toxicity (Santín *et al.*, 2015; Silva *et al.*, 2015). In addition, forest catchments are a significant source of drinking water all around the globe, therefore forest conservation is an important part of the integrated management of water resources and preservation of the ecological health of ecosystems (Santín *et al.*, 2015; Vilmi & Karjalainen, 2015). Hence, the capacity of anticipating these risks is vital for the implementation of effective post-fire mitigation measures as well as fire preparedness plans (Santín *et al.*, 2015; Silva *et al.*, 2015).

Since the ecotoxicological tests were performed under controlled and standardized conditions, the extrapolation of these results to actual environmental effects, must be made carefully. The results observed in the present study demonstrate that both species respond differently to the different AEA, with some variations that should be taken into account in lines of different ash, wildfire properties and local characteristics. In addition, the physiological changes induced are also expected in the natural environment, which will have potential consequences in the entire ecosystem, despite the supposition that no direct toxicity is expected for higher trophic levels (Silva *et al.*, 2015).

There is great interest in using diatom morphological aberrations in biomonitoring. Teratologies may be a valuable tool to assess ecosystem health and it can be assumed

that their frequency and severity are related to the magnitude of stress. However, like in the present study, the detection and mostly the quantification of teratologies is still a challenge. Like us, not all investigations have succeeded in showing a relationship between the proportion of abnormal valves and contamination level along a gradient of exposure, but the type of deformity should be considered in biomonitoring due to the fact that not all offer corresponding information (Lavoie *et al.*, 2017).

In natural conditions, abiotic and biotic parameters, not addressed in this study, can impact the organisms' response to wildfires. The general ecological signal provided by these kinds of studies may suggest that the presence of stressors can affect other organisms, and consequently the ecosystem services, function and integrity (Lavoie *et al.*, 2017). Further research should be done in different organisms from different trophic levels, in order to understand the complexity of the potentially malicious ecological effects of wildfires. In order to evaluate their real impact in the ecosystems, long-term toxicity tests should be conducted.

6. References

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Annex I – Table of taxa and correspondent number of valves per site in both campaigns (15/nov/18 and 05/jun/19). X represents observed but not counted taxa (XX for more abundant).

		15/nov/18			5/jun/19		
Taxa		***Up***	***Burnt***	***Down***	**Up**	**Burnt**	**Down**
*Achnanthidium minutissimum (*Kützing) Czarnecki	ADMI	*58*	*48*	*97*	191	84	78
*Achnanthidium minutissimum (*Kützing) Czarnecki *anormale*	ADMT	-	-	*65*	34	4	50
Cocconeis lineata Ehrenberg	CLNT	-	-	-	x	-	-
Cocconeis placentula Ehrenberg var*. placentula*	CPLA	-	-	*6*	x	4	2
Encyonema minutum (Hilse) D.G.Mann	ENMI	-	-	*6*	x	xx	15
*Eolimna minima (*Grunow) Lange-Bertalot	EOMI	-	-	-	37	15	27
*Eunotia bilunaris (*Ehrenberg) Schaarschmidt	EBIL	*x*	-	-	x	-	x
*Eunotia exigua (*Brébisson ex Kützing) Rabenhorst	EEXI	-	*1*	-	x	x	1
*Eunotia minor (*Kützing) Grunow	EMIN	*5*	*x*	*4*	4	4	4
Eunotia myrmica Lange-Bertalot	EMYR	*x*	-	-	-	-	-
*Eunotia pectinalis (*Kützing) Rabenhorst	EPEC	-	-	*1*	5	2	x
*Fragilaria perminuta (*Grunow) Lange-Bertalot	FPEM	-	-	-	19	45	11
*Fragilaria dilatata (*Brébisson) Lange-Bertalot	FDIL	-	-	-	x	-	-
Fragilaria gracilis ØstrUp	FGRA	*xx*	-	*16*	x	2	x
*Fragilaria rumpens (*Kützing) G.W.F. Carlson	FRUM	-	-	-	22	105	6
*Fragilariforma bicapitata (*A.Mayer) D.M. Williams & Round	FFBI	-	-	-	-	11	1
*Frustulia vulgaris (*Thwaites) De Toni	FVUL	-	-	-	-	x	-
*Gomphonema exilissimum (*Grunow) Lange-Bertalot & E.Reichardt	GEXL	*xx*	-	-	x	x	x
Gomphonema minusculum Krasske	GMIS	-	-	-	2	x	x
*Gomphonema minutum (C.*Agardh) C. Agardh	GMIN	-	-	-	10	4	xx
Gomphonema pala E. Reichardt	GOPA	-	-	*x*	-	-	-
*Gomphonema parvulius (*Lange-Bertalot & E.Reichardt) Lange-Bertalot & E.Reichardt	GPVL	*x*	*x*	-	-	-	-
*Gomphonema parvulum (*Kützing) Kützing	GPAR	*xx*	-	-	11	21	7
*Gomphonema pumilum (*Grunow) E.Reichardt & Lange-Bertalot	GPUM	*2*	-	*27*	xx	-	10

Gomphonema rhombicum Fricke	GRHO	*5*	*2*	*23*	4	xx	35
Gomphonema subclavatum Grunow	GSCL	-	-	-	-	x	-
*Humidophila contenta (*Grunow) Lowe, Kociolek, J.R.Johansen, Van de Vijver, Lange-Bertalot & Kopalová	HUCO	*7*	-	-	-	-	-
*Meridion circulare (*Grev.) Ag.	MCIR	-	-	-	6	x	x
Navicula angusta Grunow	NAAN	*4*	*4*	*44*	2	4	x
Navicula cryptotenella Lange-Bertalot	NCTE	-	-	-	-	x	-
*Navicula lanceolata (*C.Agardh) Ehrenberg	NLAN	-	*x*	-	-	2	-
Navicula leptostriata Jørgensen	NLST	*x*	-	-	-	-	-
Navicula libonensis Schoeman	NLIB	-	*x*	-	-	-	-
Navicula notha J.H. Wallace	NNOT	*13*	-	*116*	5	9	2
Navicula notha J.H.Wallace anormale	NNOT	*2*	-	-	-	-	-
Navicula rhynchocephala Kützing	NRHY	-	-	-	-	-	x
Frustulia saxonica Rabenhorst	FSAX	*2*	*4*	*1*	x	x	-
*Nitzschia dissipata (*Kützing) Grunow	NDIS	-	-	-	4	8	x
Nitzschia dissipata var*. media (*Hantzsch) Grunow	NDME	-	-	-	2	4	3
Nitzschia epithemoides var*. disputata (J*.R.Carter) Lange-Bertalot	NEDT	*x*	-	-	-	-	-
Nitzschia inconspicua Grunow	NINC	*x*	-	-	-	-	-
Nitzschia palea (Kützing) W. Smith var *tenuirostris* Grunow in V. Heurck	NPAT	-	*x*	-	-	-	-
*Nitzschia palea (*Kützing) W.Smith	NPAL	*2*	-	-	x	-	-
*Nitzschia perminuta (*Grunow) M.Peragallo	NIPM	*x*	-	-	-	-	-
Nitzschia recta Hantzsch	NREC	-	*x*	-	x	-	-
*Nupela lapidosa (*Lange-Bertalot) Lange-Bertalot	NULA	-	*4*	-	-	-	-
*Diatoma mesodon (*Erhenberg) Kützing	DMES	*x*	-	-	6	16	4
Pinnularia sinistra Krammer	PSIN	-	-	-	-	x	-
Pinnularia subcapitata W.Gregory	PSCA	-	-	-	-	2	-
Pinnularia viridiformis var*. minor* Krammer	PVFM	*x*	-	-	-	-	-
*Planothidium delicatulum (*Kützing) Round & Bukhtiyarova	PTDE	-	-	-	-	7	-
*Planothidium frequentissimum (*Lange-Bertalot) Lange-Bertalot	PLFR	*x*	-	-	-	-	-

*Planothidium lanceolatum (*Brébisson ex Kützing) Lange-Bertalot	PTLA	-	-	-	x	-	-
*Karayevia oblongella (*Østrup) M. Aboal	KOBG	*278*	*321*	*40*	57	44	76
*Karayevia oblongella (*Østrup) M. Aboal *anormale*	KOTG	*13*	*19*	*6*	11	-	14
*Psammothidium subatomoides (*Hustedt) Bukhtiyarova & Round	PSAT	-	-	-	x	-	x
Surirella linearis W.M.Smith	SLIN	-	-	-	4	x	3
Surirella minuta Brébisson ex Kützing	SUMI	-	-	-	x	-	x
Surirella roba Leclerq	SRBA	-	-	*8*	x	4	4
*Ulnaria biceps (*Kützing) Compère	UBIC	-	-	-	-	2	8
*Ulnaria ulna (*Nitzsch.) Compère	UULN	*2*	*x*	*4*	x	4	2
Total number of valves		**393**	**403**	**464**	**436**	**407**	**363**

Annex II – Preparation of 1 L of Chu10 modified culture medium (Stein, 1973; Hughes & Lund, 1962)

Nomenclature	Chemical Formula	Concentration (g/100mL)	Used quantity (mL/L)
Calcium nitrate	$Ca(NO_3)_2$	2	2.87
Dipotassium hidrogenorotosfofate treehidratated	$K_2(HPO_4)$.$3H_2O$	1	1
Magnesium sulfate heptahydrate	$MgSO_4$.7H2O	2.5	1
Metassilicato disodium pentahydrate	Na_2SiO_3.$5H_2O$	2	1
Iron chloride hexahydrate	$FeCl_3$.$6H_2O$	0.013	6
Sodium carbonate	$NaCO_3$	2	1
Trace Metal Solution (in a 1 L flask)	-	-	0.5

Trace Metal Solution (in a 1 L flask)			
Nomenclature	**Chemical Formula**	**Concentration (g/100mL)**	**Used quantity (mL/L)**
Iron(III) chloride hexahydrate	$FeCl_3$ $6H_2O$		3.15g
Copper sulfate pentahydrate	$CuSO_4$ $5H_2O$	1	1
Sodium molybdate dehydrate	Na_2MoO_4 $2H_2O$	0.63	1
Zinc sulfate heptahydrate	ZnSO4 $7H_2O$	2.2	1
Cobalt(II) chloride hexahydrate	$CoCl_2$ $6H_2O$	1	1
Manganese(II) chloride tetrahydrate	$MnCl_2$ $4H_2O$	1.8	1

Annex III – Preparation of 1L of DVII culture medium (Isabelle, 2011; Stein, 1973; Hughes & Lund, 1962)

Nomenclature	Chemical Formula	Concentration (g/L)	Used quantity (mL/L)
Calcium nitrate tetrahydrate	$Ca(NO_3)_2 .4H_2O$	20	2
Dipotassium hidrogenorotosfofate treehidratated	$K_2(HPO_4) .3H_2O$	40.62	0.323
Magnesium sulfate heptahydrate	$MgSO_4.7H2O$	25	1
Metassilicato disodium pentahydrate	$Na_2SiO_3.5H_2O$	21.2	2
Sodium carbonate	Na_2CO_3	21	0.95
Vitamin B_{12} solution	-	0.0001	1
Thiamine solution	-	0.1	1
Trace Elements solution *	-	-	0.1
Fe – EDTA solution **	-	-	10

Trace Metal Solution* (in a 500 mL volumetric flask)		
Nomenclature	**Chemical Formula**	**mg**
Boric acid	H_3BO_3	1550
Copper sulfate pentahydrate	$CuSO_4 .5H_2O$	62.5
Manganese(II) sulfate monohydrate or tetrahydrate	$MnSO_4. H_2O$ / $MnSO_4 . 4 H_2O$	858.5 / 1115
Zinc sulfate heptahydrate	$ZnSO_4 .7H_2O$	143.5
Sodium tungstate dihydrate	$Na_2WO_4 .2 H_2O$	16.5
Ammonium molybdate tetrahydrate	$(NH_4)_6Mo_7O_{24}$, 4 H_2O	44
Potassium bromate	KBr	59.5
Potassium iodide	KI	41.5
Cadmium nitrate	$Cd(NO_3)_2 .4 H_2O$	77

hydrate		
Cobalt(II) nitrate hexahydrate	$Co(NO_3)_2$ .6 H_2O	73
Nickel(II) nitrate hexahydrate or Ammonium Nickel Sulphate Hexahydrate	$Ni(NO_3)_2$. 6 H_2O / $NiSO_4(NH_4)_2SO_4$. 6 H_2O	72.9 / 99
Chromium(III) nitrate nonahydrate or heptahydrate	$Cr(NO_3)_3$ .9 H_2O / $Cr(NO_3)_3$ .7 H_2O	20.3 / 18.5
Ammonium metavanadate or Aluminium sulfate hexadecahydrate	NH_4VO_3 / $V_2O_4(SO_4)_3$ .16 H_2O	5.5 / 17.5
? / potassium alumen?	$Al(SO_4)_2K$ .12 H_2O / $Al_2(SO_4)_3K_2SO_4$ .24 H_2O	237 / 237

Fe-EDTA solution**	
a)	323.52 mg of EDTA-Na_2 .2 H_2O in an 500 mL volumetric flask and adjust the volume with milli – Q water.
b)	1 mL of concentrated HCL in a 100 mL volumetric flask and adjust the volume with milli – Q water.
c)	In a 1 L volumetric flask, transfer 500 ml of (a) solution and 10 ml of (c) solution then adjust to volume with milli-Q water.

Annex IV – SIMPER results relatively to diatoms' community structure.

Table 17 - SIMPER results: autumn campaign (15/nov/18), with an average similarity of 57.19%.

Species	Average abundance	Average similarity	Similarity / Standard Deviation	Contributive percentage	Cumulative percentage
KOBG	7.03	23.51	1.66	41.11	41.11
ADMI	4.77	15.38	18.21	26.89	68.00
NAAN	1.51	4.51	10.90	7.54	75.53
GRHO	1.22	3.59	4.99	6.27	81.80
KOTG	1.33	2.87	0.58	5.02	86.83
NNOT	2.01	2.54	0.58	4.44	91.26

Table 18 - SIMPER results: spring campaign (5/jun/19), with an average similarity of 62.87%.

Species	Average abundance	Average similarity	Similarity / Standard Deviation	Contributive percentage	Cumulative percentage
ADMI	5.45	12.81	9.21	20.38	20.38
KOBG	4.16	9.15	11.60	14.55	34.92
EOMI	2.46	5.75	4.90	9.15	44.07
FPEM	2.35	4.79	7.03	7.62	51.70
FRUM	2.84	4.17	2.63	6.63	58.33
GPAR	1.72	3.76	8.77	5.97	64.30
ADMT	1.94	3.63	2.08	5.77	70.07
DMES	1.38	2.81	9.96	4.48	74.55
EMIN	0.98	2.60	45.60	4.13	78.68
NNOT	1.08	2.20	3.89	3.51	82.19
NDME	0.84	1.97	8.66	3.14	85.33
KOTG	1.07	1.47	0.58	2.34	87.67
GRHO	1.29	0.89	0.58	1.41	89.08
GMIN	0.84	0.88	0.58	1.40	90.48

Table 19 - SIMPER results: autumn campaign (15/nov/18) and spring campaign (5/jun/19) with an average dissimilarity of 58.03%.

	15/nov/18	5/jun/19				
Species	**Average abundance**	**Average similarity**	**Similarity/ Standard Deviation**	**Contributive percentage**	**Cumulative percentage**	**Species**
KOBG	7.03	4.16	5.58	1.51	9.61	9.61
FRUM	0.00	2.84	4.65	1.71	8.01	17.62
EOMI	0.00	2.46	4.11	4.62	7.08	24.70
FPEM	0.00	2.35	3.87	3.33	6.66	31.36
ADMT	0.00	1.94	3.25	2.31	5.60	36.97
ADMI	4.77	5.45	2.90	1.73	4.99	41.96
GPAR	0.00	1.72	2.84	4.33	4.89	46.85
NNOT	2.01	1.08	266	1.67	4.59	51.44
DMES	0.00	1.38	2.27	325	3.91	55.35
GRHO	1.22	1.29	1.83	1.52	3.15	58.50
KOTG	1.33	1.07	1.60	1.20	2.76	61.25
NAAN	1.51	0.56	1.52	1.13	2.63	63.88
GPUM	0.89	052	1.47	1.21	2.53	66.41
GMIN	0.00	0.84	139	1.22	2.40	68.81
NDME	0.00	0.84	1.39	6.77	2.39	71.20
NDIS	0.00	0.79	1.29	1.28	2.22	73.42
ENMI	0.31	0.64	1.22	0.89	2.10	75.52
FSAX	0.70	0.00	1.18	2.26	2.03	77.56
UBIC	0.00	0.70	1.15	1.13	1.98	79.54
FFBI	0.00	0.71	1.15	0.99	1.98	81.51
SLIN	0.00	0.60	1.02	1.32	1.76	83.27
SRBA	0.36	0.66	0.96	1.12	1.65	84.93
EPEC	0.13	0.59	0.92	1.29	1.58	86.51
FGRA	0.50	0.23	0.92	0.93	1.58	88.09
CPLA	0.31	0.56	0.84	1.18	1.46	89.55
EMIN	0.63	0.98	0.78	1.07	1.34	90.89

Annex V – PERMANOVA results relatively to the ecotoxicological tests.

Table 20 - PERMANOVA results for ADMI on low severity AEA (Pair-wise Tests): 1 – 12.5%, 2 – 25%, 3 – 50%, 4 – 75% and 5 – 100%.

Groups	t	Unique perms	P(MC)
1, 2	1.2263	7	0.305
1, 3	1.2437	10	0.272
1, 4	0.60187	5	0.572
1, 5	4.234	10	0.017
2, 3	1.6469	10	0.173
2, 4	0.97758	7	0.392
2, 5	3.5153	10	0.026
3, 4	1.5595	10	0.222
3, 5	3.3515	10	0.02
4, 5	4.3166	10	0.011

Table 21 - PERMANOVA results for NPAL on low severity AEA (Pair-wise Tests): 1 – 12.5%, 2 – 25%, 3 – 50%, 4 – 75% and 5 – 100%.

Groups	T	Unique perms	P(MC)
1, 2	6.7596	10	0.001
1, 3	1.0242	5	0.343
1, 4	7.0586	10	0.005
1, 5	5.3708	10	0.007
2, 3	5.2924	10	0007
2, 4	3.1578	10	0.033
2, 5	2.1423	7	0.097
3, 4	3.8914	10	0.013
3, 5	3.6677	10	0.028
4, 5	0.81112	5	0.437

Table 22 - PERMANOVA results for ADMI on high severity AEA (Pair-wise Tests): 0 – control, 1 – 12.5%, 2 – 25%, 3 – 50%, 4 – 75% and 5 – 100%.

Groups	t	Unique perms	P(MC)
0, 1	39.981	10	0.001
0, 2	49.883	10	0.001
0, 3	27.814	10	0.001
0, 4	3.397	10	0.022
0, 5	1.834	10	0.28
1, 2	9.1515	10	0.004
1, 3	2.1975	10	0.094
1, 4	1.2481	10	0.268
1, 5	2.1043	10	0.095
2, 3	2.0226	10	0.12
2, 4	1.8614	10	0.139
2, 5	2.5233	10	0.05
3, 4	1.5598	10	0.187
3, 5	2.3159	10	0.082
4, 5	0.96509	10	0.365

Table 23 - PERMANOVA results for NPAL on high severity AEA (Pair-wise Tests): 0 – control, 1 – 12.5%, 2 – 25%, 3 – 50%, 4 – 75% and 5 – 100%.

Groups	t	Unique perms	P(MC)
0, 1	11.257	10	0.001
0, 2	8.3374	10	0.001
0, 3	10.29	10	0.001
0, 4	18.37	10	0.001
0, 5	16.771	10	0.001
1, 2	0.12347	10	0.923
1, 3	1.4104	10	0.231
1, 4	2.696	10	0.054
1, 5	5.0196	10	0.006
2, 3	1.2553	10	0.268
2, 4	1.8504	10	0.15
2, 5	3.7646	10	0.018
3, 4	0.18226	10	0.874
3, 5	2.4333	10	0.083
4, 5	4.5067	10	0.011