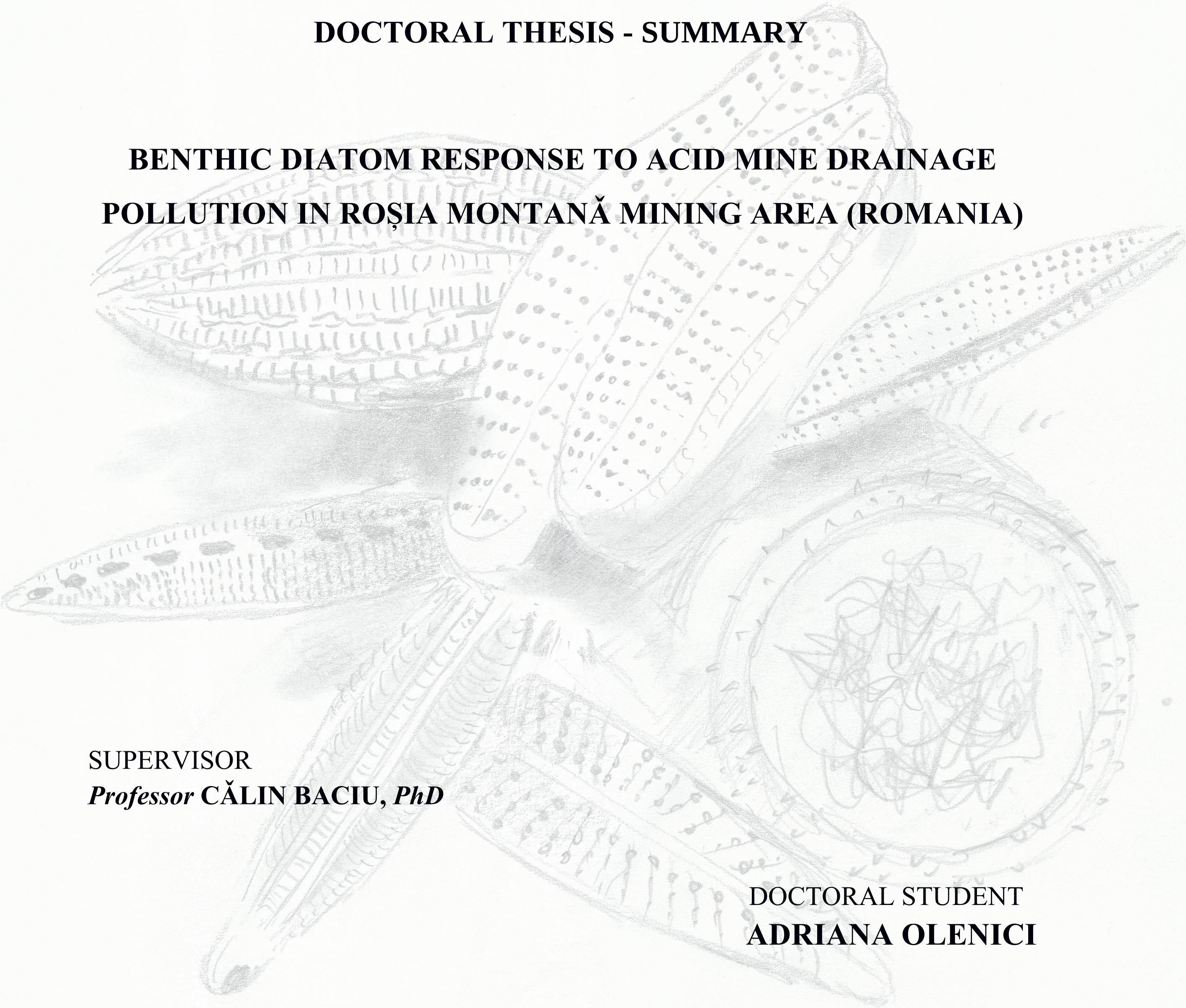
BABEŞ-BOLYAI UNIVERSITY, CLUJ-NAPOCA
FACULTY OF ENVIRONMENTAL SCIENCE AND ENGINEERING
DOCTORAL SCHOOL OF ENVIRONMENTAL SCIENCE

DOCTORAL THESIS - SUMMARY

BENTHIC DIATOM RESPONSE TO ACID MINE DRAINAGE POLLUTION IN ROȘIA MONTANĂ MINING AREA (ROMANIA)



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Keywords:

Bacillariophyta, diatom teratologies, abnormal girdle, mine waters, heavy metals, environmental stressors, geometric morphometry, water quality biomonitoring, bioindicators, diatom indices, Abrud River basin.

INTRODUCTION

1.1. Hypothesis and Objectives

In the present study, the main hypothesis is that perturbations in freshwater play a key role in the health status of the benthic diatom communities that, in turn, could be used as good indicators of, e.g. acid mine drainage pollution.

To test this hypothesis, the main objective of this PhD thesis was to analyze the response of the diatom communities of a potentially polluted catchment area, the Abrud River basin, affected by the presence of an historical mine exploitation.

The specific objectives were:

- To analyze the typology of diatom deformities observed in the study area;
- To describe a new kind of teratology affecting diatom girdle bands;
- To determine the main water physicochemical parameters in the study sites, highlighting those with major effects on the composition, structure and dynamics of diatom communities;
- To explore the relationship between the concentration of water pollutants and the occurrence of teratological forms in epilithic diatoms;
- To observe the effects caused by high concentrations of heavy metals on benthic diatom communities collected throughout the study area;
- To investigate the response of two dominant taxa, *Achnanthidium minutissimum* (Kützing) Czarnecki and *Achnanthidium macrocephalum* (Hustedt) Round & Bukhtiyarova to Acid Mine Drainage (AMD) effects in the study area;

- To find the set of environmental predictors that lead to the occurrence of abnormal diatoms in the study area and to evaluate the relationship between the degree of deformation in the valve outline and AMD-derived pollution;
- To discuss the consequences of overriding diatom teratology on diatom-based water quality assessment protocols;
- To generate a transfer function relating the abundance of abnormal cells and metal levels in waters, to be used as a biomonitoring metric;
- To evaluate and assess water quality in the study area, based on the structure of diatom assemblages.
- To identify the main heavy metal that affects the algal communities in the observed stations.
- To contribute to the diatom flora of Romania.

1.2.Brief Overview on Studies Regarding Benthic Diatoms in the Abrud River Catchment Area

Although there are many research works referring to diatom communities in the Arieș River basin (the river and its major tributaries), only a small number describe these communities within the Abrud River basin. For instance, two studies (Momeu et al., 2007; 2009) on algal, invertebrates and fish communities in the Arieș River basin (sampling surveys carried out in 2005 and 2006, respectively), included a sampling point located on the Abrud River, where no diatom taxa where recorded. A paper exclusively covering diatom communities in the Arieș River basin (Szekely-Andorko et al., 2011, samples collected during 2008) included a sampling site located also on this river, with *ca.* 100 taxa listed therein. Finally, a limnological study regarding algal and invertebrate communities (Battes et al., 2012) considered samples collected from the Roșia Montană area, with 58 diatom species identified.

1.3. Brief Overview of the Class Bacillariophyceae

Diatoms (*Bacillariophyceae*) are a group of single-celled algae present in almost all types of surface waters, including humid terrestrial habitats, even under extreme conditions.

The singularity of this group resides both in the structural particularities of the cell, especially the silica frustule, and in their major role in aquatic ecosystems. Diatoms are some of the most well-known aquatic organisms, provided their widespread use for quality assessment and monitoring applications (Hustedt, 1957; Zelinka & Marvan, 1961).

1.4. The Bioindicator Value of Diatoms

Diatoms are enormously successful organisms regarding their environmental adaptability, distribution and evolutive history. This group of algae has numerous advantages as bioindicators in aquatic ecosystems monitoring studies (Cholnoky, 1968; Lowe, 1974); making them the most frequently used algal organisms in water quality surveillance programs, as in the case of Romania (Momeu & Péterfi, 2007, 2009; Szigyarto & Bakos, 2015).

Water quality assessment methods based on the use of diatoms are well developed, their performance having been established worldwide for various types of aquatic habitats, including freshwaters, brackish waters and estuaries, lentic and lotic environments, and wetlands (Kolkwitz & Marsson, 1908; Patrick, 1949).

Monitoring procedures based on living organisms quantify the "health" status of a river, as opposed to a mere description of the chemical and physical components (Karr, 1991; Rocha, 1992). Even continuous chemical monitoring can override a high-impact event on the community key-organisms. Furthermore, it is difficult to interpret the synergic effects of chemical substances on aquatic biocenoses.

Diatoms are currently used as eutrophication indicators in lakes. While diatoms are collectively tolerant to lake productivity, individual species have specific habitat preferences and growth optima. Diatoms also help in assessing environmental conditions in rivers and streams, provided their ecological importance in these ecosystems, their ability to respond rapidly to environmental impairment, and the ease of their use. Diatoms respond directly and sensitively to many physical, chemical and biological changes in river and stream ecosystems and, since they can be found in almost all aquatic habitats, they can be used to compare streams, lakes, swamps, oceans, estuaries, and even some ephemeral aquatic habitats (Stoermer & Smol, 1999; Smol & Stoermer, 2010).

The characteristics of diatom communities have been used to assess the ecological integrity of rivers and streams (Patrick & Strawbridge, 1963), as well as to diagnose the causes for degradation. Ecological integrity is more comprehensive than biotic integrity, as it includes the physical and chemical features of the habitat.

Algal communities mirror physical, chemical and biological characteristics in aquatic ecosystems via the presence/absence of species and growth or decrease of populations, among other reactions to environmental changes. In practice, only a number of benthic algal groups are frequently used in assessing the quality of natural waters (McCormick & Cairns, 1997; Potapova & Charles, 2002), particularly for measuring saprobity and salinity degrees (Kiss, 1998; Barinova, 2017; Brabcová et al., 2017; Nautiyal & Nautiyal, 2018). If water quality is altered as a result of certain human activities and such alteration exceeds the tolerance intervals of these species, their populations will drop or disappear. Identifying the factors causing this decline requires complex additional investigations, but the "response" of the algal community will indicate certain water quality impairment and a potential pollution source.

Benthic diatom communities are used on a large scale for monitoring water quality owing to certain aspects (Lowe & Pan, 1996; Bellinger & Sigee, 2015; Kale & Karthick, 2015; Morin et al., 2016):

- In general, autotrophic benthic algae –as primary producers– have a crucial position between physical and chemical environmental factors and the other organisms of the food web, so that disruptions in the benthic algae level can severely influence the other levels in the aquatic ecosystems. Hence, some studies reveal that benthic diatom communities enable a more accurate assessment of the quality and biotic integrity of aquatic ecosystems than protozoa or invertebrates (Beyene et al., 2009).
- Diatoms are practically cosmopolitan, being found from the poles to the desert regions, both in freshwaters and in the seas, including brackish, thermal and hypersaline waters, under a wide range of environmental conditions.
- A great majority of species appear abundantly throughout the planet, and that is why many diatom-based indices have universal applicability, allowing comparative studies between different regions, which in other cases are not viable.
- The most common diatom indices are based on the identification of 400 individuals per sample. This makes the error in estimating the composition of community lower than 10%, which implies a great precision of these methods from a statistical point of view (Blanco et al., 2011).

- Both attached and motile species have a high indicator potential due to their inability to avoid pollution by means of migration, which means that they can either adapt or simply disappear.
- Benthic diatoms have relatively short life cycles, which favor quick responses to environmental changes. Benthic algal communities are usually the first to react to environmental disruptions and the first to "bounce back" upon restoration of the optimal conditions.
- Benthic diatom communities generally have higher diversities than other groups of aquatic organisms. Hundreds of species can coexist on few cm^2 of substratum, each of them with specific optima and tolerance intervals with respect to environmental factors, so that the community behaves as a whole complex biological monitoring system.
- Benthic communities have a compact structure in terms of the space they cover; therefore, no more than a few cm^2 of substrata will suffice for the collection of a representative sample.
- The collected samples are easy to handle and require little fixation, which can be a major long-term advantage, as preserved samples and fixed microscopic preparations can be reexamined at any time for subsequent investigations.
- Another advantage is the possibility of identifying taxa based only on frustule features, which are relatively easy to study under the light microscope.

Research in the field of aquatic ecology, along with the implementation of monitoring programs and the results of studies in other fields, should ultimately lead to an integrated multidisciplinary approach that includes not only diatoms, but also all the other groups of aquatic organisms (De Jonge et al., 2008).

1.5. Diatom teratology – a Tool for Metal Monitoring /Acid Mine Drainage Contamination

The morphological alterations of diatoms are non-adaptive phenotypic abnormalities caused by environmental stress that generally affect the contour of the frustule or the shape of the striae (Falasco et al., 2009a). According to the current literature, the presence of deformities in contaminated environments is considered an indication of stress; however, the mechanisms that induce deformities and quantify teratologies remain poorly understood (Lavoie et al., 2017).

Malformations are usually detected in natural diatom assemblages, but their frequency of incidence is generally low (< 0.5% according to Morin et al., 2008a; see also Arini et al., 2012). However, the proportion of abnormal valves can increase with the presence of multiple stressors (Lavoie et al., 2017).

Several studies showed a significant positive correlation between the abundance of deformed cells and environmental stress, such as drought conditions, low speed and water flow, the increase of the temperature and the intensity of light (Antoine & Benson-Evans, 1986), contamination by pesticides (Debenest et al., 2010) or decrease in water quality (Gómez & Licursi, 2003). However, the most known causes that determine the appearance of teratological forms are artificial growth conditions (Falasco et al., 2009b) and heavy metal contamination (Cantonati et al., 2014). Malformations can also be induced by other independent factors, like malfunctions of proteins responsible for silica transport and deposition (Kröger et al., 1994, 1996, 1997; Kröger & Poulsen, 2007; Knight et al., 2016), or for the structural and mechanical integrity of the valve (Kröger & Poulsen, 2007; Santos et al., 2013).

The deformities are categorized based on their type: aberrant valve outline/shape, irregular sternum/raphe, atypical striae/areolae, and mixed deformities (Falasco et al., 2009a).

Many authors consider that morphological alterations of diatoms could be useful tools to monitor environmental changes in rivers (Cattaneo et al., 2004; Cantonati et al., 2014), including those caused by the drainage of water from mines (AMD), which in recent decades has been considered an important source of environmental contamination (Letterman & Mitsch, 1978). It has been reported that AMD induces also teratologies and some authors considered the ratio of abnormal individuals to detect acid mine drainage consequences (Cattaneo et al. 2004; Lavoie et al. 2012).

The presence of deformed frustules in polluted ecosystems is often a reaction to noxious chemicals. For this reason, a great interest has emerged in using morphological abnormalities in biomonitoring studies. Teratologies open a tool box to assess aquatic ecosystem health and it can be expected that their occurrence and severity are related to the degree of stress (Lavoie et al., 2017).

In this context, cellular morphology could respond to the relationship between metal contamination and adaptation of organisms (Morin & Coste, 2006; Ancion et al., 2010). It has been often reported that diatoms respond to chemical stress, through changes in species distribution, changing the cellular volume and inducing the generation of teratologies (Morin et al., 2008b; Falasco et al., 2009b). Many studies have pointed out that the deformed shape of diatom valves in response to metal contamination are markers of this sort of pollution (Dickman, 1998; Torres et al., 2000; Gómez & Licursi, 2003). Numerous environmental pressures can be the root for the development of teratologic diatoms, and the occurrence of deformed cells can tell us about the effects of environmental variations (Falasco et al., 2009b). Morphological deformities have been reported in communities under metal pressure (Falasco et al., 2009b), and the quantitative analysis of abnormal frustules could be a tool for the monitorization of metal pollution (Morin et al., 2012).

Metal pollution of aquatic habitats due to acid mine drainage has remarkable effects on diatom teratology (Olenici et al., 2017). It is not easy to measure the degree of deformation but Olenici et al., (2017) have found a method based on geometric morphometry that allowed the discrimination between normal and abnormal individuals. Such teratologies are diverse, not yet very well studied and sometimes difficult to be appreciated using optical microscopy (Olenici et al., 2019). Actually, one of the problems of microscopic diatom observation is the nature of this material (transparent and colourless). In this regard, the work of Sánchez et al. (2018) evaluating oblique illumination techniques demonstrates that these methods allow distinguishing minute details with a similar performance that more expensive microscope Differential Interference Contrast (DIC) systems. In any case, accurate taxonomic resolution under light microscopy is critical in biomonitoring studies (Blanco et al., 2017), which points to the need of new technical and statistical tools for the correct identification at species level, mechanical approaches (such as pure morphometry-based diatom determination) having been discouraged (Blanco et al., 2017). Nevertheless, diatom automatic detection and identification has been a challenge for computer scientist (Pedraza et al., 2018) and a lot of work has been done in the application neural networks for this purpose with excellent perspectives (Pedraza et al., op. cit.). However, we must consider the natural- and environmental driven variations in diatom morphology (Olenici et al., submitted,) that constitutes a true benchmark to these technologies. Despite their ecological importance and their great diversification in world aquatic ecosystems, the diatoms of many regions of the world remain practically unknown (Blanco et al., in press a; Blanco et al., submitted). Thus, new diatom species are being continuously described in the most diverse ecosystems (Borrego-Ramos et al., 2018; Blanco et al., 2019a; Blanco et al., 2019b; Blanco et al., in press b) but it is crucial to consider also the community as a whole in environmental studies and analyze their composition as a consequence of biological interactions (Borrego-Ramos et al., 2019) or the presence of metals and other pollutants (Baciu et al., 2018).

2. *MATERIAL AND METHODS*

2.1. Study Area

Abrud River is a tributary of Arieș River (the most important right-side tributary of the Mures River, Forray & Hallbauer, 2000) and is situated in the Alba county, North-Western Romania in the Apuseni Mountains.

These mountains belong to the Alpine Carpathian Balkan system, which is in the interior of the Carpathian arc forming an isolated block (Ianovici et al., 1976; Balintoni 1994). This arc is made up by a Tertiary calc-alkaline volcanic nucleus embodying various episodes of magmatic activity in the last 14.7 million of years (Roșu et al., 2004). North-vergent Cretaceous thrust sheets of oceanic to terrestrial flysch-type sedimentary elements, placed in Palaeozoic and Precambrian basement (Leary et al., 2004).

Roșia Montană mining area is located within the Southern Apuseni Mountains, in a Metaliferi Mountains area. Metamorphic rocks, Cretaceous magmatites, Mesozoic ophiolites (Upper Palaeogene), Neogene igneous rocks (Tămaş, 2007), Mesozoic and Miocene sedimentary rocks and Quaternary sediments constitute the geological structure (Duma, 2008). Roșia Montană is the largest gold deposit in Europe, with a large reserve in Au (500-1000 Mt) and Ag (6 Gt). A Miocene-age maar-diatreme complex is emplaced into Cretaceous flysch-type sedimentary rocks, with the preponderance of black shales intercalates with sandstone and conglomerates and intruded by dacite domes. The dacitic intrusions corresponds to a Cetate Dacite and a Carnic Dacite, together with intrusions of finely disseminated pyrite and dykes that are crossing the breccias, have been decisive in the mineralization activity. Hydrothermal alteration has modified the dacite, which is the core host of the Au–Ag mineralization (Lazăr et al., 2014).

Hydrological Features

Roșia Montană is situated within the Abrud River basin, draining waters to Corna, Săliște and Roșia rivulets that are tributaries of Abrud River. The Corna Valley flows upstream of Abrud and Săliște town; Roșia Valley watershed is oriented in the west direction and flows downstream of Abrud Town. The area corresponds to a moderate steep mountainous topography (700 to 1000 m a.s.l.) being the main groundwater recharge coming from rainwater. The rivers increase the caudal due to the low permeability of the rocks and the convergence of the flow. The average flow rates in Roșia Valley was 0.16 m^3/s; in Corna Valley was 0.07 m^3/s and finally 0.16 m^3/s for the Săliște stream in the period 2001-2003 (RMCG, 2006).

AMD produced from waste dumps accumulated in ponds by mining actions contaminates all the streams from the Roșia Montană complex. Those rivers are flowing into tributaries of the Danube (Forray, 2002; Florea et al., 2005; Bird et al., 2005; Manske et al. 2006; Lăcătuşu et al., 2007; Baciu et al., 2012; Papp et al., 2018) and constitutes a challenge in the management of the problem (Gray, 1997).

The climate of the region is continental temperate affected by the altitude, with average temperatures ranging between -4.7 and 16.9 °C. Rainfalls are between 700 and 800 mm/year of rain (75%) and snow (24%) (Azzali et al., 2014).

2.2. Sampling Points

Only surface running waters were selected for this study. Sampling points were set in Cărpiniș, Roșia Montană, Abrud, Bucium Șasa, and Bucium-Sat, that is in Alba County. As such, the selected area comprises the Abrud River area and its main right-bank tributaries between Cărpiniș and Bucium-Sat. Sixteen sampling points were established in order to achieve an overview of the benthic diatom communities in the studied streams (fig. 2.2.1.).

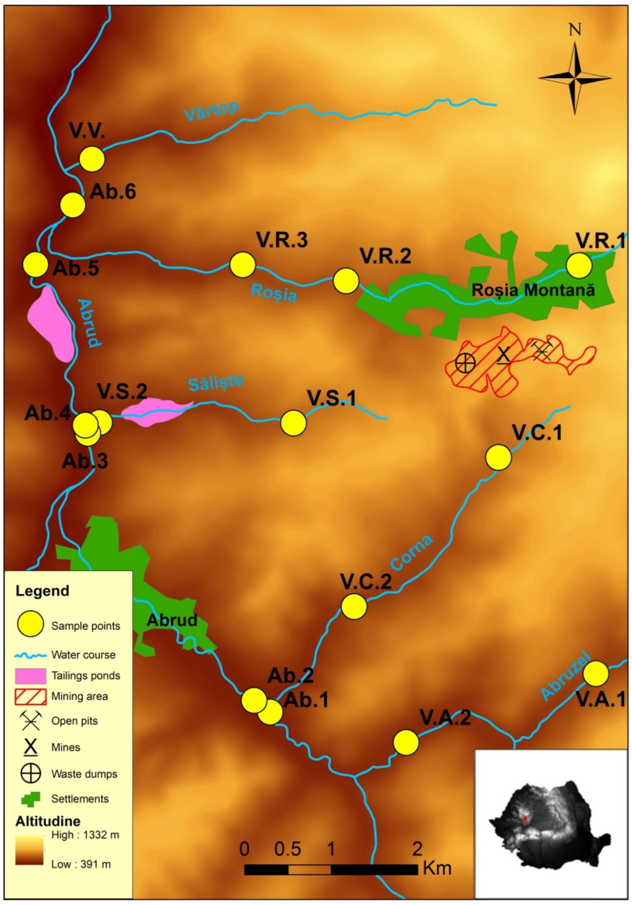


Figure 2.2.1. The map with the sampling points in the study area (V.V.=Vârtop Valley, V.R.1= Roșia Valley 1, V.R.2= Roșia Valley 2, V.R.3= Roșia Valley 3, V.S.1= Săliște Valley 1, V.S.2= Săliște Valley 2, V.C.1= Corna Valley 1, V.C.2= Corna Valley 2, V.A.1= Abruzel Valley 1, V.A.2= Abruzel Valley 2, Ab.1=Abrud1, Ab.2=Abrud2, Ab.3=Abrud3, Ab.4=Abrud4, Ab.5=Abrud5, Ab.6=Abrud6)

2.3. The Collection and Processing of Benthic Diatom Samples

Benthic diatom samples were collected and processed following European Standards EN 13946/2003 and EN 14407/2004. The collection of samples was carried out during the vegetative period (between spring and autumn), in order to determine the composition and certain structural characteristics of the benthic diatom communities. Therefore, sample collection covered spring and summer 2013, and the following sample gathering was scheduled for autumn 2013. In order to obtain representative samples and to observe the seasonal and annual dynamics of the diatom communities, samples were also collected over the course of 2014, during the same seasons as in 2013, summing 96 samples. The samples were taken from the same type of substrata (natural) in all the sampling points, more precisely stones. In the case of tributaries, sampling was carried out along the entire width of the riverbed; on the Abrud River, sample collection was performed on the shore side of the riverbed. The first processing phase for the treatment of the materials collected consisted on removing inorganic and organic contents (in order to better visualize frustule's ornamentations during subsequent microscopic examination) followed by the elaboration of permanent microscopic slides in a second phase (fig. 2.3.1.).

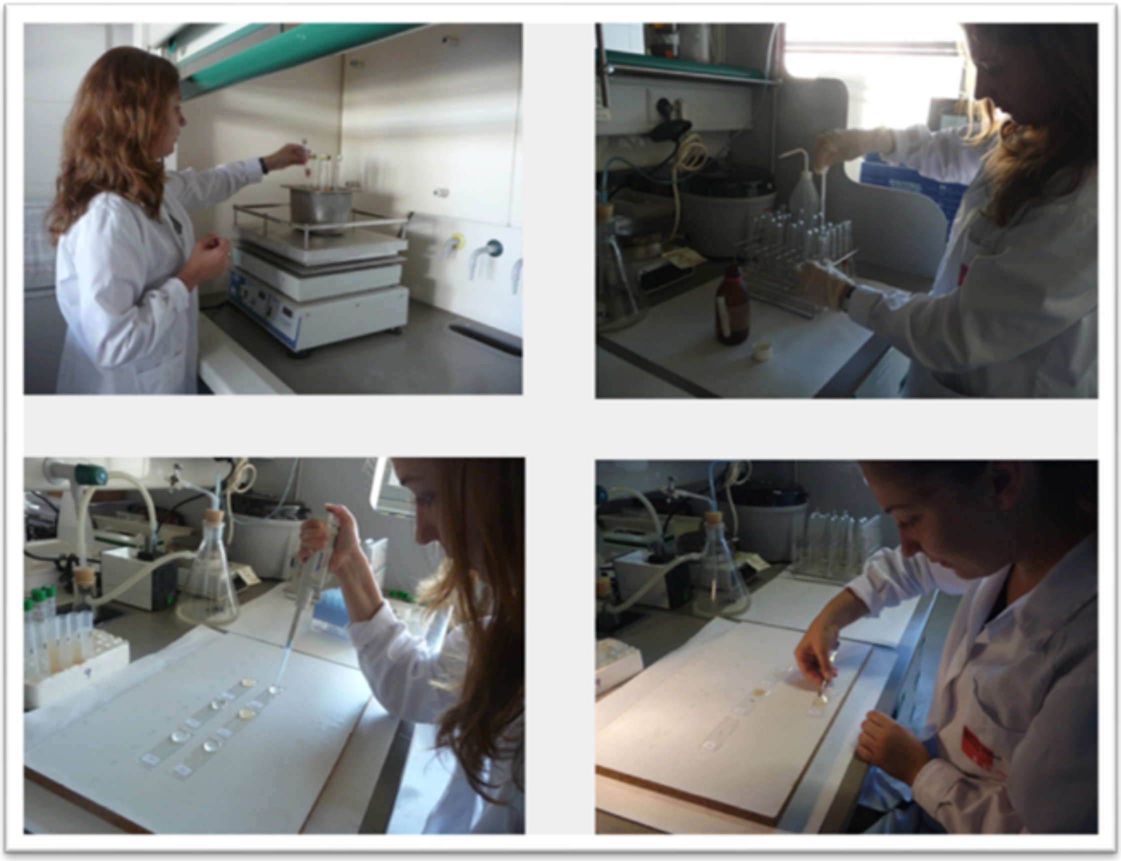


Figure 2.3.1. Diatom samples treatment in the laboratory

2.4. Measuring Physical and Chemical Parameters and Determining the Concentration of Certain Ions

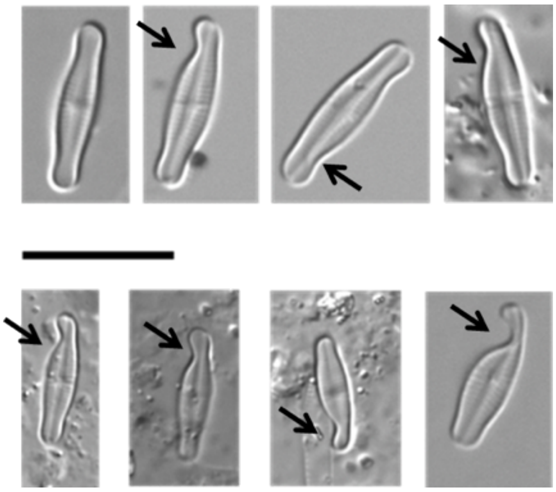
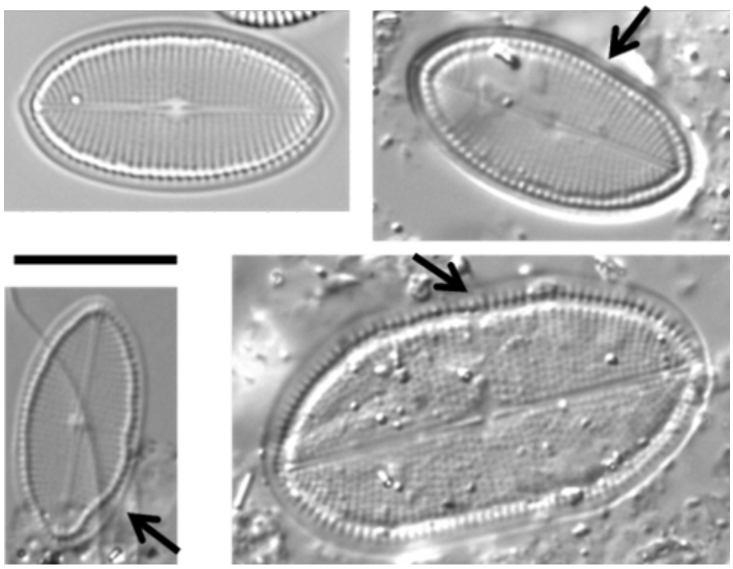
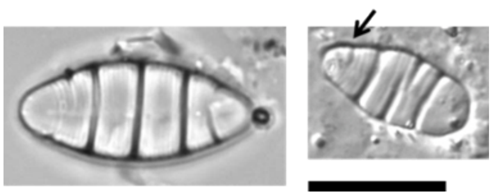
The main physical and chemical parameters of water (pH, temperature, salinity, conductivity, TDS, O_2, turbidity) were measured *in situ* concurrently with the collection of benthic samples, using a portable multimeter (350i/SET WTW) and a portable turbidity meter (WTW Turb 430IR). Water samples were also collected in order to determine the concentration of certain anions and cations in the laboratory using a Dionex ICS – 1500 ion chromatography system and to determinate the level of the concentration of some heavy metals, using the atomic absorption spectrometer ZeEnit 700.

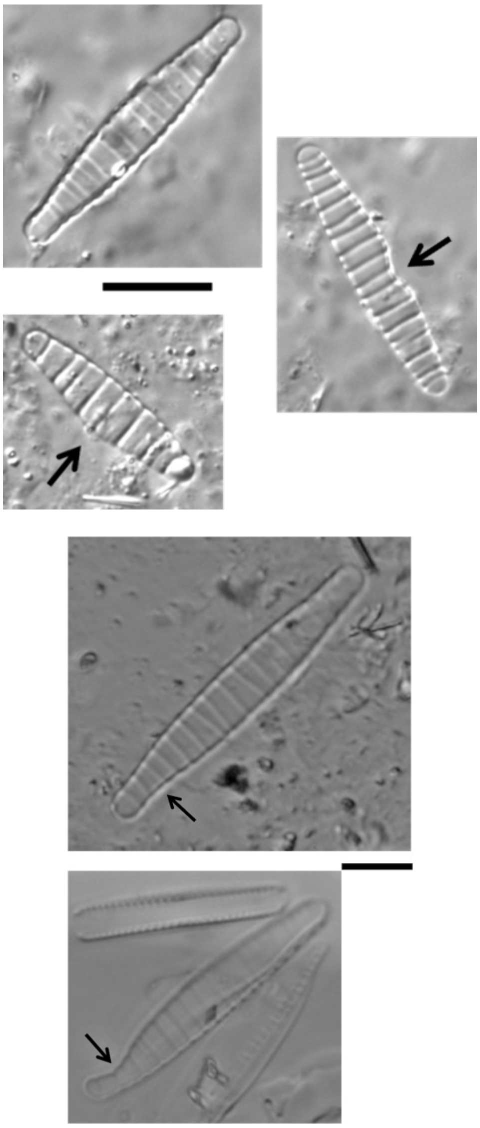
3. Results and Discussion

3.1. Teratologic Diatoms from Acid Mine Drainage Polluted Waters

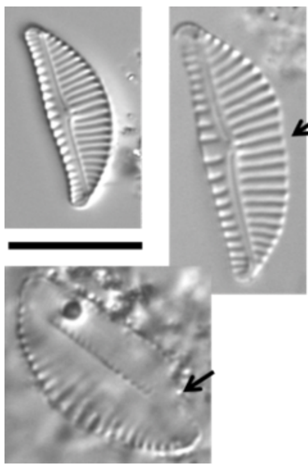
Along the study period, a wide representation of abnormal forms were recorded and have been summarized in table 3.1.1. In total, five teratological categories were detected and some representations of each type have been illustrated. A seasonal dynamic of the benthic diatom communities was observed, not only regarding the number of species or their relative abundance, but also regarding the type of identified teratology. The most repetitive abnormal type was the deformed valve outline, which agrees with previous works (Cattaneo et al., 2004; Falasco et al., 2009a,b; Lavoie et al., 2012; Cantonati et al., 2014; Tornés et al., 2018). To a lesser extent it has been observed the raphe canal system modifications (displaced fibulae) during Summer and Autumn 2013 and during Spring and Autumn 2014, and the abnormal striation patterns in Autumn 2013 and in Spring and Autumn 2014. The mixed teratologies were observed only during Spring 2014 and only one time, in Summer 2013, the deformed girdles.

Table 3.1.1. Types of diatom deformities found in the study area (Scale bar: 10 µm). Arrows indicate frustule deformities

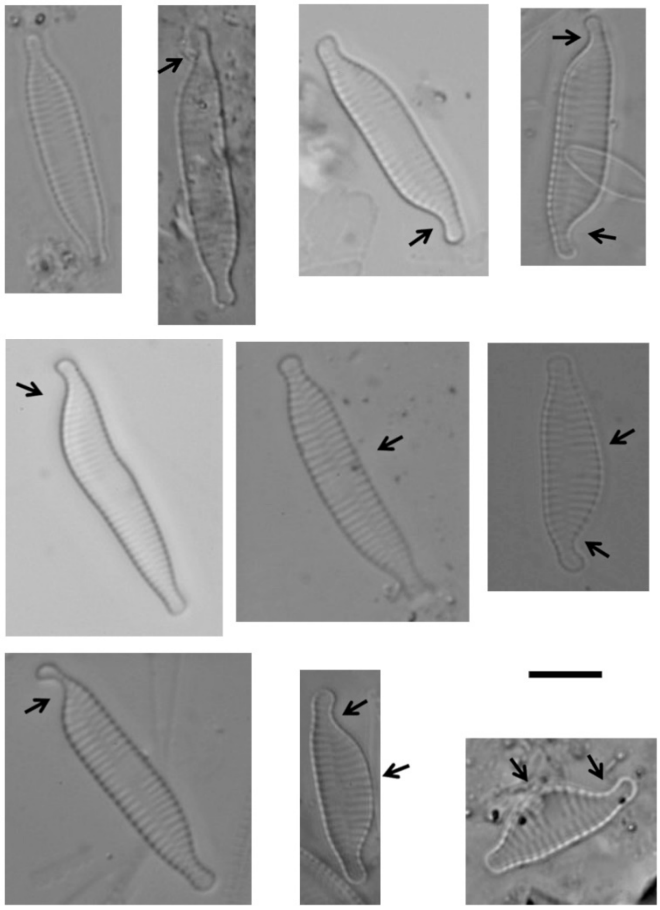
Teratology description	Normal vs. abnormal individuals
Type1: Abnormal valve outlines (different degrees of deformation)	 ***Achnanthidium minutissimum* (Kützing) Czarnecki**  ***Cocconeis euglypta* Ehrenberg**  ***Diatoma mesodon* (Ehrenberg) Kützing**



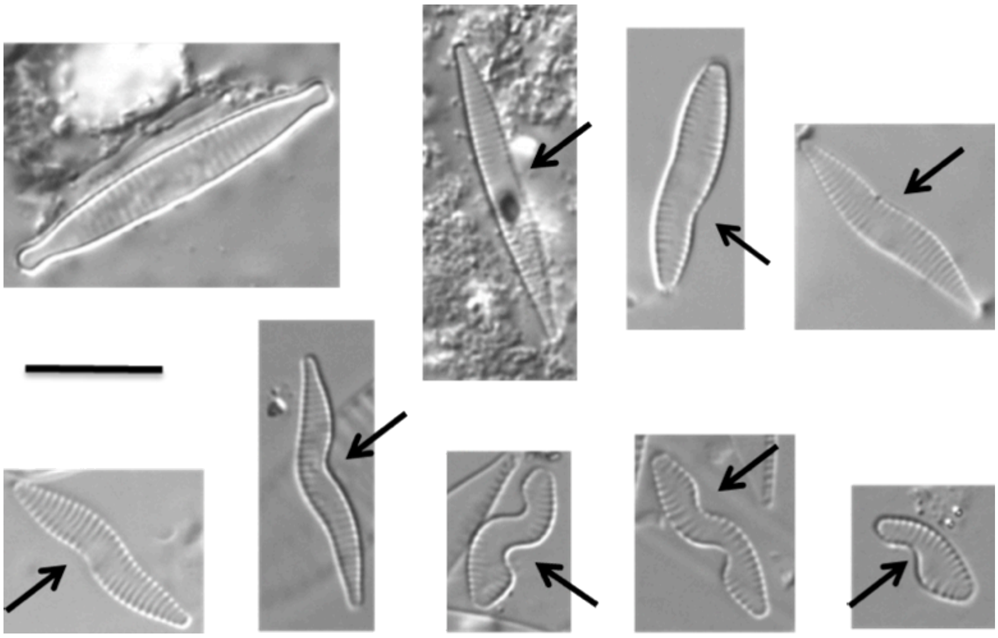
Diatoma moniliformis **Kützing**



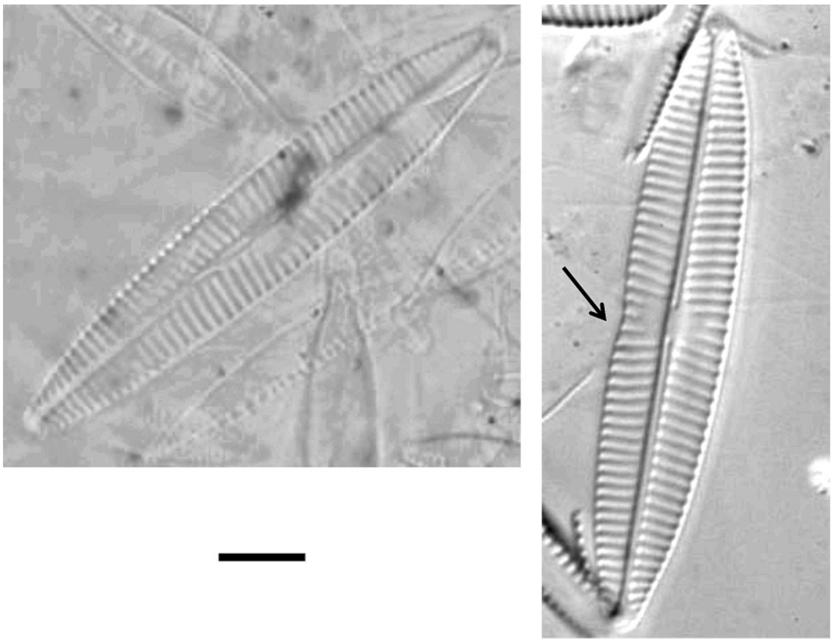
Encyonema minutum **(Hilse) D.G. Mann**



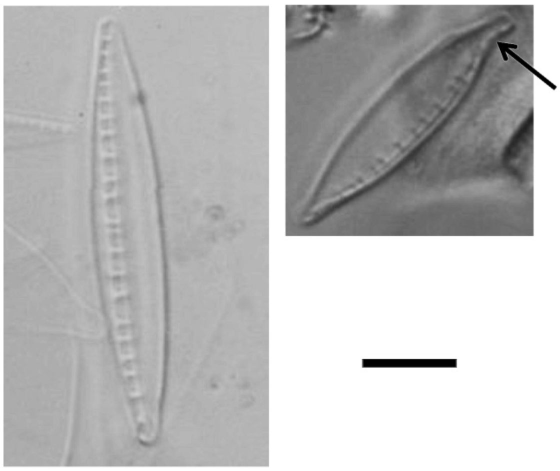
Fragilaria recapitellata Lange-Bertalot et Metzeltin



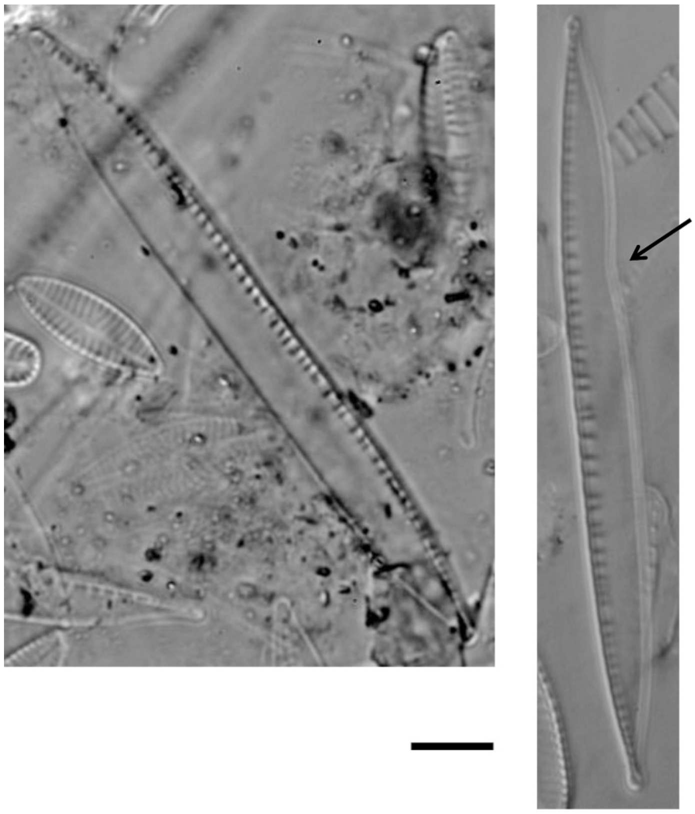
Fragilaria rumpens (Kützing) Carlson



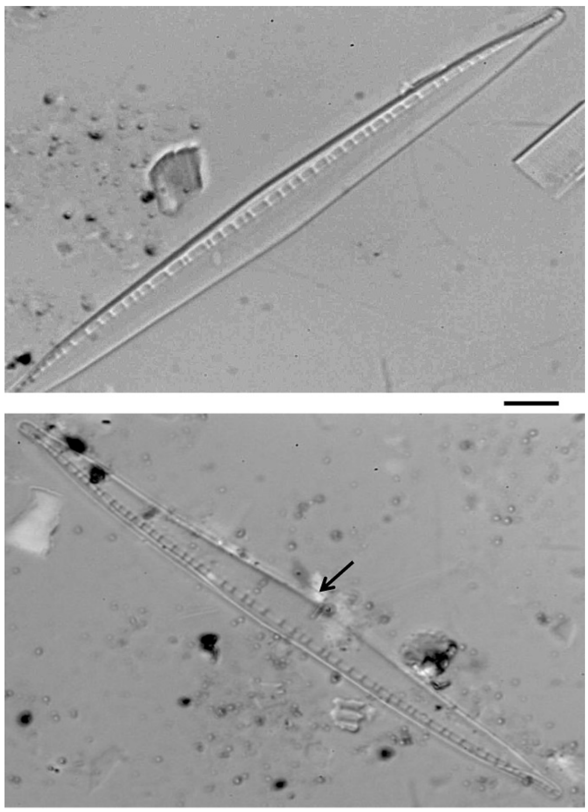
***Navicula tripunctata* (O.F. Müller) Bory**



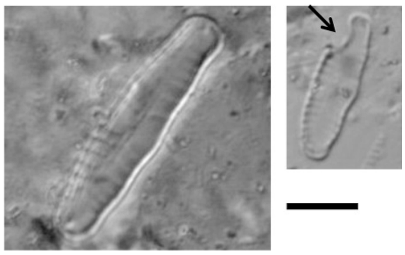
***Nitzschia dissipata* (Kützing) Grunow**



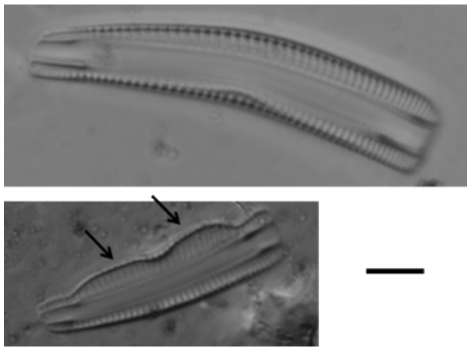
Nitzschia linearis **(Agardh) W.M. Smith**



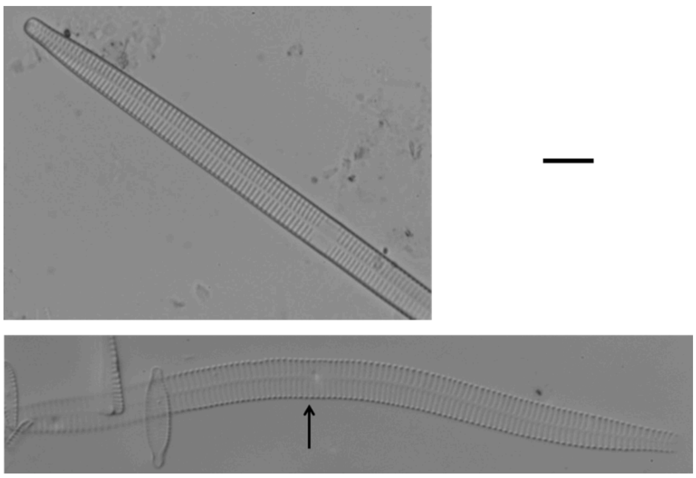
Nitzschia media **Hantzsch**



***Reimeria sinuata* (Gregory) Kociolek et Stoermer**



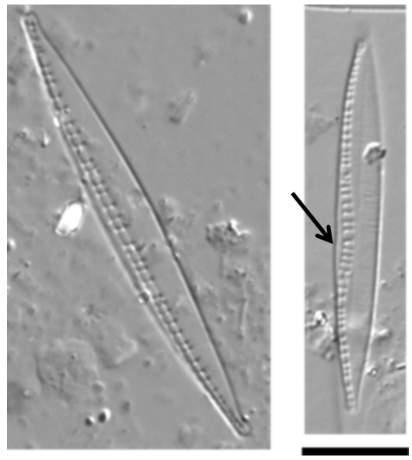
***Rhoicosphenia abbreviata* (Agardh) Lange-Bertalot**



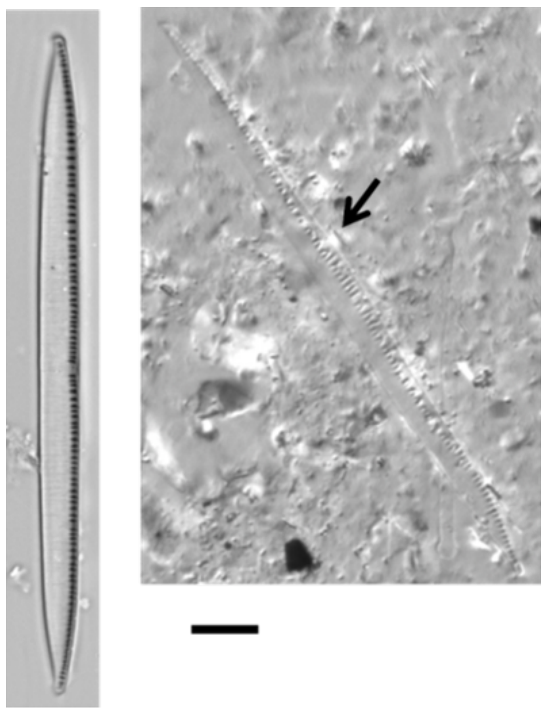
***Ulnaria ulna* (Nitzsch) Compère**

Type2:

Raphe canal system modification (displaced fibulae)



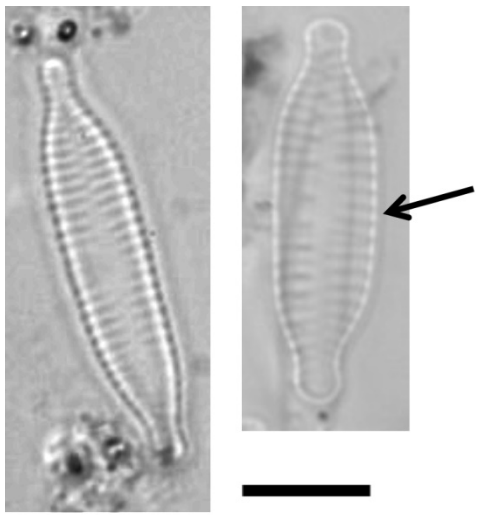
***Nitzschia dissipata* (Kützing) Grunow**



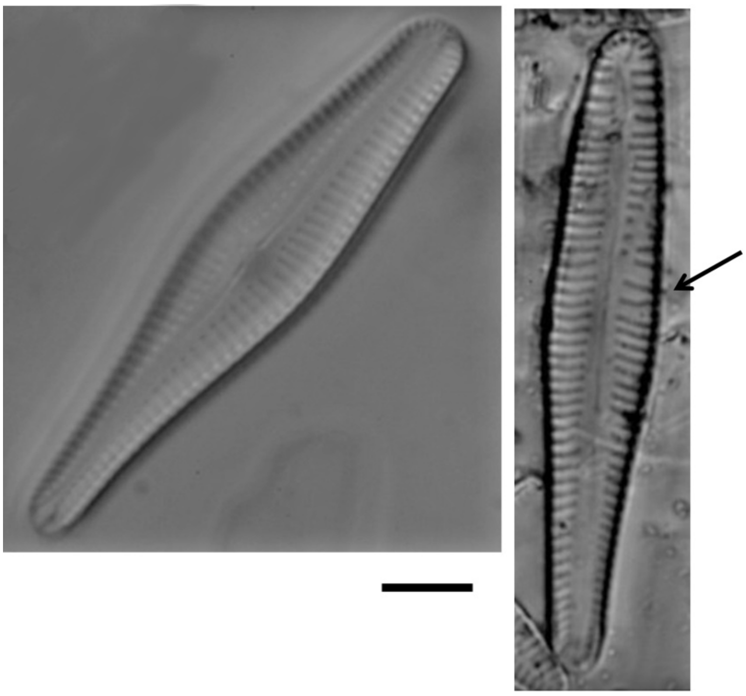
***Nitzschia linearis* (Agardh) W.M. Smith**

Type3: Abnormal striation pattern

***Diatoma vulgaris* Bory**

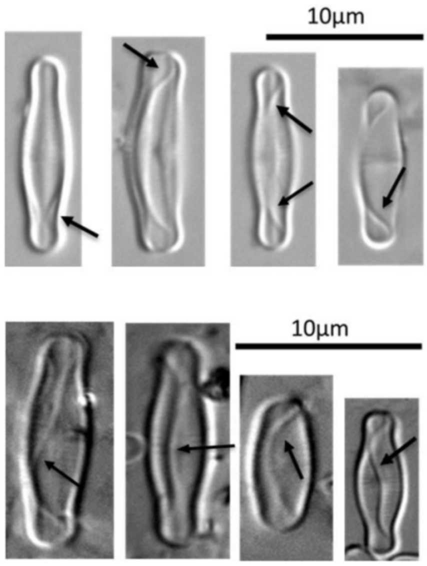


***Fragilaria recapitellata* Lange-Bertalot et Metzeltin**



***Gomphonema subclavatum* Grunow**

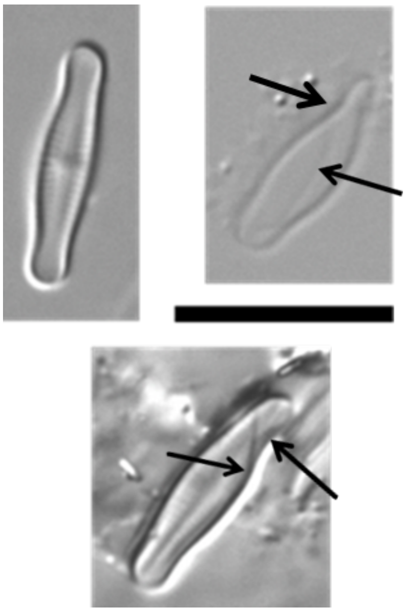
Type 4:
Deformed
girdle



***Achnanthidium minutissimum* s.l.**

(Kützing) Czarnecki (Olenici et al., 2019)

Type 5: Mixed teratology



Achnanthidium minutissimum **s.l. (Kützing) Czarnecki**

The analysis of the taxonomical composition in the affected phytobenthic assemblages showed that 37 diatom species presented abnormalities that could be considered as teratologies. The counted species (30) belonged to 14 genera (fig. 3.1.1.); *Diatoma* sp., *Fragilaria* sp. and *Nitzschia* sp. were the most represented with 4 species each one, being followed by *Gomphonema* sp. represented by 3 species.

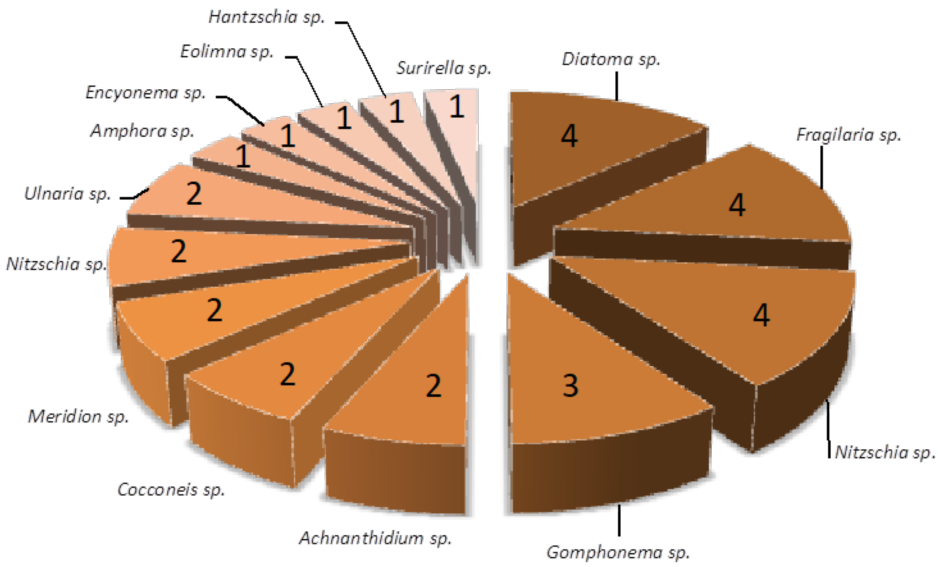


Figure 3.1.1. Number of counted species with teratological individuals sorted by genera

In order to determine the presence of heavy metals in the frustules of teratological diatoms, a energy-dispersive X-ray spectroscopy technique coupled to SEM equipment was performed. From each sample stub, 1 to 4 spectrum points were picked up choosing in different parts of the deformed frustules and in the adjacent material (figs. 3.1.2. and 3.1.3.).

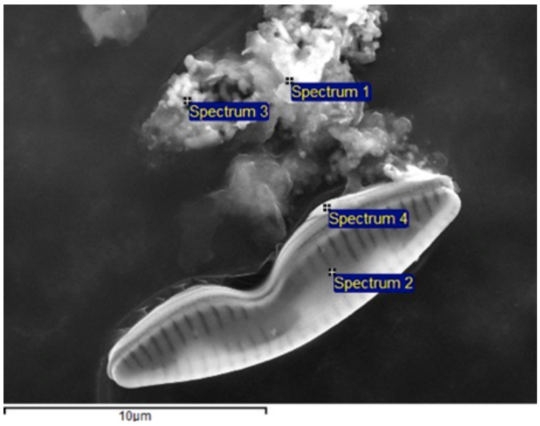
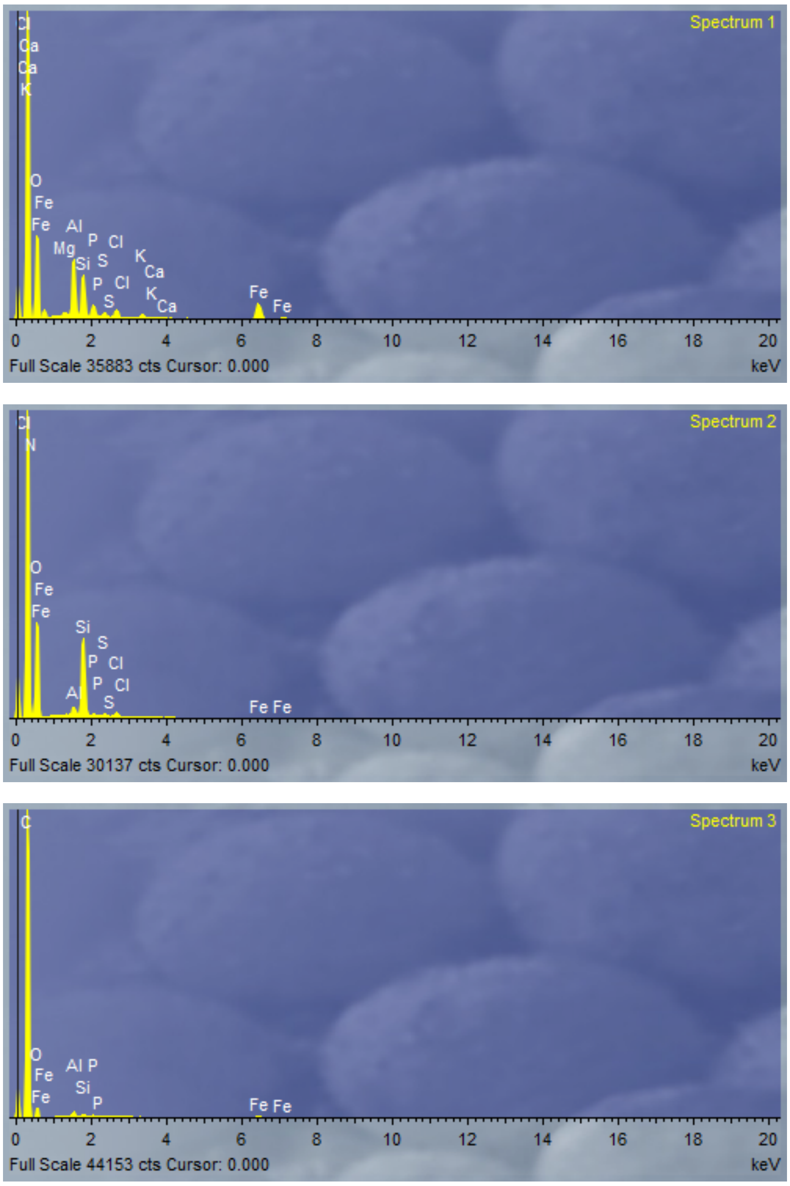


Figure 3.1.2. SEM microphotography of Fragilaria rumpens processed with energy-dispersive X-ray spectroscopy technique.



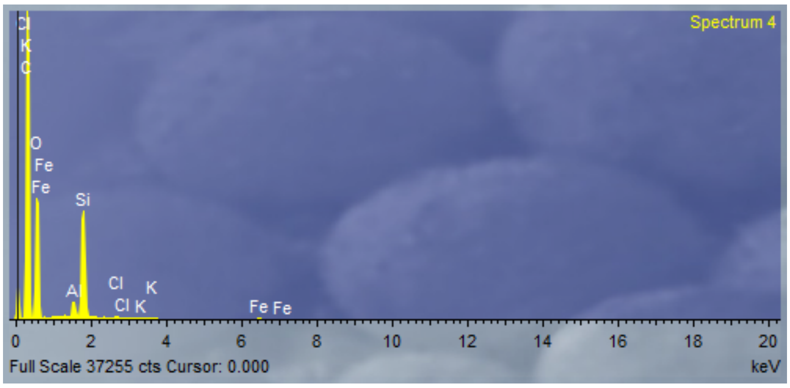


Figure 3.1.3. Chemical spectrum of the analized points in figure 3.1.2.

3.2. A New Diatom Teratology in a Heavy Metal Polluted River of Roşia Montană (Romania)

In the study area, where *Achnanthidium macrocephalum* s. str. *Achnanthidium minutissimum* s. str. were described as the dominant species, 20.53% of the cells presented a type of deformity that has not been reported previously, with a distribution of 70% and 30% respectively of the total of individuals with this type of deformation (Olenici et al., 2019). This affects the cingulum, particularly the valvocopula (the first of the girdle bands, attached to the valve), that becomes modified with a markedly undulate shape (figs. 3.2.1. and 3.2.2.).

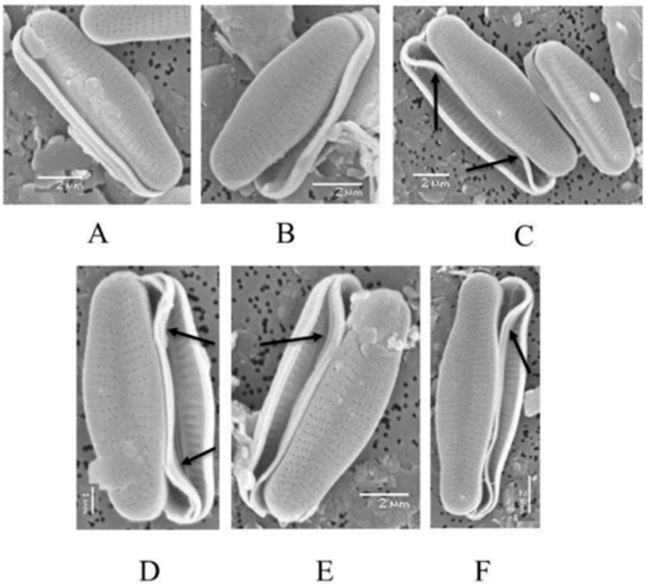


Figure 3.2.1. Deformed valvocopula seen at SEM by comparing with normal one (A and B = normal frustules; C, D, E and F = abnormal frustules identified in processed sample) (Olenici et al., 2019)

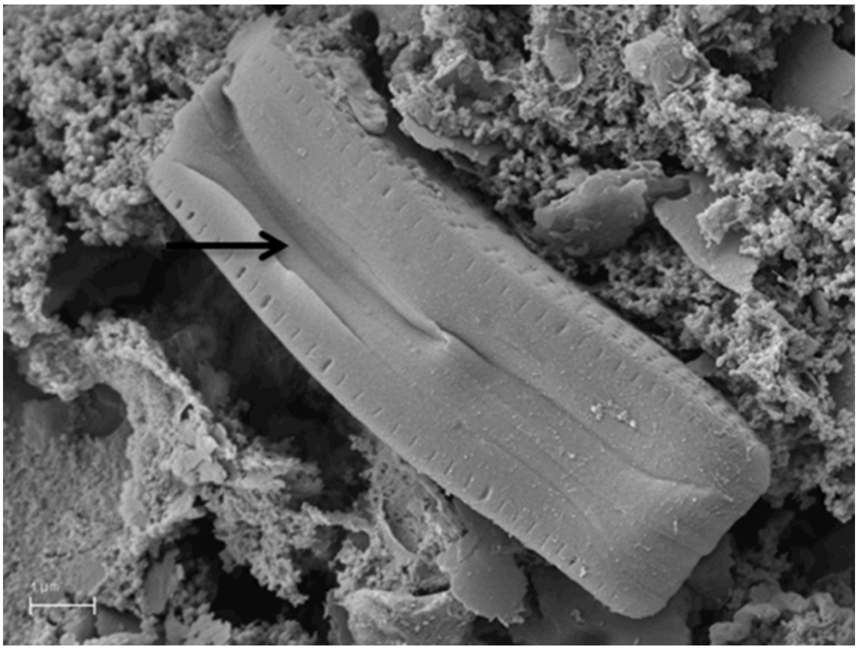


Figure 3.2.2. Deformed valvocopula seen at SEM (abnormal frustule identified in an unprocessed sample) (Olenici et al., 2019)

According to Olenici et al. (2019), two different hypotheses can be suggested to explain this kind of teratology:

a) Whereas metal contamination increases the rate of valve size diminution (which is a characteristic of diatom asexual reproduction) valve surface does not decrease as quickly as the cell volume does (Falasco et al., 2009a). Santos (2010) outlines that frustule growth is only possible by parental valve separation at the same time that new girdle bands are produced, so that the new girdle bands formed may not fit in the resulting frustules, adopting an aberrant form.

b) It has been reported that a Zn-dependent system (Jaccard et al., 2009) mediates the uptake of silicic acid by diatoms through cingulins. An excess of Zn affects the biochemical pathway of silicon metabolism (Martin-Jézéquel et al., 2003) and, in particular, the alteration of metal-induced cingulins can affect the functioning of the girdle (Karp-Boss et al., 2014).

3.3. Exploring diatom teratology using geometric morphometry

In order to assess the degree of valve deformation, geometric morphometry was used in the analyzed *Achnanthidium* populations. A sum of 543 individuals (348 *A. macrocephalum* and 195 *A. minutissimum*, both normal and teratologic cells) were photographed by means of an optic microscope. Valve morphology was displayed as a geometric setup of pseudolandmarks or reliable recognizable points in the set of individuals measured. About 40 pseudolandmarks were set at consistently dispersed points along the valve outline (fig. 3.3.1.) and digitized utilizing CLIC (Dujardin et al., 2010). The Cartesian coordinates of the pseudolandmarks were adjusted (translated, rotated and scaled) by the Procrustes generalized orthogonal least-squared superimposition method (Rohlf & Slice, 1990).

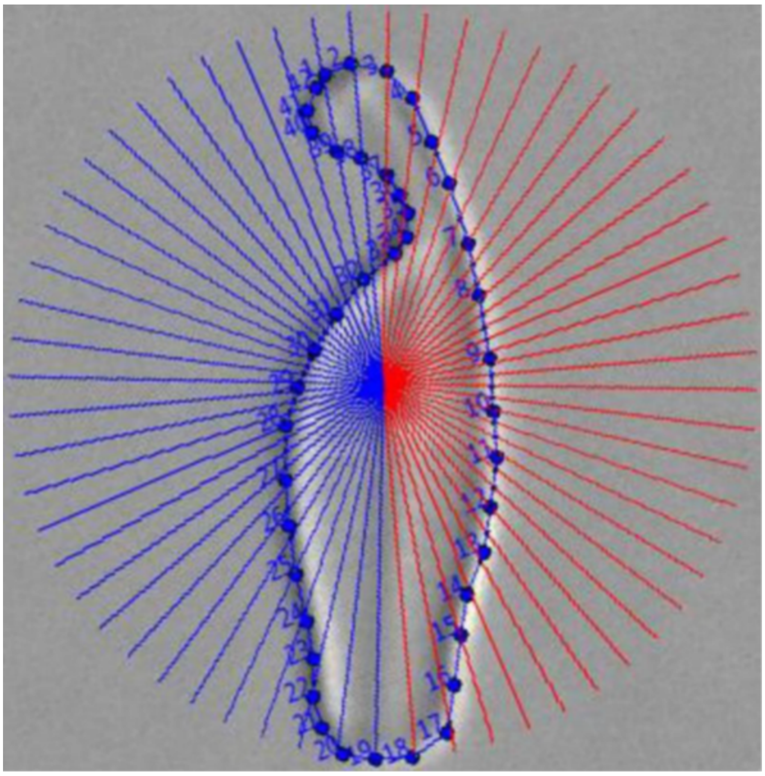


Figure 3.3.1. Position of the pseudolandmarks along the valve outline of a teratological A. minutissimum valve (Olenici et al., 2017)

Resulting data were then analyzed by multivariate methods to test for significant dissimilarities between pre-established groups through the use of a nonmetric multidimensional scaling (NMDS) analysis in Past v. 2.17 software, as described in Hammer et al. (2001). To visualize the resulting scatterplots for each predefined group, confidence ellipses were included in the output plot (figs. 3.3.2.a. and 3.3.2.b.) (Olenici et al., 2017).

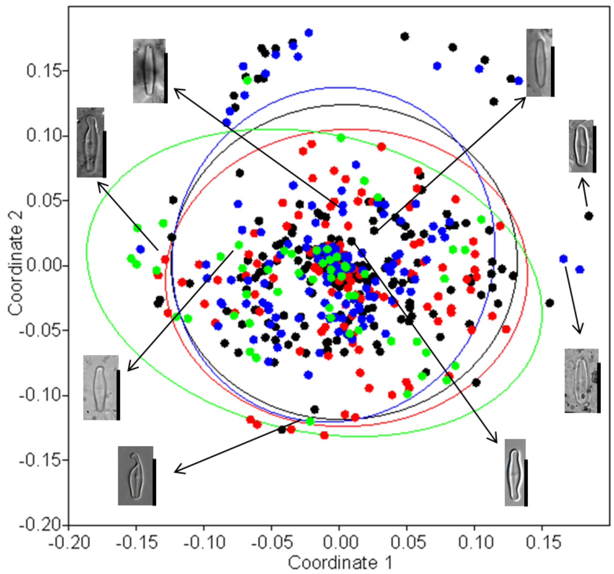


Figure 3.3.2a. Nonparametric multidimensional scaling plot of normalized coordinates for the morphological pseudolandmarks digitized on LM images of selected populations of Achnanthidium macrocephalum and A. minutissimum (• = A. macrocephalum, • = A. macrocephalum teratologic, • = A. minutissimum, • = A. minutissimum teratologic), scale bar = 10µm (Olenici et al., 2017)

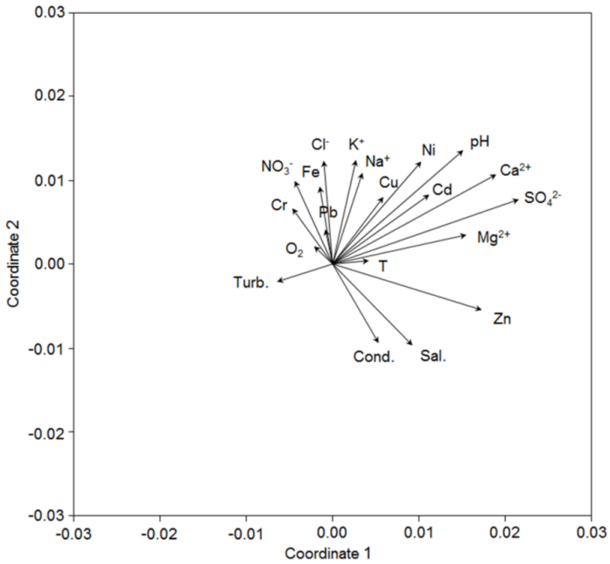


Figure 3.3.2b. NMDS plot of environmental variables from analyzed sampling sites (Olenici et al., 2017)

The NMDS analysis indicates that the aquatic physico-chemical parameters, mainly the Zn level, are related with the valve shapes of the *Achnanthidium* species. With the objective of evaluate the main environmental stressors producing frustule teratologies, a two-block partial least squares analysis (2BPLS) has been applied using as inputs the outline coordinates and the set of environmental data matrices (fig. 3.3.3.).

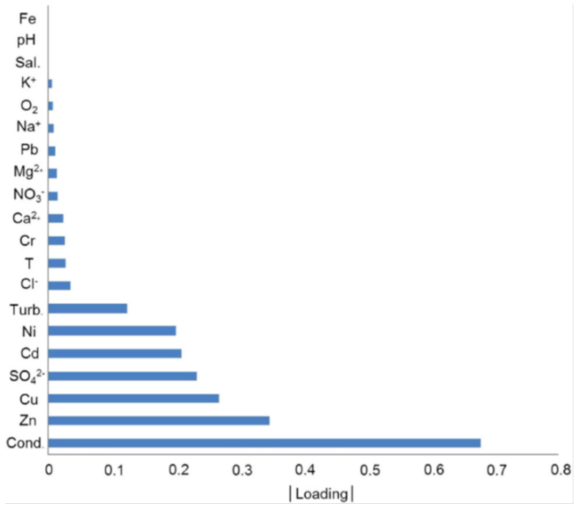


Figure 3.3.3. Relative importance of limnological variables according to the two-block partial least squares results (Olenici et al., 2017)

According to Olenici et al. (2017) the results demonstrated the efficiency of the methodological approach followed, that is geometric morphometry linked to multivariate analysis, in the quantification of the degree of deformation in diatom valves.

3.4. Variations in Diatom Morphology: Implications for Diatoms-Based Water Quality Indices

The power of diatom indices are based both on the amount of taxa analyzed for their computation and on the autecological characteristics assigned to each taxa (Blanco et al., 2007). SPI is the only metric based on the ecological profiles of virtually all known taxa at the most taxonomically fine level, including teratological forms as distinct taxa whose occurrence downweights the final score of this metric. However, it must be considered that (i) standard protocols (CEN, 2004) do not warn about the necessity of recording these abnormal valves in routine counts, and (ii) only ca. 1% of the taxa considered for SPI computation are considered teratological forms (Lecointe et al., 1993). Therefore, it can be assumed that ignoring the presence of abnormal diatoms leads to inaccurate water quality diagnoses (Olenici et al., submitted).

SPI values computed segregating abnormal valves were significantly lower, as shown in figure 3.4.1. (Wilcoxon test, W=210, p<0.001). In samples where teratological diatoms were particularly abundant (more than 30% of counted individuals), SPI overestimation can reach up to 8 units (fig. 3.4.2.), so that water impairment in these locations may remain hidden. It can be proven that normal and modified forms of the same specie have different ecological profiles with respect to key limnological variables. This is in accordance with Coste et al. (2009) and Fernandez et al. (2018) who reported that the teratological forms have different ecological profiles than normal ones. As expected, the CCA plot (fig. 3.4.3.) shows that teratological occurrences of both *Achnanthidium* taxa are related to acidity and Cd and Cu levels. This emphasis the need of assigning different autecological parameters to teratological forms in order to better reflect water conditions in polluted areas (Olenici et al., submitted).

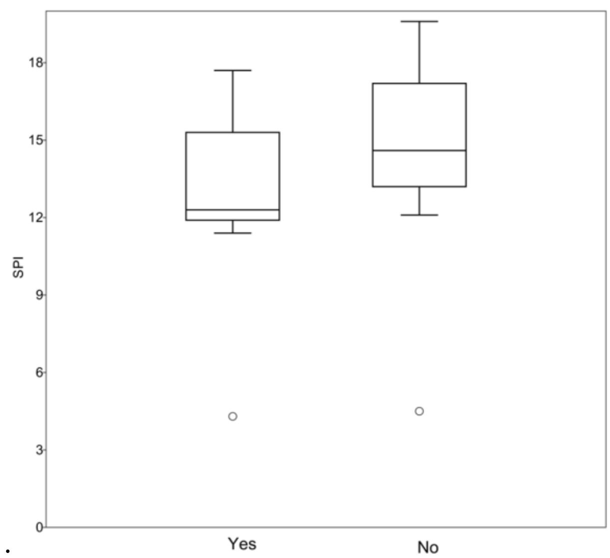


Figure 3.4.1. Boxplot of SPI values computed segregating (left) or pooling (right) teratological individuals in the Abrud AMD-polluted river (Olenici et al., submitted).

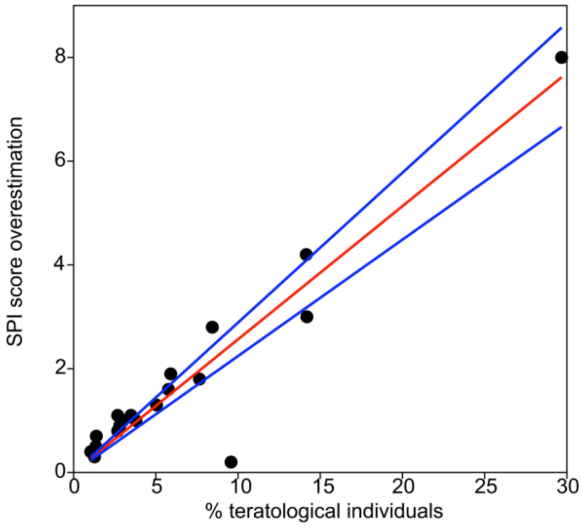


Figure 3.4.2. SPI overestimation as a consequence of overriding teratology in routine diatom counts (Olenici et al., submitted)

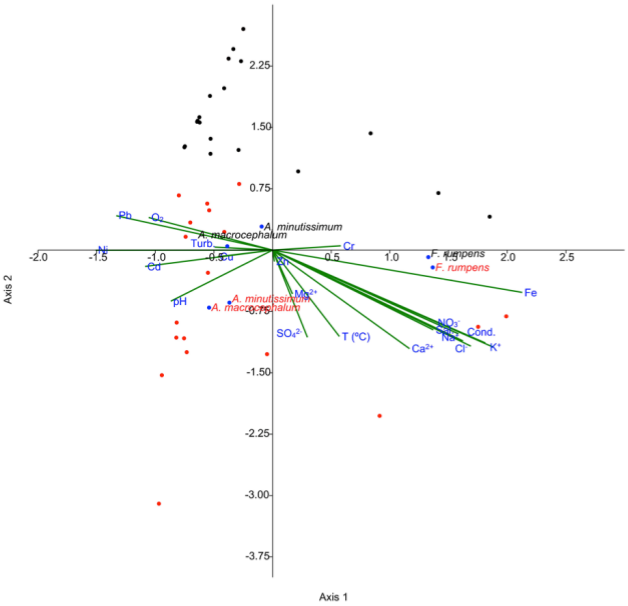


Figure 3.4.3. CCA triplot showing the autecological differences between normal (black) and teratological (red) specimens of three diatom species found in Abrud River, Romania. Black dots: control stations. Red dots: metal-contaminated stations (Olenici et al., submitted)

3.5. Monitorizing Zn Levels Using Diatoms

The creation of a transfer function, a predictive formula, from a relative large dataset can be useful to derive past chemical conditions in sediment cores, allowing the reconstruction of past events (Bennion et al., 2004). From this point of view, our results allows the generation of a transfer function that could be applied to paleolimnological records in order to obtain information about changes in the degree of exposition of these communities to heavy metals. Environmental conditions can be thus inferred for a particular habitat analyzing the inhabiting biological community, without the need of field measurements.

In order to design a biotic index for the sampled region, three steps have been followed:

1. The selection of the response variable (that is, the X axis of the response curve). In this case, the Zn concentration has been selected the as the environmental variable considering that has been the most relevant parameter in this case (Olenici et al., 2017). This heavy metal is a pollutant originated from mine activity and is tolerated in a variable manner by diatom species.
2. A field survey has been made, collecting the community to be used as bioindicator, and simultaneously measuring the variable of interest.
3. The optimum and the tolerance of each species has been calculated with respect to this variable (fig. 3.5.1.).

Once the optimum and the tolerance have been computed for all the taxa present in the community, it is possible to back-calculate variable values that would correspond to each sampling site given the relative abundances of the species occurring in the test dataset. This can be done using the weighted averages formula, also known as the Zelinka-Marvan (1961) formula:

$[Zn] = \sum_{j=1}^{n} A_j . S_j . V_j / \sum_{j=1}^{n} A_j . V_j$, where Aj, Sj and Vj are, respectively, the abundance, optimum and tolerance, of the j^{th} taxon in the sample.

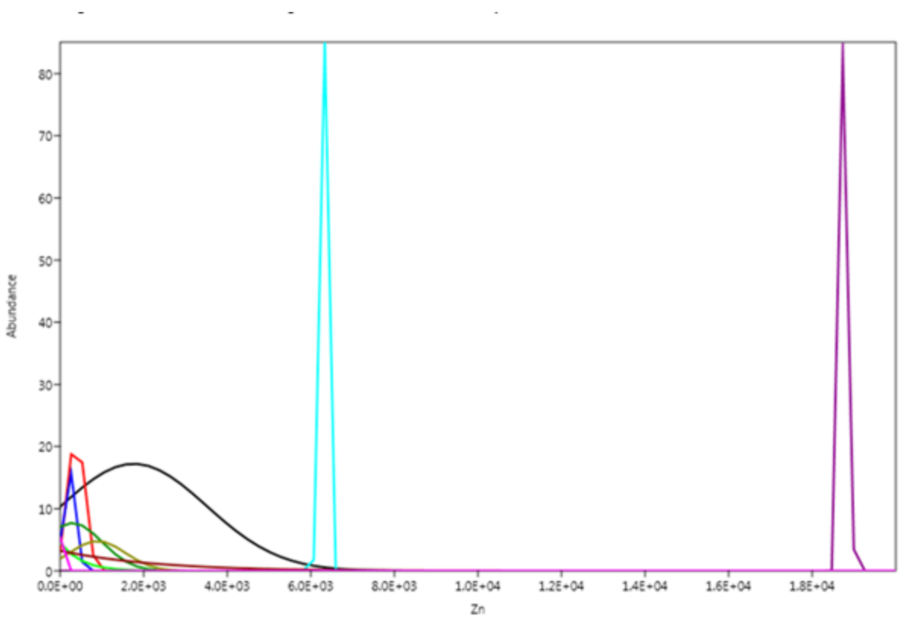


Figure 3.5.1. Species Packing (Gaussian model) for the 9 most abundant species. Each line is a "best-fit" normal curve defining the relationship between the abundance of each taxon and Zn concentrations

3.6. Contribution to the Freshwater Diatom Flora of Romania

A total of 274 diatom taxa have been identified in the catchment area of the Abrud River (Roşia Montană, Romania). These 274 taxa belong to 63 genera and represented 264 different species, plus 8 varieties, 1 subspecies and 1 form

The most represented genera have been *Nitzschia* and *Gomphonema* with 31 and 27 species, respectively. There have been also 24 identified genera that have been represented only by one species. The figure 3.6.1. shows the species distribution in the dominant genera.

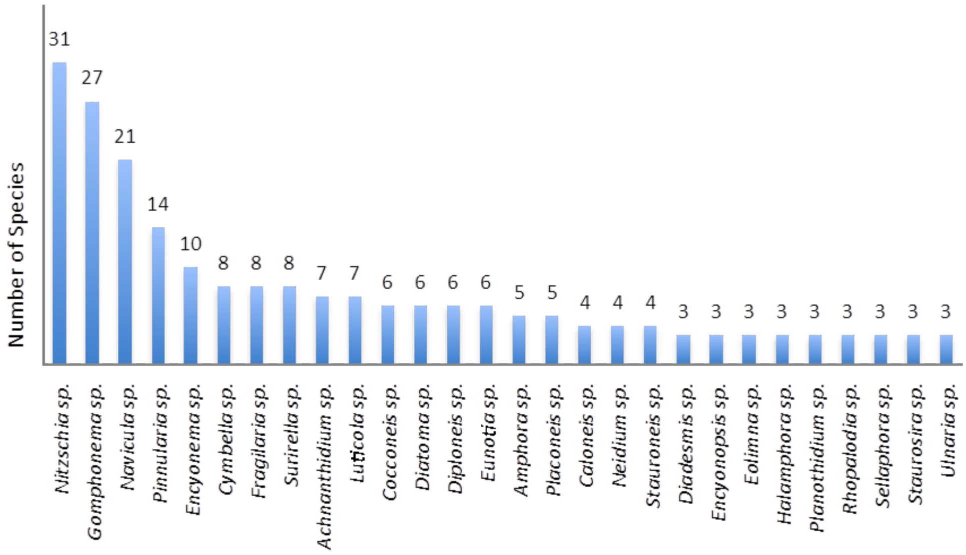


Figure 3.6.1. The main diatom genera related to the number of species of each one.

There were 17 taxa showing identification difficulties and are thus identified to the genus level. According the literature (Cărăuş, 2012; Momeu et al., 2012; Butiuc-Keul et al., 2012; Florescu et al., 2015; Szigyarto & Bakos, 2015; Buczko 2016; Szigyarto et al., 2017), a total of 35 taxa had been not recorded already in previous studies for Romania, seven more were new varieties and one more was a new form. Thus, the new species recorded represent almost 13% of the total identified species.

As a consequence of the polluted environment in the study area, changes were observed in the seasonal, annual and spatial dynamics of the diatom communities in the Abrud River catchment area, both in terms of number of species and individual relative abundances. The richness of the species varies between an extremely low number in the sampling points affected by the presence of mine waters throughout the study period and a high number in the points with good quality water (figs. 3.6.2a., 3.6.2b.).

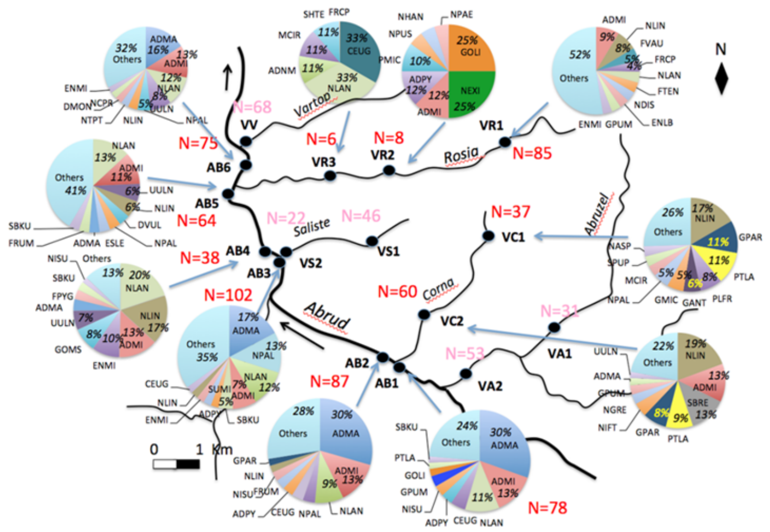


Figure 3.6.2a. Seasonal average species abundance in the sampling points of the Abrud River catchment area. Each pie plot represents the percentages of the main diatom species (OMNIDIA software (Lecointe et al., 1993). In red or pink the Nº of taxa in each station with relative abundances > 0.25 %

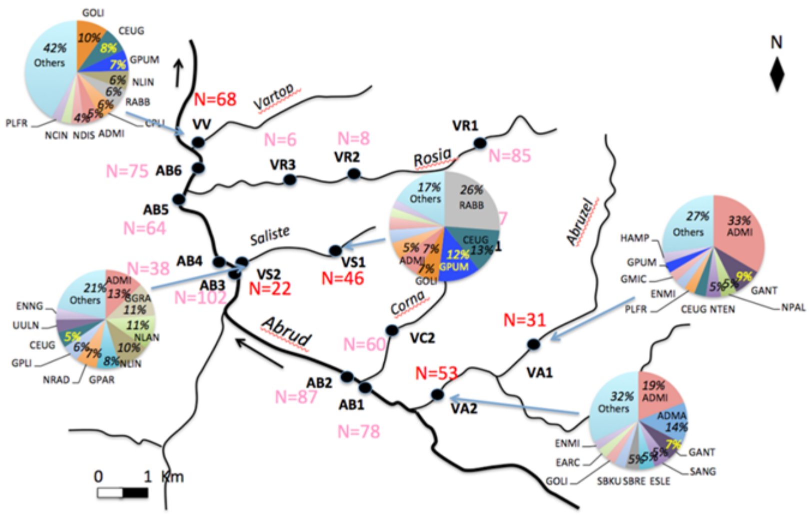


Figure 3.6.2b. Seasonal average species abundance in the sampling points of the Abrud River catchment area. Each pie plot represents the percentages of the main diatom species (OMNIDIA software (Lecointe et al., 1993). In red or pink the Nº of taxa in each station with relative abundances > 0.25 %

Downstream the town of Abrud, some dominant ß-mesosaprobic taxa were observed as well, suggesting critical organic matter levels in the water originated from untreated urban sewage, that together with the high concentrations of NO_3^-, draw attention on the low water quality. The effect was more obvious during summer 2013. The saprobic spectrum of taxa (fig. 3.6.3.) was performed following Rott et al. (1997), revealing a higher presence of β-mesosaprobic (68%) and ßα-mesosaprobic (19%) taxa, compared to oligosaprobic species (6%). The presence of polysaprobic species, which require a higher amount of organic compounds, was very low (6%). It can be observed that the level of the saprobity in the study area is a moderate one. Also, the results highlighted that the majority of the dominant species in the study area indicated a high level of trophicity in the water, being eutrophic species (van Dam *et al.*, 1994). In the same time, there were observed some dominant taxa that suggested a trophicity level form oligotrophic to meso-eutrophic and one, *Nitzschia palea* (Kützing) Smith that indicated a critical level of trophicity, being hypereutrophic. There were observed also two dominant species, like *Achnanthidium minutissimum* and *Gomphonema pumilum* (Grunow) Reichardt, that tolerate a level of nutrients in the water form low to very high (van Dam et al., 1994).

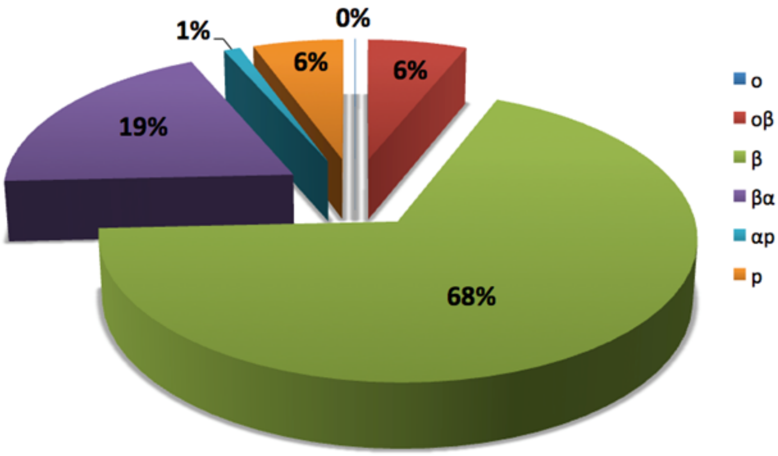


Figure 3.6.3. Synthetic saprobity classes (o = oligosaprobic, oβ = oβ-mesosaprobic, β = β-mesosaprobic, βα = βα-mesosaprobic, α = α-mesosaprobic; p = p-polysaprobic) of the 18 top dominant species averaged in all the samples

1. CONCLUSIONS

Diatoms are good indicators of the features of the water due to its ubiquity and sensitivity to environmental variables. They are easy to collect and identify at species level and the ecological profile for many species is known so that many diatom-based indexes are being used. However, diagnoses based only on a low number of indicator species do not necessarily reflect the actual status of aquatic ecosystems, so that monitoring the whole community included the teratological forms is very important.

Acid Mine Drainage (AMD) affects lotic ecosystems all over the world, being a prevalent issue concerning water quality in the Roșia Montană mining area (Romania). Accordingly, the first concern of this PhD thesis was to assess the ecological responses of the diatom communities in the Abrud River catchment area. Secondarily, the objective was to find the environmental predictors that lead to the appearance of abnormal diatoms.

Firstly, the results from the analysis of the teratologic diatoms growing on AMD-polluted waters have shown that the presence of abnormal individuals can be attributed to the fact that the diatom communities were affected by this kind of pollution released from mining works and waste rock deposits. Intermediate perturbations are responsible of the appearance of large proportions of abnormal individuals, with a variable typology of malformations, reaching almost 58% in the case *Fragilaria rumpens* (Kützing) Carlson. Also, a new type of teratology regarding the species *Achnanthidium minutissimum* and *Achnanthidium macrocephalum*, affecting the shape of the frustule cingulum, has been reported.

Automatic measures by means of geometric morphometry revealed morphological differences between normal and abnormal individuals of *Achnanthidium minutissimum* and *Achnanthidium macrocephalum*. Multivariate analyses separated the populations of these species and showed the main physico-chemical variables that have contributed to valve deformation in this context, namely conductivity, Zn, and Cu.

Secondly, the implications for diatom-based biomonitoring of variations in diatoms morphology have been tested. This work highlights an overestimation of water quality conditions caused by overriding deformed individuals in diatom-based biomonitoring studies. It can be shown that normal and teratological forms of the same species differ in ecological profiles.

In order to assess the levels of one of the key elements in the pollution of Abrud River, that is the Zn, a transfer function, was developed using the diatom assemblage as proxy. The optimum and tolerance of each species has been calculated and the resulting function can be used to derive past and present chemical conditions, allowing the reconstruction of Zn levels in freshwaters.

Finally, the contribution to the Romanian flora of this work can be summarized by the identification of 274 diatom taxa in the Abrud River catchment area, 35 of them recorded for the first time in Romanian waters. The spatial and temporal pattern variations of species richness in the study area point out the effects of water pollution on diatom communities and the differences between the main stream of the Abrud River, with species richness ranging between 38 and 102 taxa, and the tributaries with some very species-poor points in which almost no taxa were identified. Only in the clean waters upstream of Roșia Valley, a very rich diatom community (85 taxa) was found.

2. SELECTIVE BIBLIOGRAPHY

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