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Diatoms as Indicators of Water Quality and Ecological Status: Sampling, Analysis and Some Ecological Remarks

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1. Introduction

Diatoms are amazing microscopical algae whose typical feature is a siliceous coverage, called frustule, extremely diverse in shape. Diatoms live in almost all types of superficial waters. Depending on their habitats, diatoms are either planktonic (living suspended on the water), benthic (growing associated to a substrate), or both planktonic and benthic.

On one hand, planktonic diatoms usually have fine frustules and/or long appendices to facilitate floatability. Many marine diatoms, like the genus *Bacteriastrum* Shadbolt, are good examples of this characteristic. The chain formation facilitates the planktonic life too, such as in *Skeletonema costatum* (Greville) Cleve or *Fragilaria crotonensis* Kitton.

On the other hand, benthic diatoms do not need delicate structures because they live on a substrate. Therefore they do not have to worry about sinking. They can be motile when living on sediment like many species of *Navicula* Bory, grow closely attached to a substrate like *Cocconeis* Ehrenberg (Fig. 1), or live on top of auto-produced mucilaginous stalks as many species of *Gomphonema* Ehrenberg do.

The algal species that develop in an area depend on different environmental factors: salinity, temperature, pH, water velocity, shading, depth, availability of substrata to grow on, water chemistry, etc. Thus, the species that can be found in a water body will inform about some characteristics of the water. Because algae are good indicators of the features of the water, they are used to monitor water quality. Among algae, diatoms have the advantage of being easily identifiable at the species level without the need of cultures and they are very easy to collect and store due to their hard frustule. The ecological requirements of many diatom species are known and therefore many diatom-based indexes of water quality have been developed.

Such indexes have been created for benthic diatoms of continental running waters. There is a reason why benthic diatoms are more reliable for this purpose—they remain in their location unless disturbed by some catastrophic event, like a big flood. This means that they reflect the characteristics of the water from the area in which they live.

On the contrary, planktonic diatoms “get old with the water” – as the water runs, they move with it. Therefore, when a researcher collects planktonic diatoms that grew upstream, such algae may reflect other water characteristics than the ones in the sampling site.



Fig. 1. Four valves of *Cocconeis* Ehrenberg.

In this chapter we will explore the use of diatoms to monitor water quality. To achieve this, this chapter has two main objectives:

1. To be a guide for those who work with this type of algae: The chapter shows how sampling must be carried out and how samples have to be treated and analysed.
2. To warn about the risk of using the indexes alone, without any further information. There are some situations that can lead to incorrect conclusions about the health of an ecosystem if only the values of the indexes are observed. For this reason, we show two cases in which diatom indexes reflect something different from the actual state of the ecosystem.

2. Water quality and ecological status of a water body

In recent decades, a significant effort has been put forth all over the world to assess water quality attending to not only to chemical parameters (nutrients, metals, pesticides, etc.), which are obviously important, but also to biological indicators. In fact, one of the undesirable consequences of pollutants is their effect on biota. In this case, the direct study of the effects of pollution on biota is of great interest.

However, it is not so easy to define the extent to which an ecosystem is damaged. There are obvious situations in which heavily polluted waters give the ecosystems such a structure and characteristics that there is no doubt about the heaviness of the human pressures and no effort is necessary to get conclusions about the health of the system. However, there are intermediate situations where it is difficult to draw a conclusion about the water quality without further evaluation.

In this context, the European Water Framework Directive (2000) has a simple philosophy, easy to understand but difficult to put in practice: An ecosystem should be evaluated

according to how it was expected to be under no human pressure. Such an ecosystem would be considered to have a good ecological status and could be used as a pattern – the so-called reference conditions – with which other similar ecosystems can be compared. There are five levels of quality, which are identified by colours, according to the closeness of the ecosystem to its reference condition: red, very poor; orange, poor; yellow, moderate; green, good; blue, excellent. Despite these five standards of water quality levels, it is hard to know how each ecosystem would be under pristine conditions, which leaves much work to be done.

There are different biological indicators that can be used to determine the ecological status of a water body: macroinvertebrates, aquatic plants, and algae, especially diatoms, are all used in water quality research (Triest et al., 2001; Hering et al., 2006; Torrisi et al. 2010). The ecological information given by diatoms is usually summed up through one or more diatom-based indexes, which indicate the trophic level with just one number. However, the indexes alone do not determine the water quality. Moreover, the water quality is not the only factor that determines the ecological status. Other factors such as the structure of the vegetation or the hydrodynamics of the ecosystem must also be taken into account.

In summary, the use of the values of the diatom indexes alone may not be enough to determine if an ecosystem has got a good status or not and sometimes can be insufficient even to determine the water quality, as will be shown at the end of this chapter.

3. Diatoms sampling

Currently, the diatom sampling process is mostly normalised, since there are norms to ensure the accuracy of the procedure. The European Standard EN 13946 (2003), as well as the recommendations provided by Kelly et al. (1998) are well known and widely followed.

In most cases, the diatom sampling is carried out in rivers in order to monitor water quality through the calculation of one or more indexes based on these algae. Firstly, we will focus on rivers, and secondly, we will show some other variations to sample diatoms in wetlands. The following sections summarize the sampling procedure in order to serve as a quick guide.

3.1 Sampling rivers

Sampling sites must be located up and downstream of any impact in the basin of which the researchers are aware but the final selection of the sampling sites depends on the objectives of the work.

The sampling must be carried out in a lightly shadowed by riparian vegetation segment of the river of 10 m length or more. This segment has to include rapids, since stretches of river with very slow currents (<20 cm/s) allow the buildup of loosely attached diatoms, silt, and other debris. Samples have to be collected in rapids at a distance from the riverbanks to avoid waters somehow isolated from the main current that would not reflect the characteristics of the site along with any substrata that may have recently remained out of the water and could contain aerophilous diatoms.

The next step is to choose the type of substrate to sample. There are many works explaining how the sampled substrata can influence the species that can be found on the rivers

(Danilov & Ekelund, 2001; Albay & Akcaalan, 2003; Potapova & Charles, 2005; Townsend & Gell, 2005). For example, some diatoms, such as *Epithemia adnata* (Kützing) Brebisson or *Cocconeis pediculus* Ehrenberg are more likely to be found growing on plants or macroalgae rather than on rocks.

Natural rocks are the best substrate for sampling springs (Round, 1991). However, researchers should avoid any large rock difficult to remove from the river because it would make the sampling process too difficult. Pebbles are not appropriate either, because they might have come recently from upstream and they can mislead the sample. The best choice is an average-size rock of about 15 to 30 cm. However, when this type of substrata is not available, the researcher should take samples in the following order of preference: natural rocks, artificial surfaces (such as bridge pillars), artificial substrates, and plants.

Artificial substrates must be placed in the riverbed at least one month before the sampling is executed to ensure the proper development of a mature algal community. Some researches use slides, which have the advantage of giving a known surface and let the direct observation of the algal growing and the biofilm architecture under a microscope. However, it is preferable to use tiles or bricks in routine biomonitoring work because they provide rough surfaces resembling natural rocks that facilitate the settlement of algae. Unfortunately, all these substrates are often lost in the riverbed due to strong currents and human interaction with the river. Moreover, these artificial substrates can be found completely covered of fine sediments instead of algae, which is quite common since these substrata are likely to be left in rivers whose riverbed has only fine sediment and no natural rocks. A stake can be driven into the sediment through the holes of several bricks, which should be piled up at the bottom of the river. It ensures a vertical surface colonizable by algae on which little mud would sediment. Moreover, it will be easy to localize when returning to the river.

When the researcher decides to sample natural rocks, he or she should randomly collect between five and ten of them. Then, the surface of the rocks should be scrapped off and removed with a toothbrush. The brushed area of the stones, as well as the toothbrush covered with algae, can be cleaned on a plastic tray with water from the river if it is clear or with tap water if the river runs muddy. The obtained brownish suspension constitutes the sample. Tiles and bricks must be treated as natural rocks.

When sampling diatoms growing on plants, the researcher can use either emergent plants – also called helophytes, such as *Thypha* L. or *Phragmites* Adans. – or submerged plants – the so-called hygrophyes, like *Ceratophyllum* L. or *Myriophyllum* L. Not all plant species facilitate the growing of the same diatoms, in quantity and diversity. Some plants are harder to be colonised by algae for many reasons, such as being too smooth for the algae to grow, etc. For example, *Thypha* L. typically exhibits a thicker biofilm than *Scirpus* L. In general, it is recommended to always sample on the same plant species in order to make the data comparison from different sites easier. It is often impossible to do this, since the elected species may not grow in all sampling sites, but this is not a matter of extreme importance, according to Cejudo-Figueiras et al. (2010)

The algae growing on plants have to be collected by gathering leaves that are alive and stem sections, discarding either those parts that had been recently out of the water or those that are near the bottom and covered by sediment. Cuttings of about 5-cm length must be

carefully placed together in a bottle filled with tap water. The stems and leaves have to be gently scraped with a cover slip in the laboratory, while softly washed with water. The obtained brown or greenish suspension contains the diatoms that will be used in the research. This procedure is suitable with helophytes – *Typha*, *Scirpus*, *Phragmites*, etc. – but not with fine and delicate hygrophytes such as *Myriophyllum*, which would be completely smashed with the coverslip. In that case, some fragments of plants must be introduced into a plastic recipient with some water (from the river if it is clear or tap water otherwise). The recipient must be closed and shaken vigorously. The water will become muddy, brownish or greenish. The plants can be removed out of the jar and replaced with other fragments. After repeating this operation three or four times, the water from the recipient will constitute the sample and it will be blurred because of all the epiphytic algae.

3.2 Sampling wetlands

Small springs are quite easy to sample, since their riverbed is not muddy and usually have many rocks of different sizes that are easily removable. However, wetlands, as well as wide rivers, only have plants and sediments as the only available substrata, and in some cases even plants are absent. If there are plants, they should be treated as described above for rivers. As a general rule, plants have demonstrated to be better than stones in wetlands because plants provide a vertical surface mostly free of mud (Blanco et al., 2004). Stones, if present, typically have a thick layer of sediment, making them useless for sampling. However, the allocation of artificial substrata is a good choice too, if they are disposed vertically (Sekar et al., 2004).

Sediment is not a suitable substrate because diatoms growing among its particles can get nutrients from the interstitial water, having an extra source of nutrients that other algae living out of the sediment lack. This means that trophic levels would be overestimated when analysing diatoms from sediments. That is why rocks are preferred, since they cannot provide extra nutrients to the algae, forcing them to obtain the nutrients needed from the open water.

Nevertheless, some studies may recommend sampling sediment if the goal is not the application of water quality indexes but the knowledge of the algal flora. For example, there are wide marshlands in which there are large extensions of sediment (under the water or just in wet areas) free of plants. Sometimes the primary production of this sediment is very important for the whole system and, very often, a greenish highly productive biofilm can grow on it. If the researcher wants to study the algal composition of this marshland, attention must be focused on sediments, which is what characterizes these types of ecosystems, rather than any other type of substrata.

What is the best way to sample algae from sediments? If researchers want to perform quantitative studies they must sample a known surface. Half of a Petri dish must be driven into sediment to take the superficial layer, using the aid of a scrapper to facilitate the process. After homogenising the sample at the laboratory, small subsamples can be taken from it to count algae under the microscope or to measure chlorophyll values. Any subsample must be extrapolated to the volume or weight of the whole sample and to the area of the Petri dish.

Analysing sediment under the microscope is terribly weary and exasperating. If the objective of the study is to identify the species with a rough estimate of their proportions, there is a strategy that not only facilitates this task but also ensures that only living diatoms are observed. Diatoms living in sediment, like many species of *Nitzschia* Hassall, *Gyrosigma* Hassall or *Navicula* Bory (Fig. 2) are motile, which implies that the sample – or a subsample after homogenising the whole sample – can be left in the Petri dish with a thin layer of water for about 12 hours under good illumination, which acts like a lure attracting diatoms towards the light. A thin green biofilm will appear on the mud that can be carefully picked up and disposed on a slide with a drop of water to be observed under the microscope. Moving algae may adhere to some cover slips if they are left on the wet sediment, although they tend to accumulate around the slips better than under them. Therefore, the analysis of these cover slips is easier because they will have less mud.

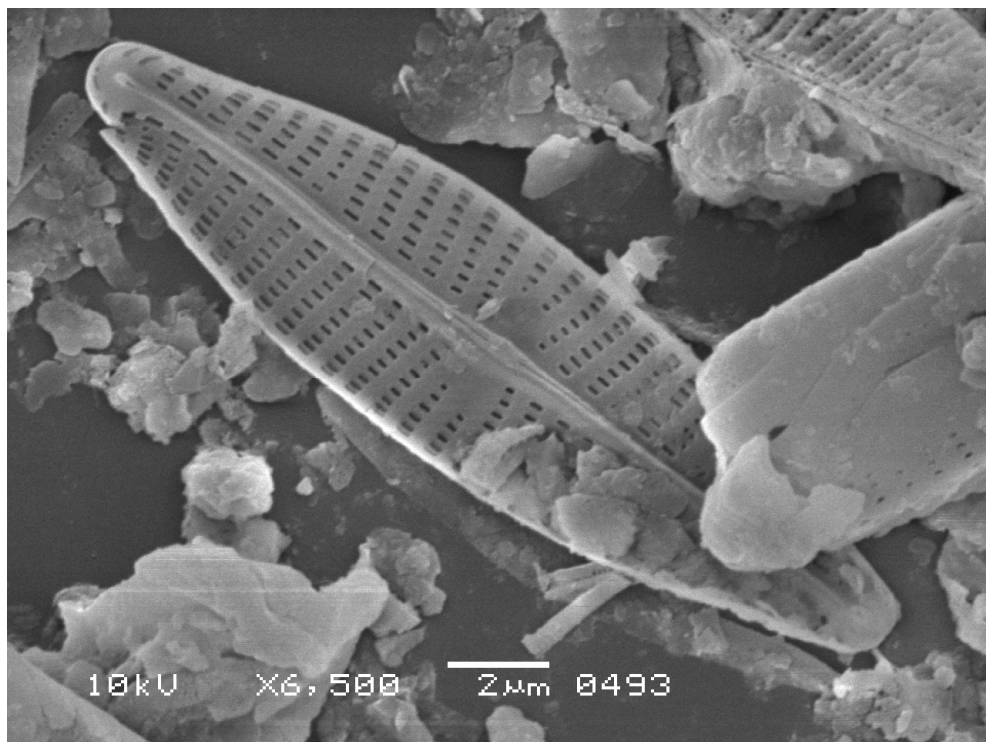


Fig. 2. A single valve of *Navicula* among particles of sediment.

In this case previously mentioned, diatoms have to be alive in order to move among the particles of the sediment. In any other case, if the collected material is not going to be treated in the lab within one day– no matter the substrate or the type of sampled ecosystem– it must be homogenized and fixed with 4% (v/v) formaldehyde.

4. Diatoms treatment

Diatoms can be identified to species level though morphological features of their frustules but these will hardly be seen without a previous treatment consisting of making all the organic matter disappear, leaving their siliceous frustule empty. There are different techniques for diatom cleaning but the hydrogen peroxide method (30% H_2O_2 solution) is the most widely used.

Firstly, if formaldehyde has been added, it should be removed. The sample has to be homogenized by shaking and a tube for centrifugation has to be filled with it. Centrifuge the sample at 2,500 rpm for 15 minutes, remove the supernatant, refill the tube with distilled water and homogenize it again. This process should be repeated three times but after the first one the supernatant must be revised to prevent any loss of diatoms.

Once the supernatant is removed for the third time, add a few drops of hydrogen peroxide to the pellet. Be careful because a lot of foam may appear! Some more peroxide has to be added carefully until half of the tube is filled with it. Then, the tube can be either left covered for several days until bubbles stop flowing or heated in a sand bath or a hotplate at about 90 °C during 1 to 4 hours. In both cases, the brownish suspension has to become whitish.

After that, add a few drops of HCl and repeat the centrifuge process previously described to remove all the hydrogen peroxide.



Fig. 3. An excessive density of valves makes the counting difficult. The yellow shape surrounds seven small valves of *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot. This species is almost impossible to detect without phase contrast. Notice that these diatoms are abundant in the sample and are widespread all over the image.

When diatoms are cleaned and suspended in water, dispense a few drops of this water on a cover slip and let it dry. Once dried, examine the cover slip under the microscope to ensure that diatoms can be easy to count. A good number of valves are eight per field but it depends on their size and shape. If there are too many, the sample must be diluted (Fig. 3); if there are only a few diatoms, add more drops and let the cover slip dry again. If there are not enough diatoms on the cover slip but there is too much inorganic matter on it, the researcher has to make a decision whether or not to add more sample to the cover slip: the addition of more sample would reduce the time of analysis but diatoms would be covered by more inorganic matter, which makes the identification of species harder.

After getting an appropriated number of diatoms on the cover slip, dispense a small drop of high refractive medium (such as Naphrax™) on a slide and place the cover slip on it, making sure that diatoms are in contact with the drop. Heat the preparation for about one minute; some bubbles will appear under the cover slip when the drop of high refractive medium boils but they will disappear as the slide cools again. Once cold, the cover slip should be closely adhered to the slide; if not, the preparation has to be heated again.

5. Diatom analysis

The next step is to identify diatoms from the sample. Some investigators obtained good results identifying to the genus level (Wu & Kow, 2002) either when samples had only a few species of each genus (Gronow, 1999) or when a quick assessment of ecological quality is required (Rimet & Bouchez, 2011). However, we recommend reaching to species level when the goal of the study is one of the following: To obtain an accurate bio-assessment, to apply diatom-based water quality indexes, or to define the ecological status of a body of water. Reaching the species level the first time that samples are analysed is time-consuming but provides significantly more information than a genus-level analysis. It also permits data aggregation in higher taxonomical groups if necessary. If other than species level is reached, the information obtained is likely to be too poor, thus forcing researchers to analyse the samples again to identify and count species. In the end, this task would be much more time-consuming.

There are genera with a large number of species and these species are water quality indicators that should be taken into consideration when analysing the samples.

For example, the big genus *Nitzschia* Hassall comprises species such as *Nitzschia fonticola* Grunow (Fig. 4), which is typically present in clean water, as well as *Nitzschia capitellata* Hustedt (one of the largest species from Fig. 3), frequently found in polluted water. The observation of *Nitzschia* in one sample does not provide information neither about water quality nor the features of the ecosystems, since *Nitzschia* can be easily found in almost any water body in the world.

Therefore, it is necessary to reach to species level when calculating diatom indexes. There are some other indexes that even require variety levels. There are many previous studies that can be referenced when determining diatoms. The most widely followed are Krammer and Lange-Bertalot (1986, 1988, 1991a, b). It is useful to have more guides and bibliography when identifying diatoms but we consider these ones to be sufficient, at least as an initial approach to diatom identification.

The scientific names from these guides are not up to date but the old names of the diatoms are listed with the founding author's name and are updated in some websites such as www.algaebase.org. In relation to synonymy, there are differences in taxonomical criteria among authors; therefore, it is important referring the authors following the scientific names of diatoms. From a taxonomical point of view, the simple determination of the genus sometimes can be insufficient to ensure its own genus identity, due to the evolution of the taxonomy. For example, *Nitzschia* Hassall is split into different genera (*Nitzschia sensu stricto*, *Grunowia* Rabenhorst, *Tryblionella* W. Smith, *Psammodictyon* Mann, etc.). Knowing that a *Nitzschia* has been found is not enough to determine if that diatom is actually *Nitzschia sensu stricto* or a member of any other genus coming from *Nitzschia*, such as *Grunowia* (Fig. 5).

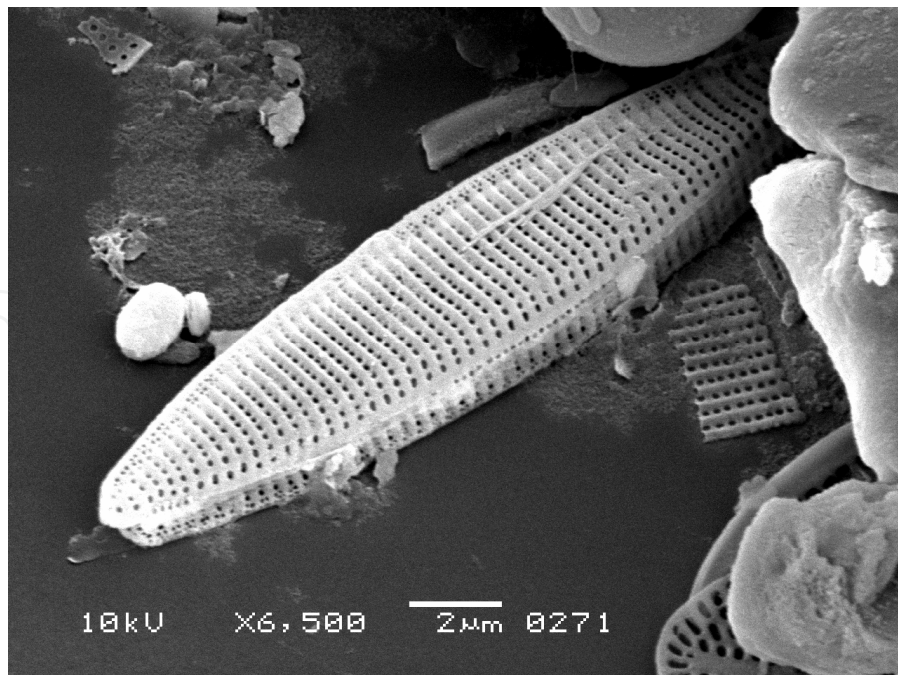


Fig. 4. External view of *Nitzschia fonticola*'s valve Grunow.

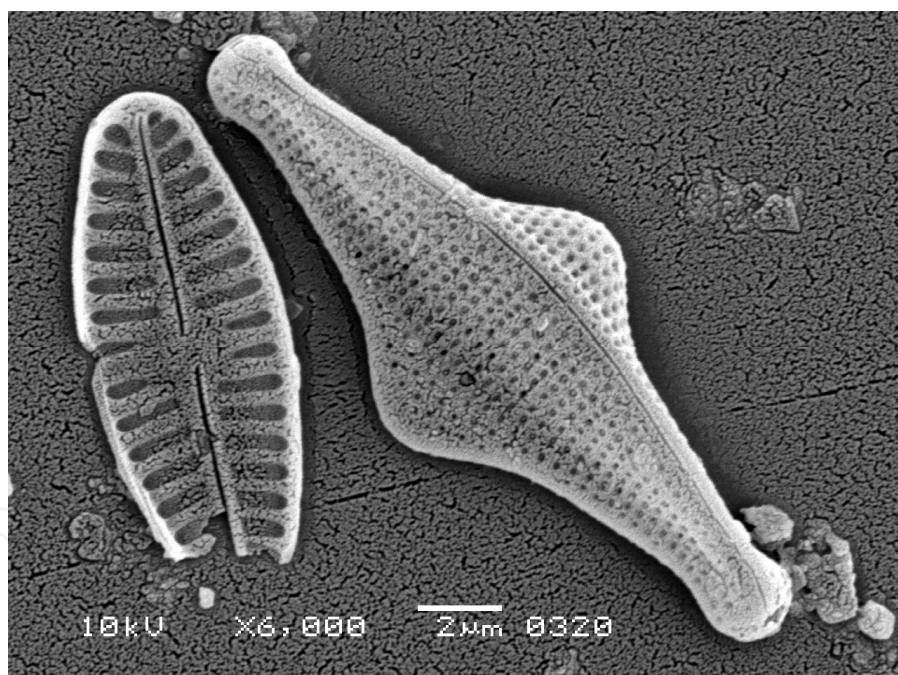


Fig. 5. The right valve is an external view of *Nitzschia sinuata* var. *tabellaria* (Grunow) Grunow, whose updated name is *Grunowia tabellaria* (Grunow) Rabenhorst.

Moreover, if species level is reached, the authors following the diatom names should be observed to update the identifications. For example, if one researcher identifies *Nitzschia constricta*, without providing any reference regarding the authors, it would be impossible to know whether he or she has actually found *Nitzschia constricta* (Gregory) Grunow (= *Psammodictyon constrictum* (Gregory) Mann) or *Nitzschia constricta* (Kützinger) Ralfs (= *Tryblionella apiculata* Gregory). A diatom analysis that determines the presence of *Nitzschia*

constricta (Kützing) Ralfs gives enough evidence to indicate that we are actually referring to *Tryblionella apiculata* Gregory, in spite of not having used updated taxonomical criteria.

The diatoms must be identified at a high magnification (100X) while using immersion oil. Non-experienced researchers tend to maximize the contrast of the image observed through the microscope to detect the limits of the valves clearly but some structures, like striae, can sometimes go unnoticed because of this excessive contrast. In these cases, it would be better to increase the lighting over the sample and to reduce the contrast in order to easily observe the diatoms in detail.

It is highly recommended to combine phase contrast with bright field techniques in order to see fine structures (striae, processes, etc.). When a microscope is not equipped with a phase contrast, put an opaque object (a pencil would be enough) between the lamp and the sample, as shown in the figure 6. Light will be deflected and it would work similarly to a phase contrast. The quality of the image can be improved if the object is slightly moved, changing its orientation in relation to the observed valve. The results of this technique are not as good as they would be when using phase contrast but it will provide similar images, plus it is cheaper than purchasing a phase contrast.



Fig. 6. An opaque object – a pencil– is placed between the lamp and the sample to simulate the phase contrast technique.

Figure 7 shows four photographs of the same valve with different types of illumination. Notice that the shape of the valve is perfectly visible in the first image, but the striae are not.

At least 400 valves have to be counted in each sample and the relative proportions of the species have to be calculated. This number provides a good estimate of the composition of the diatom flora and this is a requirement for the application of the biotic indexes.

5.1 Indexes calculation

Once the analysis is finished, the next step is to calculate biotic indexes in order to determine the water quality. Most of the widely used indexes come from the Zelinka and Marvan equation (Zelinka and Marvan, 1961), which is:

$$\text{Index} = \sum (A_i \times v_i \times j_i) / A_i v_i \tag{1}$$

Where A is the relative abundance of species i , v is the indicator value of the species and j its sensitivity.

The indexes differ in both diatom species and the values v and j assigned for each index.

Existing indexes must be tested when applied to a basin different from the ones (Prygiel et al., 1999). This testing is usually done by comparing the values given by the indexes with the physicochemical data from the same sites. The Spearman correlation between an index and chemical variables (phosphate, DIN, COD) is enough to determine whether that index can be applied to the basin or not.

There are many studies regarding this issue and it has been proved that these indexes are applicable and work in different parts of the world (Torrise & Dell’Uomo, 2006; Atazadeh et al., 2007; Taylor et al, 2007). However, most researches tend to state that it is important to test the indexes before using them to verify their accuracy (Potapova & Charles, 2007). As a matter of fact, new indexes have been developed for different basins after having found a weak correlation between chemical data and diatom indexes.

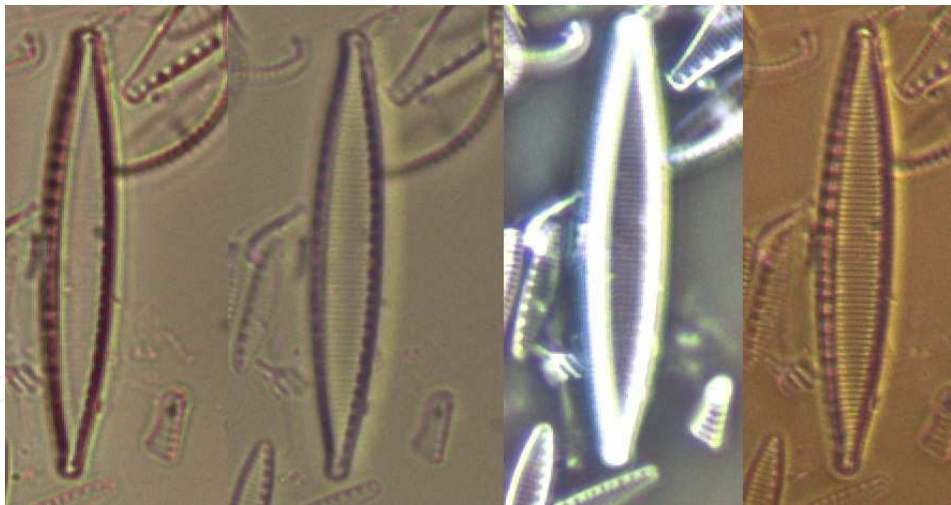


Fig. 7. Four photographs of the same valve of *Nitzschia fonticola* Grunow under different types of illumination. From left to right, bright field with a high contrast, bright field with low contrast, phase contrast, and simulation of phase contrast with an opaque object.

Index calculation is a quick and easy task if the software Omnidia, which is widely used among researchers, is available. It is an expensive computer program but it is extremely useful and worthy. It performs fast index calculations and transforms them into a scale that ranges values from 0 to 20, which facilitates comparisons. There is also a free Excel file, available at http://omnidia.free.fr/update/IBD_NEW.XLS, that permits the calculation of the IBD index. However, we recommend the acquisition of the Omnidia software.

Can diatom-based indexes be used carelessly if they correlate with the chemical data? It is not possible to do that, even though its applicability has been demonstrated all over the world. There are some situations in which indexes can present spurious values that have to be analyzed in order to avoid false conclusions. The rest of this chapter will show some examples where diatom-based indexes failed to work.

6. High index values in bad water quality and the relationship with the ecological status: The case of the Guadianar River

This case shows a real situation in which some diatom indexes, applied in a river with poor water quality and bad ecological status, return the high values that can be found in a pristine river. When facing a similar situation, researchers must consider other ecological elements in order to determine what is actually happening in the ecosystem.

The Guadianar River is located in the South of the Iberian Peninsula. The river has been suffering human impact for decades by receiving non-treated wastewaters, which have drastically reduced the quality of its waters. The diatom-based indexes showed low values indicating bad water quality due to the extended presence of organic matter and eutrophy in the water, as they were expected to do.

However, the Guadianar River has also received acidic waste with a high content of heavy metals—mainly Fe, Zn, Cd, and Cu— from a nearby mine. This impact is located upstream the organic inputs. The pollution from this mine reaches the Guadianar River through one of its tributaries, the Agrio River. This chemical pollution became more serious after a catastrophic accident in 1998, when a dam spilt tons of contaminated mud and water with high levels of heavy metals into the river.

Fortunately, the situation has improved since the accident. After hard and expensive work, today the river is a green corridor, a path communicating two different protected natural areas. Wastewater was treated in sewage treatment plants and most heavy metals were removed. Nevertheless, the water still has lower quality than desired because: 1. the eutrophy level is still too high and 2. part of the heavy metals remained within the sediment, which risk of mobilization after a big flood.

In relation to diatoms, it is interesting to analyze the composition of the flora on the areas not affected by the organic matter and eutrophy but only by the mine. We will briefly present the characteristic appearance of the river after the spilling of the mine and the subsequent cleaning, between the years 2001 to 2006. After the spill, the pH values of the water decreased and the amount of dissolved heavy metals increased drastically. This situation worsened during summer because in this season the water levels diminish, increasing the concentration of pollutants and changing the water pH to below 5.

Benthic algae were sampled in the Agrio and Guadianar Rivers following the previously explained procedure. In the Guadianar River, sampling was carried out upstream, in relation to the mine, in the confluence of the two rivers, and downstream.

The samples obtained upstream from the mine spilling showed small cyanobacteria, different green algae and many diatoms from different genera (*Melosira* Agardh, *Ulnaria* (Kützing) Compère, *Achnanthidium* Kützing, *Planothidium* Round et Bukhtiyarova, *Cocconeis*

Ehrenberg, *Cymbella* Agardh, *Encyonema* Kützing, *Navicula* Bory, *Gomphonema* Ehrenberg, *Nitzschia* Hassall, *Tryblionella* W. Smith and others). After the confluence of the Agrio River and the Guadiamar, the algal flora was completely different. Researchers observed a strong development of filamentous green algae *Klebsormidium* Silva, Mattox & Blackwell, *Ulothrix* Kützing and *Mougeotia* Agardh in the acidic waters along with large quantities of diatoms belonging to only a few species. The most characteristic species from the flora in this part of the river were *Brachysira vitrea* (Grunow) Ross and different taxa belonging to the complex of *Achnantheidium minutissimum* (Kützing) Czarnecki

Some diatom indexes were calculated in all these sites using the software Omnidia.

Table 1 shows the results of one sampling performed in April 2006, where the indexes suggest that both Guadiamar and Agrio rivers had good water quality. In terms of organic matter or eutrophy, the information given by the indexes was accurate. But we cannot consider the general quality of the water to be good, since it was highly polluted by acidity and metals.

Some features of the flora were more indicative of the actual state of the river. The observed green masses of *Klebsormidium* or *Ulothrix* are typical of acidic water with high levels of metals and are often found in these types of ecosystems (Stevens et al., 2001; Niyogi et al., 2002; Martín et al. 2004). Moreover, the richness of diatoms (see number of species in Table 1) was much lower in the Agrio River and downstream of the confluence of both rivers than in the upper part, upstream of the mine.

The genus *Achnantheidium* Kützing, very abundant in these rivers, is also noteworthy. This genus comprises small pennate diatoms. The most relevant ecological feature of these diatoms is an extraordinary ability to colonize, as a pioneering species, any system submitted to some kind of perturbation that leads to the disappearance of the flora of a site. This perturbation can be natural, like a flood after a heavy rain. Likewise, this genus can dominate in some samples just because the biofilm is not mature (Ács et al., 2004).

Moreover, these diatoms can live under a wide range of environmental conditions and resist different types of pollutants. On one hand, *Achnantheidium saprophilum* (Kobayasi & Mayama) Round & Bukhtiyarova was first described in waters polluted with organic matter (Kobayasi & Mayama, 1982), whereas *Achnantheidium minutissimum* (Kützing) Czarnecki (Fig. 8) and *A. biassolettianum* (Grunow) Round & Bukhtiyarova are considered indicative of clean water by the diatom indexes. The exact differentiation among these species and some others is often difficult (Potapova & Hamilton, 2007; Fig. 9) and their utilization in the indexes can sometimes be misleading (Martín et al., 2010).

On the other hand, different authors have found these diatoms growing in waters polluted by metals, as in the Guadiamar River (Sabater, 2000; Seguin et al., 2001; Szabó et al., 2005; Luís et al., 2011). Actually, this genus is frequently seen in this basin, independent of the presence of the mine, since it was abundant upstream. However, at the same time, this genus is the one that better resists the inputs from the mine. In fact, not only did *Achnantheidium* not disappear from the affected sites – as did *Amphora pediculus* (Kützing) Grunow, for example –, but also grew more than any other diatom. This developing can be linked to the fact that *Achnantheidium* may not be favoured by heavy metals and acidity but less disfavoured than other diatoms. Thus, *Achnantheidium* can grow without almost any

competitors and dominate the biofilm. This could be the reason why some authors even consider *Achnanthyidium* to be an indicator of heavy metal pollution (Nakanishi et al., 2004), although *Achnanthyidium* is not exclusive to these environments.

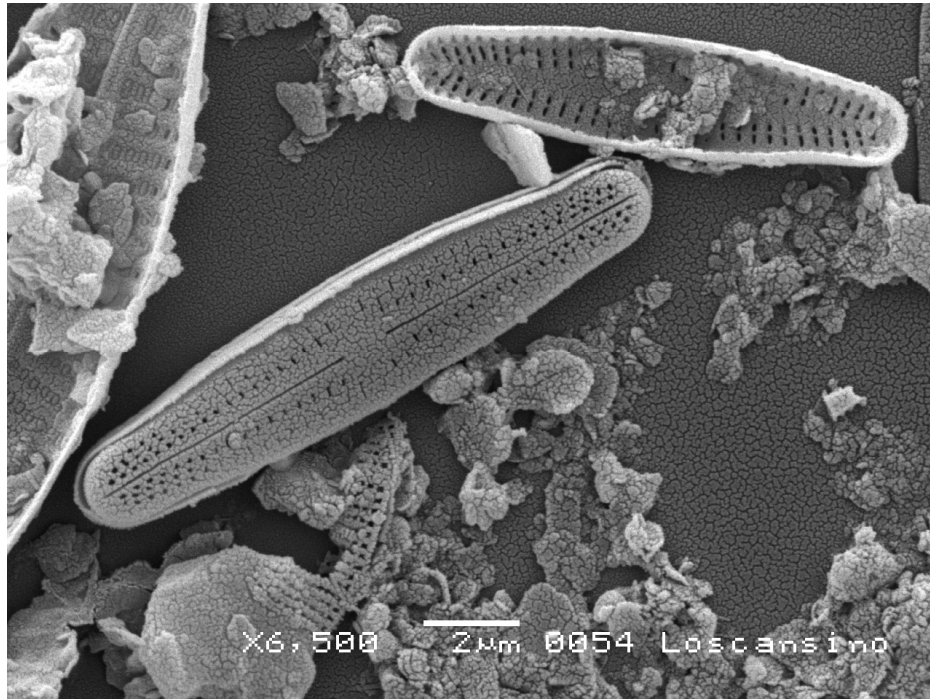


Fig. 8. Internal and external view of two valves of *Achnanthyidium minutissimum* (Kützing) Czarnecki.

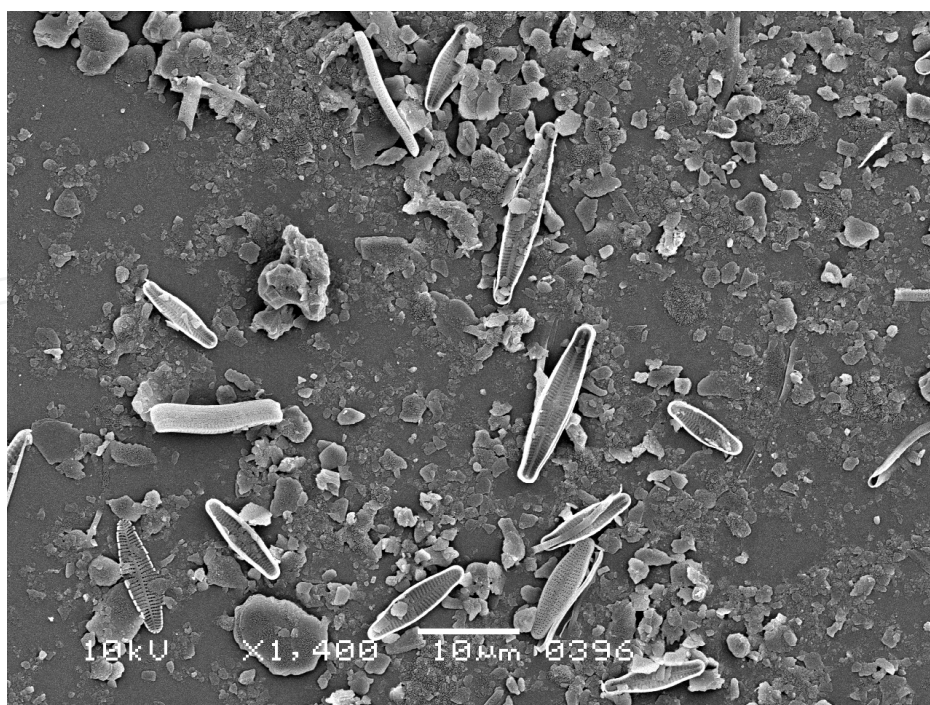


Fig. 9. A group of valves of *Achnanthyidium*. Despite the different sizes, they might belong to the same species.

SPECIES	Upstream of the mine	Agrio River	Confluence of both rivers	Downstream of the confluence
<i>Achnanthidium affine</i> (Grunow) Czarnecki	0,0	0,0	4,8	0,0
<i>Achnanthidium jackii</i> Rabenhorst	3,5	0,0	21,6	1,1
<i>Achnanthidium minutissimum</i> (Kützing) Czarnecki	30,4	52,7	0,3	0,0
<i>Achnanthidium saprophilum</i> (Kobayasi et Mayama) Round & Bukhtiyarova	0,0	34,4	9,3	73,9
Complex of <i>Achnanthidium minutissimum</i>	33,9	87,0	35,9	75,0
<i>Amphora pediculus</i> (Kützing) Grunow	22,3	0,0	0,9	0,0
<i>Amphora veneta</i> Kützing	0,0	0,0	2,4	0,0
<i>Brachysira vitrea</i> (Grunow) Ross	0,0	10,9	30,2	0,0
<i>Cocconeis pediculus</i> Ehrenberg	2,0	0,0	0,3	0,0
<i>Cocconeis placentula</i> var. <i>euglypta</i> (Ehrenberg) Grunow	6,3	0,0	0,0	0,0
<i>Cyclotella meneghiniana</i> Kützing	2,2	0,0	0,3	0,2
<i>Cymbella amphicephala</i> Naegeli	0,0	0,2	9,3	0,2
<i>Encyonopsis microcephala</i> Krammer	4,1	0,0	0,0	0,0
<i>Eolimna minima</i> (Grunow) Lange-Bertalot	0,0	0,0	0,3	19,8
<i>Gomphonema angustum</i> Agardh	0,0	0,0	2,1	0,0
<i>Gomphonema olivaceum</i> (Hornemann) Brébisson	6,5	0,0	0,3	0,0
<i>Nitzschia palea</i> (Küt.) W. Smith	0,0	0,0	2,1	0,0
<i>Staurosirella pinnata</i> (Ehrenberg) Williams & Round	2,6	0,0	0,0	0,0
<i>Ulnaria acus</i> (Kützing) Aboal	0,0	0,0	2,1	0,0
Total number of species	42	10	45	16
IPS	16.4	16.6	15.9	11.0
IBD	13.2	13.6	10.6	6.7
EPI-D	14.9	17.5	12.8	12.3

Table 1. Species found in the river Guadiamar and Agrio during April 2006 and their percentages. Only species that contribute to the 80% of the total of diatoms in any site are included in the table and these contributions are shown in the grey cells. The percentages of the species belonging to the complex of *Achnanthidium minutissimum* are added and shown, as well as the total number of species and the values of IPS, IBD and EPI-D indexes. The colors indicate the category of water quality determined by the indexes, according to this key: red, very poor; orange, poor; yellow, moderate; green, good; blue, excellent.

A large abundance of deformed valves also indicates that the river was affected by heavy metals (Fig. 10). Some authors have also found these features in diatoms living under high levels of metals (Morín et al., 2008; Tapia, 2008).

This example shows how indexes can sometimes led us to inaccurate conclusions in situations in which pollutants are not nutrients or biodegradable organic matter. In the case of pollution by heavy metals, other features of the flora (growing of green macroalgae resistant to this type of pollution, low richness and diversity, abundance of tolerant diatoms, high percentage of deformed valves, etc.) are more reliable than biotic indexes to detect the impacts of the pollutants.

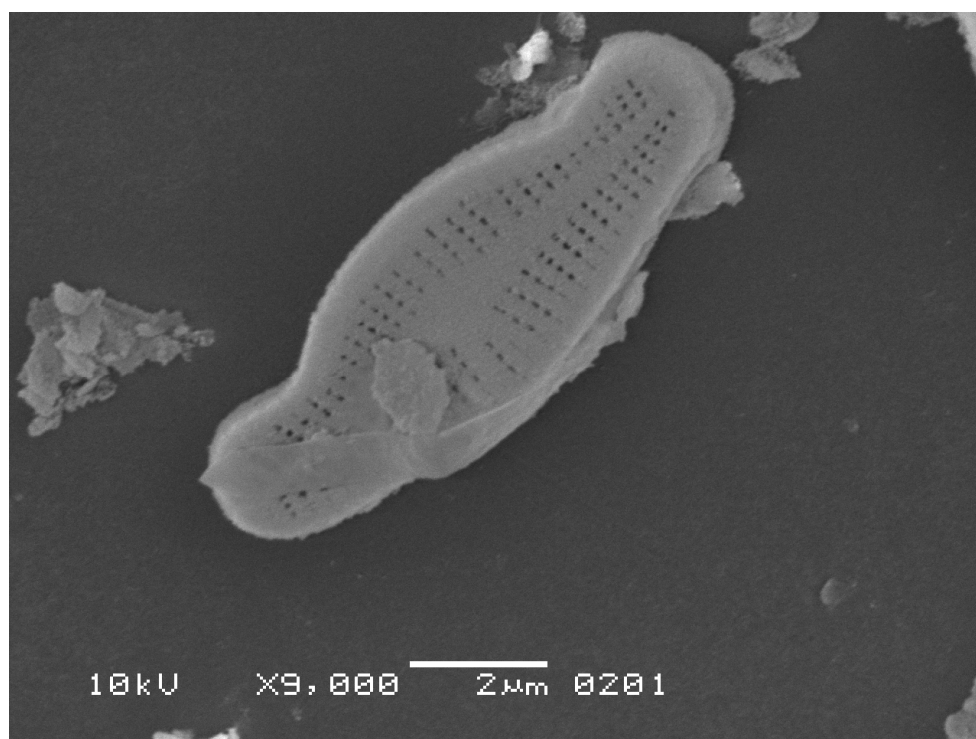


Fig. 10. A deformed valve of *Achnanthesidium* from the Guadamar River, downstream from the mine.

7. Low indexes values but good ecological status: The case of El Picacho pond

This second case study shows the opposite situation with respect to the previously analysed case study. The diatom-based indexes were high in the Guadamar and Agrio Rivers despite the very poor water quality. In this second case, we find an ecosystem with a very good ecological status that can be underestimated by diatom indexes.

In general, a good ecological status of a water body requires good water quality. Nevertheless, some water bodies pass through different periods during its annual succession. One period can include, without man's influence, high levels of eutrophy, thus giving bad values of water quality by diatom indexes. But if it concerns the nature of the ecosystem, we cannot consider that it has a poor ecological status.

We illustrate this point with the following example. There is a small pond in the mountainous areas in the South of Spain called El Picacho (Fig. 11). It is located in a protected area (Los Alcornocales Natural Park) and has almost no human impact. Its flora and fauna are basically as they are supposed to be under no human pressures. Every spring, its crystalline waters are full of submerged plants, mainly *Myriophyllum alterniflorum* de Candolle and *Ranunculus peltatus* Schrank, the latter covering the whole pond with little beautiful white flowers. The growing of the submerged vegetation is so large that, at the end of the spring, there are not open waters at all.

The pond starts to dry when summer comes until there is no water left in the middle of the season. During the drying, the vegetation starts to die. Decaying plants produce an elevation of the organic matter and eutrophy, and the algal species that develop are indicative of this situation.



Fig. 11. El Picacho pond in August 2011.

We sampled the pond twice during summer 2011, in June and August. The most abundant submerged species was *Myriophyllum alterniflorum* De Candolle, so it was the chosen substrate for sampling diatoms. The sampling procedure and the work carried out in the laboratory were the same as previously described in this chapter.

Table 2 shows the species found in each sample, the values of the biotic indexes IPS, IBD and EPI-D, and the water quality class determined by the indexes. As in the anterior case, the indexes were calculated using the Omnidia software.

The pond does not suffer any human impact. In this sense, it can be considered as a reference site for other small Mediterranean ponds with a similar annual succession. If the pond can be considered a reference site because of having no impacts, one could expect to have high values of the indexes (green or blue), but the indexes do not actually have a good or excellent rating. In June, when the drying has just started, the values are good (green) for EPI-D and moderate (yellow) for IPS and IBD, although they are in the limit of the poorer category (yellow). However, in August, when there is only a little water left in the pond, the values of the indexes are worse.

According to this, El Picacho pond does not achieve the European Water Framework Directive requirements, at least with respect to this indicator. But the truth is that the pond has a high ecological status because of its natural, mostly untouched condition.

This does not mean that the indexes do not work. In fact, they do work, since the water during this season is actually eutrophic. The values of the indexes, if desired to determine ecological status, have to be analysed in relation to the ecosystem characteristics and nature so as not to reach a misleading conclusion.

SPECIES	% JUNE	% AUGUST
<i>Gomphonema gracile</i> Ehrenberg	20.3	1.1
<i>Gomphonema parvulum</i> (Kützing) Kützing	29.8	9.4
<i>Nitzschia gracilis</i> Hantzsch	17.9	71.2
<i>Nitzschia palea</i> (Kutzing) W.Smith	2.1	6.8
<i>Ulnaria acus</i> (Kützing) Aboal	8.8	3.2
IPS	12.3	9.9
IBD	10.5	9.5
EPI-D	13.2	11.9

Table 2. Species found in El Picacho during summer 2011 and the respective percentages of each. The species that contribute less to the 5% of the total in both June and August have been deleted in order to reduce the table size. The values of IPS, IBD and EPI-D have been calculated using the Omnidia software. Colours indicate the category of water quality determined by the indexes, according to this key: Red equals very poor quality, orange-poor, yellow-moderate, green-good, and blue-excellent.

8. Conclusion

The diatom-based indexes are widely used and have proved to work in many areas of the world. However, they should be tested before being applied in a basin that was never previously studied. They are mainly used to detect organic pollution and eutrophy.

The sampling procedures to ensure a good calculation, treatment and analysis of the samples are normalised.

However, these indexes are only a working tool, meaning that the information that they provide should be compared to other sources of information from the same sites, such as chemical analysis, diversity of diatoms, and other elements of the flora, among others. Otherwise, indexes could be misinterpreted when estimating water quality and the ecological status of an ecosystem.

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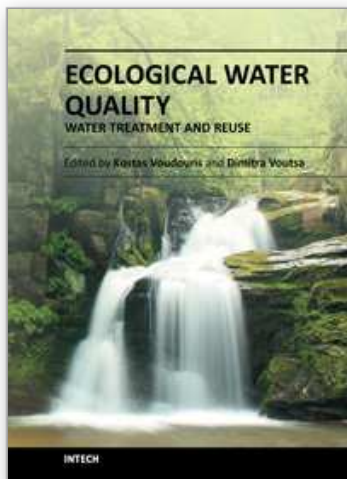
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