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# Changes in the phytoplankton community structure in a monomictic temperate lake

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**ABSTRACT**

**Changes in the phytoplankton community structure in a monomictic temperate lake**

This study focused on the phytoplanktonic community of lake Sanabria (NW Spain) during the mixing period. We integrated the classic phytoplankton counting method and the pigment analysis via high performance liquid chromatography, to obtain a global image of the community structure, which was very similar among the study zones and unaltered despite a temporal increasing of biomass during this period. The diatom *Asterionella formosa* Hassall (around 80 %), and consequently the fucoxanthin were the species and the secondary pigment more abundant, respectively. This dominance contrasted with the studies performed in 90´s, where the community structure was distributed among chlorophytes, cryptophytes, diatoms, dinoflagellates and cyanophytes, with no clear and persistent dominance of any of the main taxonomic groups and an occasional presence of *Asterionella formosa*. However, the classical variables, such as nutrients or chlorophyll *a* concentration, used for trophic definition have barely changed since the studies of 90's. It is possible that other parameters, such as the residence time or the effect of the increase in the temperature since 90´s could have influenced the phytoplankton community structural changes observed.

**Key words**: *Asterionella formosa*, diatoms, HPLC, pigments, Sanabria

***RESUMEN***

***Cambios en la estructura de la comunidad fitoplanctónica de un lago templado monomíctico***

*El estudio se centró en la comunidad fitoplanctónica del Lago de Sanabria durante la época de mezcla. Se utilizaron, de manera integrada, el método clásico de recuento de fitoplancton y el análisis de pigmentos por cromatografía liquida de alta resolución (HPLC) para obtener una imagen general de la estructura de la comunidad, la cual resultó ser muy similar tanto espacial como temporalmente, a pesar de la existencia de un incremento estacional en la biomasa durante este periodo. La diatomea* Asterionella formosa *Hassall (en torno al 80 %) y, consecuentemente, la fucoxantina fueron la especie y el pigmento secundario más abundantes, respectivamente. Esta dominancia contrastó con los estudios realizados en la década de los 90 en los que se reflejaba que la estructura de la comunidad se repartía entre clorofíceas, criptofíceas, diatomeas, dinoflagelados y cianofíceas, sin que existiera una dominancia clara y persistente de ninguno de los grupos taxonómicos principales, y donde Asterionella formosa solo aparecía de manera ocasional. Sin embargo, las variables clásicas que definen el estado trófico, como la concentración de nutrientes o de clorofila* a*, no han sufrido apenas cambios. Es posible que otros parámetros, como el tiempo de residencia del agua o el efecto del incremento de la temperatura desde entonces, puedan tener relación con el cambio observado en la estructura de la comunidad fitoplanctónica.*

**Palabras clave**: *Asterionella formosa, diatomeas, HPLC, pigmentos, Sanabria*

## INTRODUCTION

Phytoplankton is the basis of the trophic chain of aquatic autotrophic ecosystems. Hence, composition and dynamics of the phytoplankton community affect higher trophic levels (Reynolds, 1987), and the global functioning of the whole system. Traditionally, light intensity and availability of nutrients, particularly nitrogen and phosphorous, have been pointed as the main driving factors determining phytoplankton composition in lentic freshwater systems (Yang *et al.*, 2008). In this sense, more recent studies tend to emphasize the importance of the synergistic effect that could exist within other ecological variables, such as the characteristics of the mixture (Becker *et al.*, 2010). Phytoplankton stands out for its importance as an indicator of trophic and ecological status of water (Rakocevic-Nedovic & Holler, 2005; Padisák *et al.*, 2006; Pasztaleniec & Poniewozik, 2010), and has become a key factor for eutrophication evaluation according to European institutions (OCDE 1982), culminating with the establishment of the Water Framework Directive (WFD 00/60/EC; EC Parliament and Council, 2000), which considered it as an indispensable tool for biomonitoring epicontinental water quality.

Knowledge of the phytoplankton community can be achieved through different techniques. From the classic microscopy ones, through pigment analysis or flow cytometry, to molecular based techniques. All of them have advantages and disadvantages; the integrated use of the maximum number of them can be used to obtain a realistic global image of the phytoplankton community. High-performance liquid chromatography (HPLC) allows the separation and quantification of pigments, such as chlorophylls and carotenoids, which, together with the specificity of some of these pigments for certain taxonomic groups, allows for inferences about the community through the application of chemotaxonomy. The use of this technique in freshwater is not so usual as it is marine or estuarine waters, (Descy *et al.*., 2009; Simmons *et al.*, 2016), but it has shown effectiveness monitoring the phytoplankton community in lakes (Fietz & Nicklisch, 2004; Descy *et al.*, 2005; Picazo *et al.*, 2013). HPLC allows rapid and automated analysis of lipophilic photosynthetic pigments (Jeffrey *et al.*, 1999) with a high differentiating power of the higher taxonomic categories (Hou *et al.,* 2011). On the other hand, microscopic techniques are able to discriminate at the level of genus or species, but present difficulties in the identification of nanoplankton and picoplankton, commonly well represented in oligotrophic systems, and also the reproducibility is lower than with chromatographic techniques (Goericke & Montoya, 1998; Fietz & Nicklisch, 2004).

Freshwater phytoplankton study stands out in lentic systems, particularly lakes and reservoirs that harbour well-structured resident communities. Lake Sanabria is the biggest natural lentic water system in Spain and the first limnological studies were developed in it at the beginning and the middle of 20th century (Taboada, 1913; Margalef, 1955). During the decade of the 1970s, a greater awareness for natural patrimony conservation arose; a special plan of landscaping was established and legal figures created, resulting in the declaration of the natural park of Lake Sanabria and surroundings in 1978. During the following decades, particularly during the 1990s, scientific activity in the lake intensified (Aldasoro *et al.*, 1991; Vega *et al.*, 1992; De Hoyos, 1996; De Hoyos *et al.*, 1998; De Hoyos & Comín, 1999; Negro *et al.*, 2000), with detailed descriptions of phytoplankton dynamics in this system. In the 21st century the studies focused on natural and artificial systems of smaller size in the lake surroundings (Negro *et al.*, 2003), sedimentary records (Luque & Julià, 2002; Rico *et al.*, 2007; Jambrina-Enríquez *et al.*, 2014), and which established a retrospective comparison of the community (Pahissa *et al.*, 2015).

In recent years, a social based polemic has taken place considering a study by Guillén, (2015), which integrates a series of reports and non-published investigations that question the oligotrophic or oligo-mesotrophic state of this mountain lake. These declarations have been strongly debated by the scientific community, which claimed its opposition based on detailed studies considering the trophic state of the lake (Aldasoro *et al.*, 1991; Pahissa *et al.*, 2015). Despite there being no well-founded evidence supporting either the contamination or the

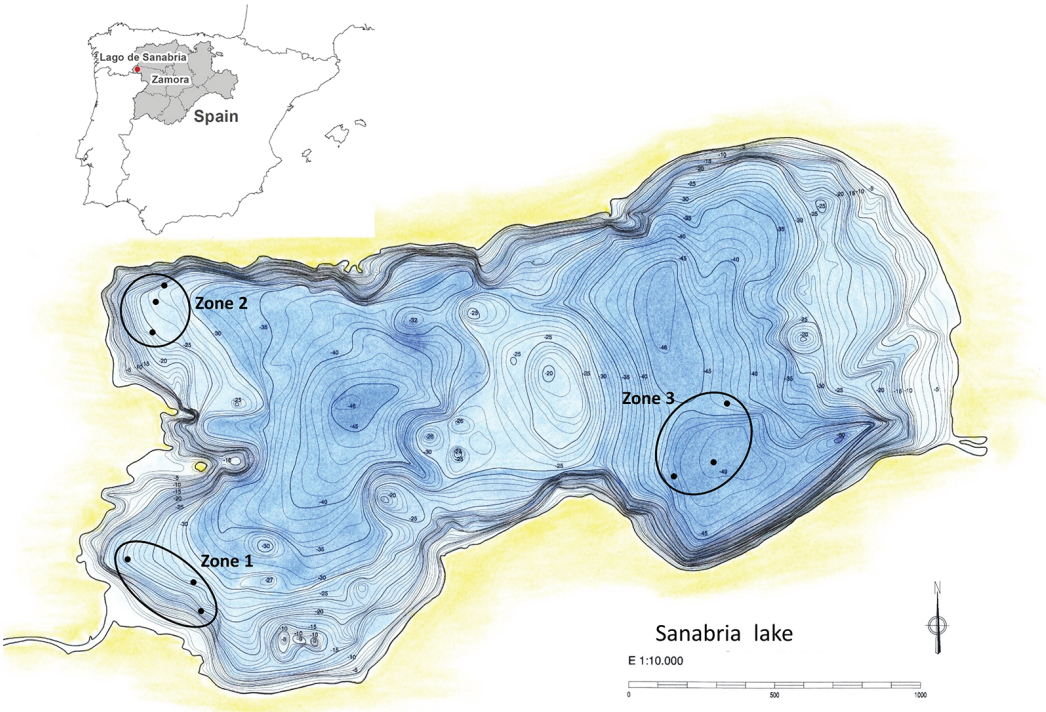


**Figure 1.** Sampling station map adapted from Vega *et al.* 2005. Sampling sites (black stars). Zone 1: stream input influence; Zone 2: livestock influence; Zone 3: lacustrine and reference zone. *Mapa con las estaciones de muestreo adaptado a partir de Vega* et al. *2005. Puntos de muestreo (estrellas negras). Zona 1: influencia de la pluma fluvial; Zona 2: influencia de la presencia de ganado; Zona 3: zona lacustre de referencia.*

eutrophication of the lake, monitoring of the ecological variables seems to be advisable. Fires, change of land use in the catchment area, increase of nutrient imputes related to a higher human presence, along with a change of water turnover due to rather anthropic modifications of the course or actual climatic scenarios, imply a potential alteration of the lakes natural state. Therefore, the objectives of this work are to: i) characterize the phytoplankton community of Lake Sanabria during the mixing period (January-March), through the integrated use of chemotaxonomy (HPLC) and the classical phytoplankton counting method; ii) compare the phytoplankton community with that described in 90's; iii) compare the community composition between three different zones, with varying threats regarding the nutrient supply, with the prediction of observing differences in the phytoplankton community between these zones.

## MATERIALS AND METHODS

### Study site

Lake Sanabria is located in the NW part of Spain (42° 07' 30" N, 06° 43' 00" W), at 1000 m above sea level. It occupies a glacial depression of the Tera River valley, which belongs to the Duero River basin and originates at the confluence of the Segundera and Cabrera mountain ranges. Lake Sanabria has its origins in the last glaciation, through the morrenic closing of the valley. It is composed by two smaller basins separated by an intermediate threshold of depth (Aldasoro *et al.*, 1991). The total area of the lake is 3.47 $km^2$ and the volume is 96 289 887 $m^3$. Other relevant morphometric parameters: maximum depth is found in the eastern basin with 51 m, maximum width of 1530 m is observed across this basin, and the shoreline length is 9518 m

(Vega *et al.*, 2005). The lake is categorised within the Spanish typology as an acidic, deep, and middle mountain lake.

According to the characteristics of the mixing, Lake Sanabria is a warm monomictic and holomictic lake, with a mixing period generally extended from the ending of November, beginning of December, until March when water starts to stratify. In the winter months, an average homoeothermic state is found between 4 to 7 ºC; while across stratification, an epilimnetic maximum temperature is observed around 24 ºC, during the month of August with a 1.7 ºC/m decrease in temperature throughout the metalimnion constituting the thermocline (Vega *et al.*, 1992).

The geology of the lake is dominated by plutonic and metamorphic rocks (granite, quartzite and gneiss) conforming precipitous walls that end in a plane bottom. These materials, in absence of contamination, determine the oligotrophic state of the lake (Rico *et al.*, 2007). Mineralisation is, therefore, low, with conductivity mean values near 14 µS/cm, and pH values of approximately 6.5, a slightly shifted equilibrium towards the acidic form of $CO_2$. In addition, oxygen has a winter maximum concentration near 100 % saturation without any anoxic period measured at any point of the year (De Hoyos, 1996). Within the threshold of oligotrophy, nutrient inputs come from three main sources: sediment resuspended during the winter mix, runoff from the basin through the main tributaries of the lake: Tera, Cardena, and Segundera rivers, and finally the discharges of anthropic origin that concentrate primarily during summer due to a greater tourist pressure particularly in the western basin, where Ribadelago is located (Aldasoro *et al.*, 1991).

**Data collection**

Abiotic and biotic data collection took place from January to March (mixing period) of 2017. We decided to choose the mixing period, due to the homogeneity of the conditions along the whole study period, and trying to sample in the period of the year with the less human impact in the lake. A random sampling by zones was carried out, monthly and the data presented here are from January, February and March. The three zones studied were determined in order to detect areas of the lake exposed to possible sources of anthropic alteration (Zones 1 and 2) and without them (Zone 3). In addition, for its correct definition a bathymetric map was used (Fig. 1), avoiding areas near the shoreline and shallow depth. The first zone was chosen because of its proximity to the mouth of the river Tera after passing through the locality of Ribadelago, the second corresponded to the recent observations of livestock activity near the North-western shore margins. Finally, the third zone represents a strictly lacustrine area, which has been widely studied for harbouring the maximum depth, constituting a reference to the presumably unaltered state of the lake. Within each of these zones, three replicates (A, B, C) were taken using a system of random choice of Cartesian coordinates.

In relation to the abiotic data, the maximum depth was measured with a laser meter; also, the depth of the Secchi disk was measured. Conductivity and pH were measured at each station in a single surface sample while oxygen concentration, percentage saturation, and temperature were measured at intervals of 0.5 m to five m depth, at intervals of one m from five to 20 m, and at the points of greatest depth in intervals of five m from 20 m to the bottom. For this purpose, the probes corresponding to each variable (WTW: Multi350i; WTW: Oxi197) were used. Likewise, conductivity, temperature and pH were measured in the Tera River, at Ribadelago. As for the biotic data, surface direct water samples and net samples (both in the first 0.5 m depth) were taken for different purposes. Two hundred and fifty mL of direct samples were taken and fixed with one mL Lugol, while an approximate volume of 500 mL was preserved as a living sample in a Nalgene® bottle to observe alive the community in order to help with the identification. For the analysis of pigments, 5 L of water were taken in opaque bottles. Net samples were filtered through a 20 µ m mesh, sweeping a similar surface at each sampling station for three minutes. The resulting sample was preserved both *in vivo* and fixed with Lugol.

**Characterization of phytoplankton community**

*Microscopy*

The phytoplankton community was characterized from the Lugol-fixed surface samples following the Utermöhl method (Utermöhl, 1958) according to the WFD. Additionally, living subsamples of net and direct samples were observed under light microscopy at various magnifications to determine the presence of species that no resist well the fixation. For the 50 mL settled samples, cells were identified and counted under a Nikon Diaphot TMD (Nikon Corporation, Tokyo, Japan) inverted microscope. For the bigger-sized taxa, transects of 4 cm of the chamber area were examined at 100x magnification in each sample, collecting a minimum of at least 500 individuals of the dominant taxa; while for the smaller-sized taxa, two or three cm transects were examined at 400x magnification, reaching up to 50 individuals at least. Species biovolume was calculated using formulas from the Baltic Marine Environment Protection Commission (HELCOM; Olenina *et al.*, 2006).

*High-performance liquid chromatography*

Water samples gathered for the pigment analysis were filtered with gentle vacuum (< 150 mmHg) onto Whatman GF/F glass-fibre filters (Whatman International Ltd.), filters were immediately

**Table 1.** Summary of superficial abiotic data from the vertical profiles of each replicate and month. ZSD: Secchi disk depth; Conduct: conductivity; Ox: oxygen concentration; Ox sat: oxygen saturation; T: temperature. *Resumen de los datos abióticos superficiales extraídos de los perfiles verticales para cada replica y mes. ZSD: Profundidad disco de Secchi; Conduct: conductividad; Ox: concentración de oxígeno; Ox sat: saturación de oxígeno; T: temperatura.*

| Month | Sampling zone | Hour | | ZSD | Max. Depth | Conduct. (µS/cm) | pH | Ox. (mg/l) | Ox. Sat. % | T (ºC) |
|---|---|---|---|---|---|---|---|---|---|---|
| JANUARY (24/01/2017) | River | – | | – | – | 11 | 6.90 | – | – | 1.10 |
| | 1 | 10:10 | Mean | 5.20 | 12.50 | 13.30 | 5.83 | 9.50 | 86.33 | 6.27 |
| | | | SD | 0.26 | 4.17 | 0.96 | 0.32 | 0.10 | 0.58 | 0.06 |
| | 2 | 11:25 | Mean | 5.00 | 18.40 | 11.47 | 5.85 | 9.50 | 86.33 | 6.40 |
| | | | SD | 0.50 | 5.61 | 0.45 | 0.22 | 0.10 | 1.53 | 0.00 |
| | 3 | 13:30 | Mean | 5.70 | 50.57 | 12.53 | 5.90 | 9.30 | 85.00 | 6.73 |
| | | | SD | 0.61 | 1.33 | 1.56 | 0.17 | 0.10 | 1.00 | 0.15 |
| FEBRUARY (22/02/2017) | River | – | | – | – | 12.70 | 7.00 | – | – | 3.80 |
| | 1 | 9:48 | Mean | 5.27 | 13.23 | 14.10 | 6.78 | 10.67 | 97.00 | 6.33 |
| | | | SD | 0.25 | 4.16 | 2.44 | 0.19 | 0.06 | 0.00 | 0.06 |
| | 2 | 10:58 | Mean | 5.00 | 20.37 | 15.23 | 6.60 | 10.63 | 97.00 | 6.57 |
| | | | SD | 0.00 | 2.22 | 0.45 | 0.10 | 0.06 | 0.00 | 0.21 |
| | 3 | 12:37 | Mean | 5.27 | 48.40 | 13.03 | 6.40 | 10.50 | 95.67 | 6.57 |
| | | | SD | 0.21 | 2.87 | 0.87 | 0.17 | 0.00 | 0.58 | 0.15 |
| MARCH (08/03/2017) | River | – | | – | – | 8.70 | 6.40 | – | – | 4.10 |
| | 1 | 9:58 | Mean | 5.23 | 14.70 | 12.80 | 6.03 | 10.93 | 96.33 | 5.60 |
| | | | SD | 0.40 | 3.46 | 1.00 | 0.49 | 0.21 | 1.15 | 0.53 |
| | 2 | 10:59 | Mean | 5.00 | 22.20 | 13.73 | 6.27 | 10.53 | 95.33 | 6.43 |
| | | | SD | 0.00 | 4.25 | 2.35 | 0.46 | 0.06 | 0.58 | 0.15 |
| | 3 | 12:30 | Mean | 5.47 | 48.20 | 12.73 | 6.30 | 10.53 | 97.33 | 6.75 |
| | | | SD | 0.25 | 3.30 | 1.54 | 0.20 | 0.06 | 2.31 | 0.21 |

frozen at -80 °C until their analysis. Frozen filters were extracted under low light in three mL of 90 % acetone and ground with a glass stick for five min. The resulting slurry was filtered with a Teknokroma PTFE (0.22 µm pore size) syringe filter (Teknokroma, S.C.C.L.), and the filtrate was kept refrigerated until analysis. Just before injection, 0.5 mL of extracts was diluted in 0.2 mL of distilled water to avoid peak distortion (Zapata & Garrido, 1991). Pigments were analyzed by HPLC following the method of Zapata *et al.* (2000), modified by reducing 10-fold the concentration of pyridine (final concentration 0.025 M) in eluent A (Seoane *et al.*, 2009), using a Waters Symmetry C8 column (150 x 4.6 mm, 3.5 µm particle size, 100 Å pore size) (Waters Corporation) and a Waters 2996 photodiode array detector (350-750 nm; 1.2 nm optical resolution) interfaced with a Waters 2475 multi λ fluorescence detector (Waters Corporation). Pigments were identified by their retention times and absorbance spectra. Retention times were compared with those of pure standards obtained commercially from DHI (Hoersholm) and those reported in Jeffrey (1997) and Zapata *et al.* (2000). HPLC calibration by external standard was performed using chlorophyll and carotenoids standards obtained commercially from DHI. The molar extinction coefficients obtained from Jeffrey (1997) were used for pigment quantification.

*Cultures and live samples*

Live samples and net samples were observed under light microscopy (100x, 200x and 400x magnifications) with the goal of facilitating the identification of the species that could not be well defined in the lugol-fixed samples. Also, cultures were stablished with this purpose, using F/2 culture medium (Guillard & Ryther, 1962).

*Data treatment*

First, we analysed the dynamic of the phytoplankton community through the realisation of succession graphics (Microsoft® Excel® 2011) for the higher taxonomic categories, and for the concentration of what we found to be the relevant pigments throughout the studied period. Mean values were included for each zone and month of study. The spatial and temporal variability of the multivariate compositional and pigment structure of assemblages were examined by a distance-based permutational multivariate analysis of variance (PERMANOVA, Anderson *et al.*, 2008) considering composition and abundance of taxa, higher taxonomic categories, and pigments concentration. The experiment was designed with two factors: Month (random with three levels; January, February and March) and Zone (fixed with three levels; Zones 1, 2 and 3) with a significant level of $\alpha = 0.05$. For both kinds of taxonomical analyses, data were square root transformed to balance the contributions of rare and dominant taxa and categories, while the Bray-Curtis index was used to determine similarity between pairs of samples. For pigment concentration, data was standardised by the maximum and an Euclidean distance matrix was generated. Also, Pearson correlations were carried out between the relevant pigments and the chlorophyll *a*. Finally, to visualise these results, samples were graphically organised through a multidimensional scaling (MDS) and Draftsman plot correlations were established between relevant pigments.

## RESULTS

### Abiotic data

The abiotic data are shown in Table 1. The maximum depth varied between 8.1 m at site 1C in January and 52 m at site 3C in March. Depth of the photic zone, calculated from Secchi disc, showed values in all areas around 14 m, which in the zone 1 and in the first station of the zone 2 represents the whole column water, due to their depth minor than 14 m. Conductivity ranged between 11 and 15.8 µS/cm, with the values measured at the mouth of the Tera River being slightly lower, with a minimum value of 8.7 µ S/cm in March. In addition, pH presented values between 5.8 and 7 with a mean value of 6.21. The concentration of oxygen, as well as the percentage of saturation, showed similar values, around 10 mg/L and 90 %, respectively, being slightly lower in January than in February and March. Finally, the temperature presented values with a variation

**Table 2.** Presence absence checklist of the species observed in the counts or in live samples. 1 = presence and 0 = absence Asterisks indicate rare taxa. *Listado de presencia y ausencia de especies observadas en los recuentos o en las muestras no fijadas. Los asteriscos indican taxones infrecuentes.*

| | January | | | February | | | March | | |
|---|---|---|---|---|---|---|---|---|---|
| | Zone | | | | | | Zone | | |
| **CHLOROPHYTA** | **1** | **2** | **3** | **1** | **2** | **3** | **1** | **2** | **3** |
| *Ankistrodesmus falcatus* (Corda) Ralfs | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 |
| *Ankistrodesmus fusiformis* Corda | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| *Ankistrodesmus* sp. Corda* | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| *Ankistrodesmus spiralis* (W.B.Turner) Lemmermann* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *Ankyra* sp. Fott | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| cf. *Elakotothrix gelatinosa* Wille* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| cf. *Sphaerocystis schroeteri* Chodat | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Chlamydomonas* sp. Ehrenberg | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Crucigenia quadrata* Morren | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Crucigenia tetrapedia* (Kirchner) Kuntze | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Crucigeniella pulchr*a (West & G.S.West) Komárek* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| *Dictyosphaerium* sp. Nägeli | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| *Elakatothrix gelifacta* (Chodat) Hindák | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Gonium* sp. O.F.Müller* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| *Kirchneriella* sp. Schmidle | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Monoraphidium* cf. *contortum* (Thuret) Komárková-Legnerová | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Monoraphidium* sp. Komárková-Legnerová | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Oocystis lacustris* Chodat | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Oocystis* sp. Nägeli ex A.Braun | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Pediastrum privum* (Printz) Hegewald* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Pediastrum tetras* (Ehrenberg) Ralfs* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Quadrigula closteroides* (Bohlin) Printz | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| *Scenedesmus* sp. Meyen* | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| *Tetradesmus obliquus* (Turpin) M.J.Wynne | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *Tetraedron caudatum* (Corda) Hansgirg | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| **BACILLARIOPHYTA** | | | | | | | | | |
| *Asterionella formosa* Hassall | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Aulacoseira* cf. *granulata* (Ehrenberg) Simonsen | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Aulacoseira distans* (Ehrenberg) Simonsen | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *Aulacoseira* sp. Thwaites | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Centric diatom | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Centric diatom 100 µm* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| cf. C*yclotella* sp. (chains) (Kützing) Brébisson* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cyclotella* glomerata H.Bachmann | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Eunotia* sp. Ehrenberg* | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| *Fragilaria* cf. *acus* (Kützing) Lange-Bertalot* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |

Cont.

**Table 2. (cont.)**

| | January | | | February | | | March | | |
|---|---|---|---|---|---|---|---|---|---|
| | Zone | | | | | | Zone | | |
| **BACILLARIOPHYTA** | | | | | | | | | |
| *Fragilaria* sp. Lyngbye* | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| *Gomphonema constrictum* Ehrenberg* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| *Navicula* sp. Bory* | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 |
| *Nitzschia* sp. Hassall* | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 |
| Pennate diatom | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Pinnularia* sp. Ehrenberg * | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *Tabellaria fenestrata* (Lyngbye) Kützing | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| *Tabellaria flocculosa* (Roth) Kützing | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| **ZYGNEMATOPHYCEAE** | | | | | | | | | |
| *Closterium sp.* Nitzsch ex Ralfs | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| *Cosmarium* cf. *contractum O.Kirchner* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Euastrum ansatum* Ehrenberg ex Ralfs* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *Mougeotia sp.* C.Agardh* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Spondylosium planum* (Wolle) West & G.S.West | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Staurastrum anatinum* Cooke & Wills | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| *Staurastrum brachiatum* Ralfs ex Ralfs | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| *Staurastrum paradoxum* Meyen ex Ralfs* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *Staurodesmus* sp. Teiling | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Xanthidium antilopaeum* Kützing | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **CYANOBACTERIA** | | | | | | | | | |
| *Anabaena* sp. Bory ex Bornet & Flahault | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| cf. *Oscillatoria* sp. Vaucher ex Gomont | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| *Chroococcus minutus* (Kützing) Nägeli | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| *Cyanotetras crucigenielloides* Komárek | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| *Merismopedia glauca* (Ehrenberg) Kützing* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| undefined cocoids | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| **CHRYSOPHYCEAE** | | | | | | | | | |
| *Bitrichia* sp. Woloszynska* | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 |
| *Dinobryon bavaricum* Imhof* | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| *Dinobryon divergens* O.E.Imhof | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| *Mallomonas* sp. 1 Perty | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| *Mallomonas* sp. 2 Perty | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| **CRYPTOPHYTA** | | | | | | | | | |
| *Cryptomonas* cf. *erosa* Ehrenberg | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Cryptomonas* cf. *marssonii* Skuja | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Plagioselmis nannoplanctica* (H.Skuja) G.Novarino, I.A.N.Lucas & S.Morrall | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **DINOPHYTA** | | | | | | | | | |
| *Peridinium* sp. Ehrenberg* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| *Peridinium umbonatum* Stein* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |

lower than 2 °C, presenting a tendency to superficial heating of the water in the samples taken towards noon. The results of the environmental variables showed a clear mixing of the water.

**Biotic data**

From the microscopic identification of the samples, 72 different taxa were found (Table 2). In this way, the microscopic analysis of the community identified the Chlorophyta division as the best represented with a total of 25 taxa, followed by diatoms (Bacillariophyta division) with 19 taxa. In relation to the biovolume (Fig. 2), considerable spatial and temporal homogeneity in the structure of the community with a progressive increase in the total biovolume from January to March was observed. Diatoms, contributed most of this increasing biovolume, specifically the species *Asterionella formosa* Hassall, which dominated the community with a percentage of contribution in biovolume temporarily increasing from 70 to 85 %. Other diatoms observed were the taxa: *Aulacoseira* sp. Thwaites, *Tabellaria fenestrata* (Lyngbye) Kützing, and *Tabellaria flocculosa* (Roth) Kützing. Likewise, a centric diatom with about 100 µm of diameter stands out for its exclusive presence in the 2C March sample, notably increasing the total biovolume of the diatoms in this particular area (Fig. 2). As for the rest of the higher taxonomic groups, the green algae followed the diatoms in biovolume, underlining taxa such as: *Monoraphidium* sp. Komárková-Legnerová, *Crucigenia tetrapedia* (Kirchner) Kuntze, *Crucigenia quadrata* Morren, or *Oocystis lacustris* Chodat for chlorophytes and taxa such as *Staurodesmus* sp. Teiling, *Staurastrum anatinum* Cooke & Wills, *Cosmarium* cf. *contractum* O.Kirchner, or *Spondylosium planum* (Wolle) West & G.S. West for the desmids. The other taxonomic groups maintained low biovolumes, but were nevertheless considerable in terms of the number of cells per L. Taxa such as *Plagioselmis nannoplanctica* (H.Skuja) G.Novarino, I.A.N.Lucas & S.Morrall (Cryptophyta), *Chroococcus minutus* (Kützing) Nägeli (Cyanobacteria), or *Dinobryon divergens* O. E.Imhof (Chrysophyceae) were the most abundant in these

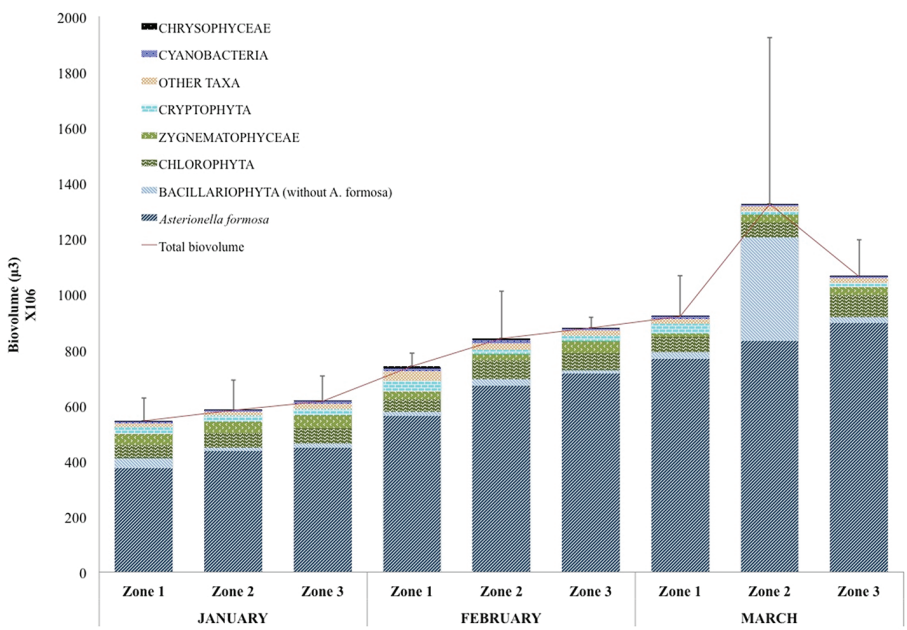


**Figure 2.** Phytoplankton community succession in terms of biovolume (µm$^3$) throughout the months of study. Mean values of the higher taxonomic groups are included for each zone. *Sucesión de la comunidad fitoplanctónica en términos de biovolumen (µm$^3$) a través de los meses de estudio. Se incluyen los valores medios de los grupos taxonómicos superiores.*

other groups. We also found that the sample taken in December (data not shown) were also totally dominated by *A. formosa*, and the dominance continued in the same year in August (unpublished data).

Regarding the results of the pigment analysis by HPLC, the values of chlorophyll *a* ranged between 1.7 µg/L in the zone 3 in January and 3.5 µg/L in the zone 2 in March. In addition to the chlorophyll *a*, we found four relevant pigments that dominated the community along the entire period: fucoxanthin, chlorophyll *b*, lutein, and alloxanthin (Fig. 3). Fucoxanthin stands out among the other accessory pigments, sharing a similar pattern to chlorophyll *a* (Fig. 4), and showing the influence of diatoms on the variation of chlorophyll *a* and, therefore, on the total biomass of the system. According to Pearson correlations, the only significant case found was between chlorophyll *a* and fucoxanthin. Chlorophyll *b* and lutein experienced a decrease from January to February and March, while alloxanthin maintained slightly lower concentrations in all areas and over the months, with values around 1.3 x $10^{-4}$ and 5.9 x $10^{-5}$ µmol/L.

PERMANOVA detected significant differences for the month factor in the three cases studied. However, significant differences were not observed for the Zone factor (Table 3). The MDS reflects the spatial arrangement of the samples for the month factor, considering the composition and abundance of taxa. The grouped samples can be discriminated according to each of the three months studied (January-March). The eccentric position of the M2C sample of March mainly responds to the high biomass of a rare taxa, a 100 µm undefined diatom. (Fig. 5).

Considering the relevant pigment concentration (µmol/L) according to the Month factor (Fig. 6), it can be seen how it coincided with the analyses previously presented. January samples remain even more isolated from the rest, forming a perfectly distinguishable group; while a smooth transition between February and March samples is observed, being less scattered and jumbled than in the case of the composition and abundance of taxon analyses. Also, in figure 6 the deviation of sample M2C (March) appears to be less pronounced than in Figure 5.

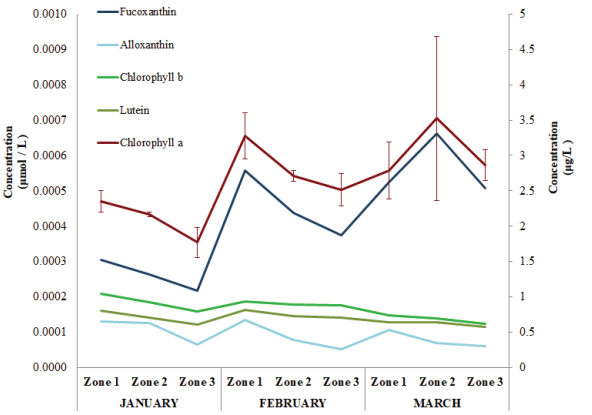


**Figure 3.** Pigmentary succession of the phytoplankton community. Mean concentration (µmol/L) values are shown for the most relevant pigments: fucoxanthin, alloxanthin, chlorophyll *b*, and lutein, while chlorophyll *a* is presented in µg/L concentration values in a secondary axis together with its standard deviation values. *Sucesión pigmentaria de la comunidad fitoplanctónica. Se presentan los valores de las concentraciones medias (µmol/L) de los pigmentos más relevantes: fucoxantina, aloxantina, clorofila* b *y luteína, mientras que la concentración de clorofila* a *se presenta en el eje secundario en µg/L junto con sus valores de desviación estándar.*

Finally, these results show the existence of a significantly homogeneous community at the spatial scale for the studied zones, in combination with a temporal rise in chlorophyll *a* that seems to be associated with an increase in the total biovolume of the samples, despite maintaining the general structure of the community.

## DISCUSSION

The marked dominance of *A. formosa* contrasts with what was previously expected from the biological quality reports that have been carried out by the Duero hydrographic confederation (CHD, http://www.mirame.chduero.es). Since the monitoring started in 2006, this species began to appear in net samples corresponding to May 2010, as well as in the counts of July 2013 and September 2016 campaigns, and in both cases the abundance remained two orders of magnitude below the values detected in this study. In addition, De Hoyos *et al.* (1999) did not mention it as one of the dominant diatoms in their study about the phytoplankton community in their study

which spanned for three years, and, finally Pahissa *et al.* (2015), even showed that diatoms were the dominant group in summer period, it was due to the presence of *Aulacoseira* and *T. flocculosa*. Not only *Asterionella formosa*'s abundance has been totally different to the past studies, also the dominance of diatoms contrasts with some reference works (Aldasoro *et al.*, 1991; De Hoyos, 1996, 1999; Negro *et al.*, 2000) that reflect that diatoms do not seem to have ever dominated the community, maintaining until 2012 low abundances of fundamentally centric

**Table 3.** Main results of the PERMANOVA test to evaluate the effect of the factors Zone and Month on the composition and abundance of taxa, higher taxonomic categories, and concentration of pigments (µmol/L). *P* value that was considered significant $p < 0.05$. *Resultados principales de la prueba PERMANOVA para evaluar el efecto de los factores Zona y Mes para la composición y abundancia de taxones, categorías taxonómicas superiores y concentración de pigmentos (µmol/L).*

| **Composition and abundance of taxa** | | | | |
|---|---|---|---|---|
| Source | Df | MS | Pseudo-F | *P* |
| **Zone** | 2 | 277.44 | 0.9239 | 0.5892 |
| **Month** | 2 | 1323.70 | 5.9465 | 0.0001 |
| **Residual** | 18 | 222.61 | | |
| **Higher taxonomic categories** | | | | |
| Source | Df | MS | Pseudo-F | *P* |
| **Zone** | 2 | 91.179 | 2.1334 | 0.1002 |
| **Month** | 2 | 458.400 | 14.9230 | 0.0001 |
| **Residual** | 18 | 30.717 | | |
| **Pigments** | | | | |
| Source | Df | MS | Pseudo-F | *P* |
| **Zone** | 2 | 4.4175 | 2.6776 | 0.0813 |
| **Month** | 2 | 71.9350 | 73.8060 | 0.0001 |
| **Residual** | 18 | 0.9746 | | |

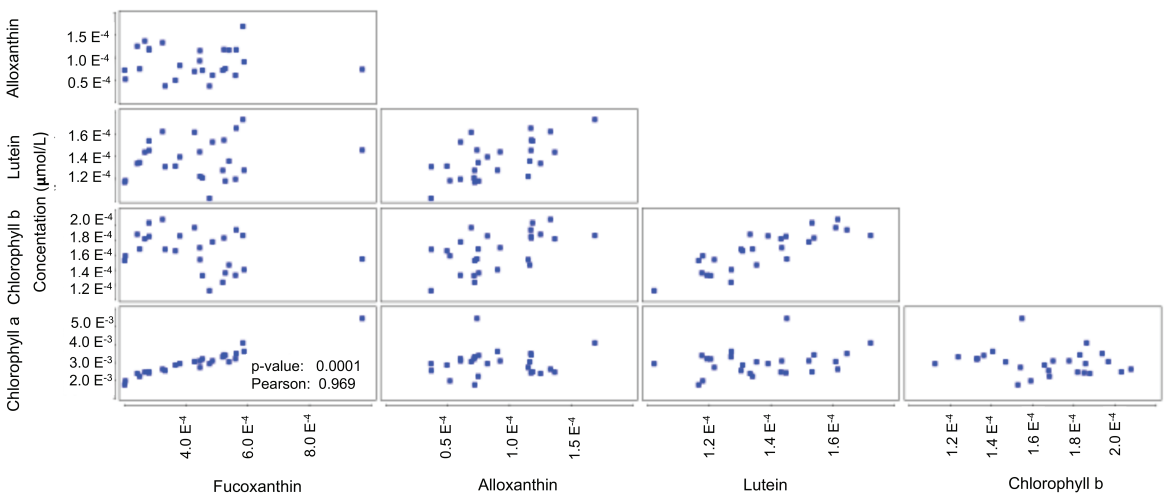


**Figure 4.** Draftsman plot correlations between chlorophyll *a* and the rest of the significant pigments: fucoxanthin, alloxanthin, lutein, and chlorophyll *b*. Pearson correlation and *p* values are shown for the unique case that presented significance; between chlorophyll *a* and fucoxanthin. *Correlaciones Draftsman plot entre la clorofila* a *y el resto de los pigmentos mayoritarios: fucoxantina, aloxantina, luteína y clorofila* b. *Los valores de la correlación de Pearson, así como el* p-*valor se presenta únicamente en la correlación que resultó significativa; entre la clorofila* a *y la fucoxantina.*

species like *Aulacoseira distans* (Ehrenberg) Simonsen or *Cyclotella glomerata* H. Bachmann. However, *A. formosa* has been detected since 90´s in water bodies associated with the lake. In the period 1991-1992, it presented a contribution of more than 0.5 % of the Valparaíso reservoir, located downstream of the lake (Negro *et al.*, 2000). It was also identified in some other small natural water systems in its geological environment (Negro *et al.*, 2003), so translocation phenomena may have happened.

The absence of significant differences for the zone factor during the months of study for both taxonomic and pigmentary analyses suggests spatial homogeneity, qualitatively and quantitatively, in the general structure of the phytoplankton community. Highly contrasting with what was initially expected from the Aldasoro *et al.* (1991) study that proposed a seasonal controlled patchiness for the horizontal distribution of chlorophyll *a*. This homogeneity was slightly altered by the differential appearance of minority taxa, frequently lowly represented in terms of biovolume. In this context, the exception was the exclusive appearance of the centric diatom of 100 µm of diameter in the sample 2C of March. This alga presented a moderate cell/L concentration; however, due to the large average diameter of the individuals, the biovolume that is explanatory for the total system biomass (Felip & Catalan, 2000) obtained very high values, close to those of *Asterionella*. The fact that it only appeared in one of the nine samples of March could imply the necessity of a greater random replication in order to reach a greater degree of knowledge of the structural complexity of the community and, presumably, independently of the contemplated zone factor in this study. Likewise, although the water mixture is intense and complete, minimising the possibilities of niche diversification in the planktonic zone (Jäger *et al.*, 2008), the performed analysis only examined the surface of the water column. Integrated sampling could lead to more faithful results to extrapolate to the whole planktonic area. Regarding the month factor, in all cases, the differences were greater between January and the other two months of study, that presented more similar values. However, for the three performed data analyses, increasing values of biovolume and

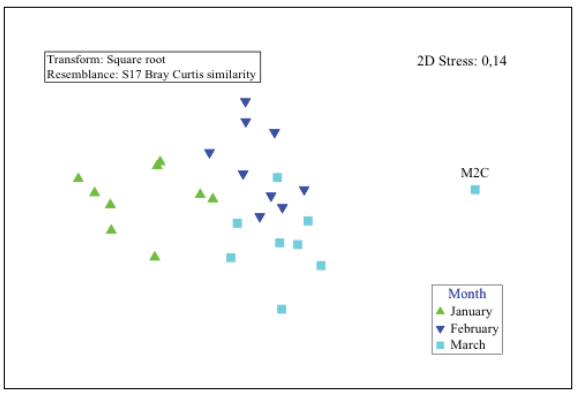


**Figure 5.** Multidimensional scaling for the composition and abundance of taxa. Disposition of samples is shown for the Month factor. M2C = Sample C from zone 2 in March. *Análisis de escalamiento multidimensional para la composición y abundancia de los taxones. La disposición de las muestras se presenta para el factor Mes. M2C = Muestra C de la zona 2 del mes de marzo.*

chlorophyll *a* were detected from January to March, which suggested a temporary change over the mixing period in the system biomass. The observed correlation between chlorophyll *a* and fucoxanthin (Fig. 4) corresponds to the detected increase in *A. formosa*. In contrast to the availability of nutrients offered by water mixing, this fact could point towards the change in solar radiation as the main potential driving factor that would condition this increase in biomass.

Since 2012 a series of unpublished statements, supported by the Douro international biological station (EBI), later collected in the self-published monograph (Guillén, 2015), have been warning of a supposed contamination and eutrophication process of Lake Sanabria. These declarations exposed some supposed symptoms that alluded to a loss of quality in the physical and chemical state of the water, which was mainly identified through a change in water colouration that, as it is affirmed, would have concerned a deficient depuration system of the sewage. This deficiency may have contributed to the excessive growth of *T. fenestrata* and its pronounced dominance until the end of period 2012-2014, when it seemed to drastically remit, supposedly due to a chytrid infestation of this dominant alga. This sudden dominance appeared to be backed up by the

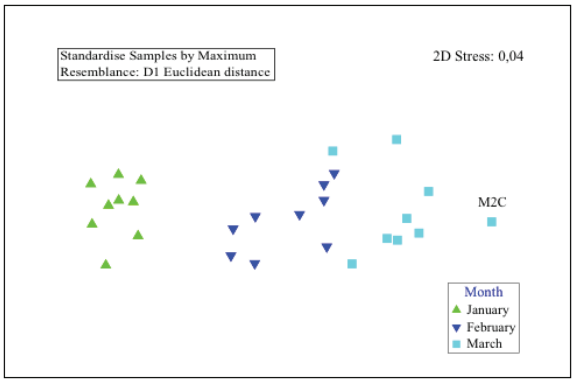


**Figure 6.** Multidimensional scaling for the pigment concentration. Disposition of samples is shown for the Month factor. M2C = Sample C from zone 2 in March. *Análisis de escalamiento multidimensional para la concentración pigmentaria. La disposición de las muestras se presenta para el factor Mes. M2C = Muestra C de la zona 2 del mes de marzo.*

reports of the CHD during this period. Apart from the dominance of *T. fenestrata*, Guillén (2015) points out some other irregularities in the community in comparison to the period 1987-1989 studied by De Hoyos (1996). Among these irregularities during the period 2012-2014, a complete disappearance of chrysophytes and cryptophytes, a negligible presence (less than 1 %) of the chlorophytes (chlorophyceae) and continued presence of euglenophytes were observed. In addition, a lack of presence of conjugated algae, abundant in these acidic water bodies, with the replacement of *S. anatinum* by an indicator of mesotrophy, such as *Staurastrum pingue* Teiling, was also noted. All of these observations would lead to an impoverishment in the biodiversity of the phytoplanktonic community, altering the trophic chains and, eventually, affecting the global ecological quality status of the lake.

As for the general community structure, we did not fully share the observations described in Guillen (2015); in fact, although the dominance of diatoms persists, fundamentally to the detriment of the abundance of green algae, which obviously means that a great change in the general structure of the community has taken place in comparison to the period 1987-1989, the general composition of the rest of the taxonomic groups found in this study remains intermediate between the period 1987-1989 and the period 2012-2014. Despite their low abundance, chrysophytes remained present during the whole study, particularly the genus *Mallomonas* Perty. Cryptophytes are well represented in terms of cell/mL through the genus *Cryptomonas* Eherenberg and the species *P. nannoplanctica*, which holds an average concentration of approximately 30 cell/mL. The chlorophyceae class reaches up to 7 % of the global biovolume average, being the best represented in terms of number of species, the conjugated algae were always present constituting the 4 % of the global biovolume average of the study period, standing out over taxa such us: *Staurodesmus*, S. *planum*, or *S. anatinum*. Finally, we did not identify the presence of euglenophytes. All of these observations could suggest a tendency to the return of the previous conditions after a drastic structural change or a completely new adjustment in the community structure.

About the diagnostic variables measured during this study, pH maintained the expected values, with a slight tendency to acidity, due to the characteristics of the geological environment. Mean conductivity in the lacustrine zone was 12.6 µS/cm, close to the 14.6 µS/cm mean value registered by Vega *et al.* (1992). Global mean of Secchi disk depth, 5.20, remained in the mesotrophic threshold (6-3) (OCDE, 1982), despite not being the most accurate variable due to its inverse relation to turbulence and suspended matter. Mean chlorophyll *a* went from 2.09 µg/L in January to 3.05 µg/L average in March, which is between the oligotrophy and the oligomesotrophy thresholds. As for other relevant data, values of total phosphorus contributed by the CHD presented a concentration of 6.7 µg/L PT in September 2016 and 11.4 µg/L PT in July 2016, values slightly higher than those detected at the origin of these reports of CHD. These values are below the threshold limit of oligotrophy (< 10 µg/L) in September, slightly higher in July, in the transition to the expected values for a mesotrophic system. Despite not being able to provide the annual means as advisable when defining the trophic status, these data do not seem to reflect a notorious process of contamination or eutrophication at present, much less a true loss of the natural state of the lake.

The presence of *A. formosa* and other pennate diatoms has classically been associated with eutrophication processes, thriving when nutrient supply increases (Olsén & Willén, 1980; Reynolds, 2000). In the case of *A. formosa*, some studies relate its increased abundance with an increase in nitrate (Bozniak & Kennedy, 1968) and phosphate levels (Bigler *et al.*, 2007). Nevertheless, this alga can be frequently found in temperate water bodies of different trophic states (Negro *et al.*, 2000; Wyngaert *et al.*, 2015), which may question the diagnostic capabilities of their autoecological features. Bertrand *et al.* (2003) elaborated a detailed study of the responses of *A. formosa* to environmental factors in a reservoir complex, indicating that the main conditioning to the appearance of the species were the morphometric features and the retention time of the system. In addition, they observed a great negative relation of its presence to turbulence and hydrodynamics with preference for lower temperatures (< eight ºC) and low phosphate levels (< one µg/L). Variables not contemplated in this study, such as the characteristics and frequency of the mixing episodes as well as the rate of water renewal could be influencing the dynamics of the community (Lindenschmidt & Chorus, 1998; De Hoyos & Comín, 1999; Bertrand *et al.*, 2003). In fact, comparing the residence times of the water exposed in De Hoyos & Comín (1999), from 1942 to 1992, with the values of the last years (Vega pers. comm.), it can be observed that the residence time is almost double, with 0.76 years as the average of the period 1942-1992 and 1.31 years in the last five years before our sampling. Likewise, timing alterations on the phytoplanktonic dynamics due to extreme inter-annual alterations in the climate, like time series of winter NAO (North Atlantic Oscillation) index could be triggering a response in phytoplankton (Weyhenmeyer *et al.*, 2001, 2002). Most of the lake rainfall is due to Atlantic fronts during winter, which with a relatively high inter-annual variability are strongly controlled by NAO (Visbeck *et al.*, 2001; Trigo *et al.*, 2002, 2004) in this particularly sensitive Northwest Iberian Peninsula (Jambrina-Enriquez *et al.*, 2014). These facts added to the global change frame where drought episodes, change of land use, and fires are becoming more frequent and could be creating a completely new scenario of environmental factors that are to be seriously taken into account.

Overall, a new structure of the phytoplankton community has been observed in Lake Sanabria during the mixing period of 2017, but not substantial changes in the classic and diagnostic variables of the trophic state, that can advise of an alteration of the ecological status compared with the communities described in the 1990's and with those reported for routine monitoring. A homogeneity of the general structure of the community along the period and at the three different selected zones has been noted, discarding the human impact at least in the winter period. We suggest further and continuous monitoring of this important ecosystem and contemplate the effect that other ecological variables such as changes in hydrodynamics and climate change could produce.

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