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Seasonal dynamics of phytoplankton and microbiological communities during sporadic fish die-offs in the Bir M'Cherga reservoir (Tunisia)

Myriam AMMAR [a,b], Katia COMTE [b] & Monia EL BOUR [a]*

[a]Laboratoire de Pathologies des Animaux Aquatiques, Institut National des Sciences et Technologies de la Mer, 2025 Salammbô, Tunisia

[b]UMR 7245 MCAM CNRS-MNHN, Muséum National d'Histoire Naturelle, 12 rue Buffon CP 39, F-75231 Paris Cedex 05, France

Abstract – Proliferations of cyanobacteria are becoming increasingly widespread in many artificial reservoirs, and may have detrimental effects on ecosystem functioning, especially when the water sustains commercial fisheries. This is the case of the Bir M'Cherga reservoir in North Tunisia, where sporadic fish die-offs have been recently reported. A two-year survey investigated a dual-community structure including both phytoplankton and the main fecal bacterial indicators facing to environmental factors.
Low abundances of fecal indicator bacteria were recorded, indicating that no direct human contamination had occurred. However, the ecological status of this reservoir did show signs of degradation, *Bacillariophyceae* being superseded by Cyanobacteria, with a "nearly-exclusive" dominance of *Planktothrix agardhii* lasting several months, in association with *Planktolyngbya limnetica* and *Pseudanabaena limnetica*. In contrast, the rapid decline of *P. agardhii* observed in spring 2007 favored greater phytoplankton diversity, with the summer occurrence of *Cylindrospermopsis raciborskii*. Even though no significant relationships were identified between fish mortality and biological factors, the very presence of two bloom-forming and potentially toxic cyanobacterial species may be viewed as a potentially serious issue with regard to water use and fish farming in the Tunisian reservoir.

Tunisian reservoirs / seasonal dynamics / bloom-forming cyanobacteria / *Planktothrix agardhii*

Résumé – Des phénomènes de proliférations cyanobactériennes s'observent à la surface des écosystèmes limniques, et ce quel que soit le continent étudié. Or ces fortes biomasses engendrent un profond dysfonctionnement de l'hydrosystème, notamment lorsque ces étendues d'eau servent à la pisciculture. Une étude a ainsi été réalisée sur la structure de la double communauté phytoplanctonique et des bactéries fécales en réponse aux conditions environnementales, dans le réservoir de Bir M'Cherga (Tunisie), à la suite de mortalités inexpliquées de poissons.
Une dégradation de l'état écologique des eaux a été confirmée par le remplacement des *Bacillariophyceae* par les Cyanobactéries, avec une dominance quasi-exclusive de *Planktothrix agardhii* pendant plusieurs mois, associée à *Planktolyngbya limnetica* et *Pseudanabaena limnetica*. Un rapide déclin de *P. agardhii* au printemps, a favorisé une augmentation de la diversité phytoplanctonique, avec l'apparition estivale de *Cylindrospermopsis raciborskii*.

* Corresponding author: kcomte@mnhn.fr

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Bien qu'aucune corrélation n'ait pu être directement établie entre ces mortalités épisodiques et les facteurs biologiques, la seule présence d'espèces de cyanobactéries à fort potentiel invasif et toxique justifie une vigilance accrue de la qualité des eaux et du peuplement piscicole dans les eaux du réservoir Bir'M Cherga.

Réservoirs tunisiens / dynamiques saisonnières / cyanobactéries à efflorescences / *Planktothrix agardhii*

INTRODUCTION

Water quality can be affected by a wide range of interacting biotic and abiotic factors acting at different temporal and spatial scales. However, human activities are acknowledged as being the main cause of water quality degradation (*e.g.* Vörösmarty *et al.,* 2010). The local impact of human beings on chemical and microbiological water quality results mainly from the discharge of sewage, and of agricultural, industrial, and urban wastewater. While all the aquatic systems are impacted by increased water use, lakes and/or reservoirs are particularly predisposed to poor water quality as, unlike rivers and estuaries, they do not undergo regular flushing. As a result, nutrients, exogenous charges, and/or pollution entering the water system accumulate and remain there over prolonged periods of time.

Artificial reservoirs, which are generally characterized by a relatively small size, long residence time, and a location in highly urbanized and/or agricultural areas, tend to undergo more rapid and excessive nutrient charges, leading to the eutrophication (Vörösmarty *et al.,* 2010). This deterioration becomes far more inevitable if aquaculture activities (*i.e.* fish farming) are set up in these waterbodies, resulting in an additional increase in endogenous N and P sources (Borges *et al.,* 2010). One of major consequence of this input, is the proliferation of cyanobacteria in eutrophic waters (Scheffer *et al*., 2003) worldwide. The massive occurrence and persistence of these organisms are serious issues as they have bloom-forming abilities, which can impair water quality, increasing its turbidity and particulate matter content, leading to blocked water filters and tainted drinking water (Geoffrey, 2000). In general, cyanobacterial blooms go hand-in-hand with associated disturbances, including tainting of the taste and color of the water, and possible hypoxia and/or anoxia of the water column (Twomey *et al.,* 2002). In addition, some common cyanobacterial species are able to produce toxic compounds such as hepatotoxins (*i.e.* microcystins) or neurotoxins (Chorus & Bartram, 1999), and many other secondary metabolites that can threaten all living organisms in the food web, from ciliates to fish (Combes *et al*., 2013; Ernst *et al*., 2001), and ultimately human health (Chorus & Bartram, 1999; Halstvedt *et al*., 2007).

As a result, water quality monitoring has become a priority for many countries, as it can help to protect water bodies from acute disruption and various forms of contamination (*i.e.* biological, physical, and chemical) and thereby avoid exposing human health to toxin-producing and pathogenic microbes. However, a recent study (Merel *et al.,* 2013) reported the lack of monitoring campaigns in the Eastern Europe and Africa, which could potentially lead to an underestimation of the prevalence of toxic cyanobacterial blooms and the diversity of toxins worldwide.

This is true of countries located on the Southern side of the Mediterranean Sea, such as Tunisia, where thirty artificial reservoirs are currently in use (Ben Mammou & Louati, 2007) for various services, including flood management, supply of domestic water, irrigation, agricultural and industrial activities in its vicinity, and fish farming. These include the Bir M'Cherga reservoir, which was built in 1971 to manage the flood events that periodically threaten the southern parts of Tunis City and to supply water for various needs. It is also the main fish-farming reservoir in Tunisia, which plays a significant role in the national economy (DGPA 2010). Five fish species are farmed in the reservoir, *Cyprinius carpo*, *Mugil cephalus & Liza ramada*, *Barbus callensis* and *Pseudophoxinus callensis* (Djemali, 2005).

However, since 2004, some episodic but unexplained fish die-offs affecting all species indiscriminatingly have been reported, particularly between March and June (El Bour, pers. comm.). While a few studies reported the presence of toxic cyanobacteria species in the M'Bir Reservoir, including the first observation of *Cylindrospermopsis raciborski* (Woloszynska) Seenayya & Subba Raju in Desikachary 1972, in this aquatic system, and the presence of *Planktothrix agardhii* (Gomont) Anagnostidis & Komárek 1988 and *Microcystis aeruginosa* (Kützing) Kützing 1846 (see Fathalli *et al.,* 2010, 2011) during the 2004 to 2005 period, no studies of phytoplankton assemblages or the seasonal dynamics of potentially harmful microbial species and their relationships to various physico-chemical variables have been carried out in this reservoir, despite its ecological and economic importance for Tunisia.

This lack of ecological data is of concern for the environmental management, monitoring, and control of water quality, as subtle environmental changes and/or short-term fluctuations (Wu *et al.,* 2013) may be sufficient to extend the persistence of a bloom-forming species in water, which may increase the explicit risk of potential toxin production depending on the prevalence of the cyanobacterial species involved. Considerable efforts have been undertaken to clarify the environmental stressors that may affect species distribution and flash bloom-forming species, but so far the causal factors (*i.e.* abiotic and biotic) that influence cyanobacterial blooms and toxin production remain largely elusive (Tran *et al.,* 2013) as many factors may act in concert and synergistically affect water worldwide (N'Dong *et al.,* 2014).

This worrying issue is as topical as ever and, indeed, recent studies tend to indicate that, with regard to climate change (*i.e.* global warming), eutrophication and increasing water temperature are two of the main factors promoting the successful expansion of potentially toxic species such as *C. raciborskii* (Paerl & Huisman, 2009) and *P. agardhii* (O'Neil *et al*., 2012). A better understanding of the environmental factors controlling the occurrence of these organisms and changes in the composition of algal communities is clearly required and may help to predict the specific development of harmful species that could compromise water quality and public health.

Consequently, we set out to identify: i) the seasonal dynamics of the twofold biological communities (phytoplankton and major fecal bacterial indicators) and their relationships to standard environmental factors. ii) the possible causal factors including biological and/or chemical variables that could be linked to episodic fish deaths in artificial reservoirs. To the best of our knowledge, this is the first report in which a twofold-community structure (phytoplankton and fecal bacteria) has been investigated in relation to sporadic fish deaths.

MATERIALS AND METHODS

Study site

The Bir M'Cherga reservoir is located near Tunis in the South-Eastern Zaghouan region (36° 30'46" N; 10° 00'46" E) (Fig.1). Built in 1971, the reservoir covers an area of 2000 ha (DG/EGTH, 2005), and has an average depth of 7 m (Djemali, 2005). The water residence time has been estimated to be 0.75 year (Daoud *et al*., 2009). The Bir M'Cherga reservoir is intended to regulate flow in the Oued Miliane and prevent flooding of the low-lying areas of Tunis City. It also provides water to irrigate 1300 ha of agricultural land, and to cater for the needs of domestic

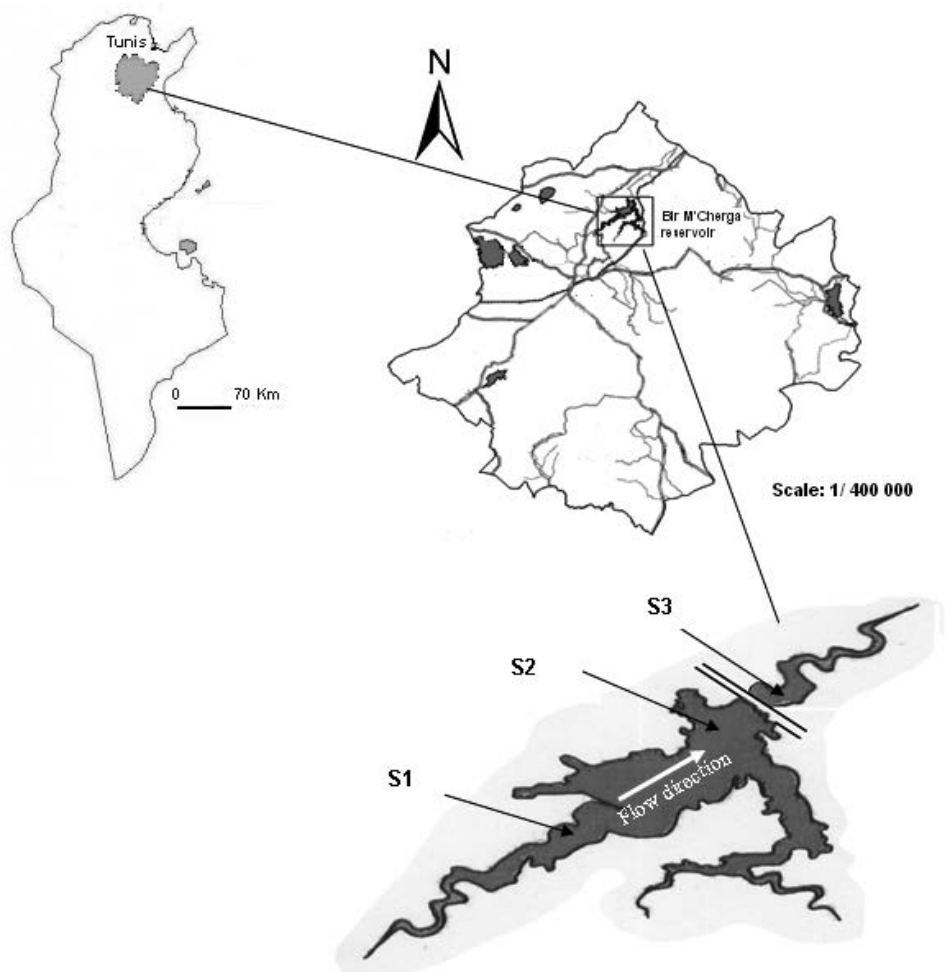


Fig. 1. Study sites of the Bir M'Cherga reservoir and sampling site locations (S1, S2, S3). The arrows indicate the direction of flow.

animals. The watershed (1263 km^2) of the Bir M'Cherga reservoir is mainly rural and has a population of 7203 (INST, 2004).

As in many other Tunisian reservoirs, the amount of sediment present has increased considerably in recent years, rising from 6.2 10^6 to 11.4 10^6 m^3 according to the estimates published from 1987 to 2002 (Ben Mammou & Louati, 2007). This reservoir also constitutes the largest aquaculture reservoir in Tunisia (Djemali, 2005).

Sub-surface samples (depth *c.a.* 30 cm) were collected monthly from February 2007 to January 2009 (n = 24) at three sampling stations (S1, S2, and S3) (Fig. 1). S1 is a shallow station (depth: 1.5 m) located at the entrance to the reservoir (36°28'54"N; 9°58'43"E), S2 is located at the deepest point of the reservoir (depth: 14.5m) (36°30'38"N; 10°00'34"E), and S3 is a shallow station (depth: 2m) located downstream of the dam (36°30'47"N; 10°00'47"E).

Environmental parameters

Average meteorological data was provided monthly (air temperature and global irradiation) or daily (wind speed, and precipitation) by the Zaghouan meteorological station, which is located 20 km north of the reservoir (Tunisian National Institute of Metrology). Average values (over periods of 1, 2, 3, 4 or 5 days or 1 month) of wind speed and precipitation were calculated to provide integrated daily values prior to sampling.

Water temperature, oxygen saturation, pH, conductivity, and salinity were measured *in situ* using a multi-parameter probe (MultiLine P4 SET fitted with the SenTix 41, CellOx 325 and TetraCon 325 probes; WTW, Weilheim, Germany). Water samples for the determination of the concentrations of nutrients and chlorophyll *a*, and the phytoplankton community composition were collected in acid-washed sampling bottles, and stored at 4°C prior to analysis.

Samples used to determine dissolved inorganic nutrients (NO_3^--N, NO_2^--N, PO_4^{3-}-P, NH^{4+}-N, $Si(OH)_4$) were filtered through GF/F fiberglass filters (Whatman, Maidstone, England), stored at –20°C and analyzed colorimetrically using an Autoanalyzer 3 (Bran+Luebbe, Norderstedt, Germany). Ammonium (NH^{4+}-N), nitrite (NO_2^--N) and nitrate (NO_3^--N) were measured according to Koroleff (1976), Benschneider & Robinson (1952) and Wood *et al.* (1967), respectively. The silica ($Si(OH)_4$) concentration was determined following Mullin & Riley (1955). Orthophosphate (PO_4^{3-}-P) was analyzed according to the method of Koroleff (1976). The detection limits were 0.01 µM for NO_2^--N and PO_4^{3-}-P, 0.05 µM for NH^{4+}-N and $Si(OH)_4$, and 0.1 µM for NO_3^--N.

Biotic parameters

The phytoplankton community

Chlorophyll *a* concentrations were determined by filtering 300 mL of raw water (GF/C filters, Whatman, Maidstone, England) followed by acetone extraction (Lorenzen, 1967) and subsequent spectrophotometric measurements using a UV-visible spectrophotometer (Jenway 6705, Jenway, UK).

For phytoplankton identification and counting, 30 mL of water were immediately fixed using 5% (v/v) formaldehyde. Phytoplankton species were identified using an Optiphot 2 light microscope (Nikon Instruments Inc, Melville,

USA) and standard taxonomic keys (Komárek & Anagnostidis, 1998; Komárek & Anagnostidis, 2005). Taxon abundances were determined by the Utermöhl method (1958) on the basis of 400 cell counts.

Species biovolumes were estimated according to the Dia & Reynaud (1982) and Sun & Liu (2003) calculation formulae**,** using a microscope coupled to a Digital Sight DS-L1 image acquisition system (Nikon Inc). Measurements were performed on at least 30 distinct individuals. Phytoplankton diversity was estimated using the Shannon index (Shannon & Weaver, 1963).

Bacteriological analysis

Water samples were collected using sterile bottles and stored at 4°C. Counts of cultivable heterotrophic bacteria were performed using the Standard Plate Count Agar (PCA) method after incubating for 48 h at 37 ± 0.5°C (American Public Health Association, 1980). Fecal bacterial abundances (total coliforms and fecal enterococci) were estimated following the Most Probable Number (MPN) method (Olson, 1978) modified by Sabatini *et al.* (2004). The samples were incubated for 48 h at 37 ± 0.5°C. To estimate *Vibrionaceae* abundances, 0.1 mL of raw water was taken and inoculated onto a thiosulfate citrate bile salt sucrose (TCBS) solid medium (88.1 $g \cdot L^{-1}$; see Bolinches *et al.* 1988), and incubated at 37 ± 0.5°C for 48 h. The yellow or green colonies of *Vibrionaceae* formed were then counted.

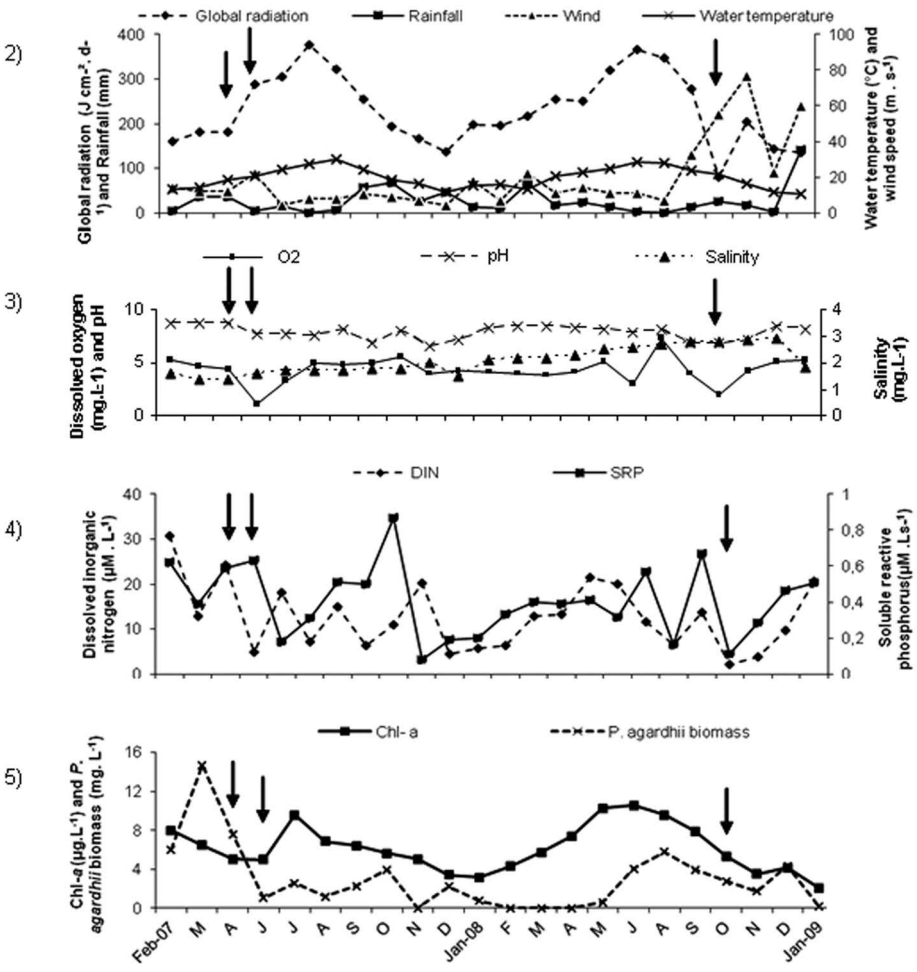
Statistical analyses

As the data (*i.e.* environmental and biotic variables) were not all normally distributed (previously checked for all datasets), the non-parametric tests were performed on Statview III software (Roth *et al.,* 1995). Spearman's correlation was run to determine the relationships between environmental variables and the main phytoplankton and fecal bacterial species. In addition, environmental variations between the three stations (the between-station effect) were assessed by the Friedman test.

RESULTS

Environmental parameters

Meteorological conditions including rainfall, wind speed, total radiation and air T° (Fig. 2) were typical of a Mediterranean climate, including low rainfall (Table S1), dry seasons (except in January 2009), and a rather warm air T° throughout the years (mean values of 18.9°C) linked to the fluctuations in the overall radiation (Fig. 2). The wind speed reached a mean value of 12 m s^{-1} with a higher value in the winter of 2008, sufficient to produce mixing of the entire water column and to resuspend sediments during these months. Table 1 reveals some significant differences between the environmental parameters at the three stations, with a higher nutrient charge (*i.e.* Total N, NO_2, NO_3 and P), higher salinity and a lower pH at S1, than at the other two. As sporadic fish deaths were recorded exclusively in the reservoir (*i.e.* S2 and S3), S1 was not included in the further detailed chemical analysis (data



Figs 2-5. Seasonal dynamics of the main physicochemical variables (**2, 3, 4**) and Chlorophyll *a* and *P. agardhii* biomass (**5**) in M'Bir reservoir (mean of the 2 study stations). The vertical arrows indicate the sporadic fish deaths detected during the 2007-2009 period.

not shown). Figures (Figs 2-5) show the temporal fluctuations of main several abiotic factors. Briefly, the mean concentrations of dissolved inorganic nitrogen (DIN), consisted mainly of NO_3^--N at both stations, ranging from 4 to 28.0 µM, with the highest values observed during the winters (Fig. 4). The SRP concentration (*i.e.* soluble reactive phosphorus) showed successive fluctuations that appeared to follow rain events, but after a time lag which was not determined here (Fig. 4). Oxygen concentrations were below 2 $mg \cdot L^{-1}$ during two months (June 2007 and October 2008), suggesting that the water was hypoxic with potential anoxia of the water column (Fig. 3).

Table 1. Environmental differences between the three stations in the M'Bir Cherga reservoir from February 2007 to January 2009. The station effect was analyzed using the Friedman test analysis (n = 22 sampling dates).

Factors	Source of variation *(between-station)*			Df	F	*P* value
	S1	*S2*	*S3*			
Water temperature (°C)	20.1[a]±6.47	19.7±5.85	19.3±5.85	2	1.226	0.553
pH	7.6±0.73	7.9±0.65	7.7±0.69	2	25	<0.001***
Salinity ($mg \cdot L^{-1}$)	2.9±0.92	2.1±0.51	2.0±0.50	2	16.021	<0.001***
O2 conc. ($mg \cdot L^{-1}$)	5.0±1.42	4.4±1.23	4.3±1.04	2	9.116	0.0105*
$N\text{-}NH^{4+}$ (µM)	5.80±4.72	5.41±4.32	5.27±3.87	2	5.33	0.069
$N\text{-}NO_3^-$ (µM)	19.8±23.7	6.50±5.27	11.01±13.8	2	11.053	0.004**
$N\text{-}NO_2^-$ (µM)	2.31±2.71	0.69±0.43	1.15±1.44	2	12.944	0.0015**
Total nitrogen (µM)	49.5±45.5	27.70±12.07	33.60±18.70	2	25.083	<0.001***
SRP (µM)	0.48±0.28	0.40±0.20	0.46±0.23	2	2.883	0.236
Total phosphorus (µM)	7.58±18.27	3.45±1.81	3.48±0.99	2	7.186	0.027*
Silica (µM)	10.1±9.13	9.35 ±10.73	10.3±10.05	2	4.587	0.1009
Chlorophyll a ($\mu g\ L^{-1}$)	6.24±3.01	6.30±2.26	7.70±4.36	2	4.085	0.129

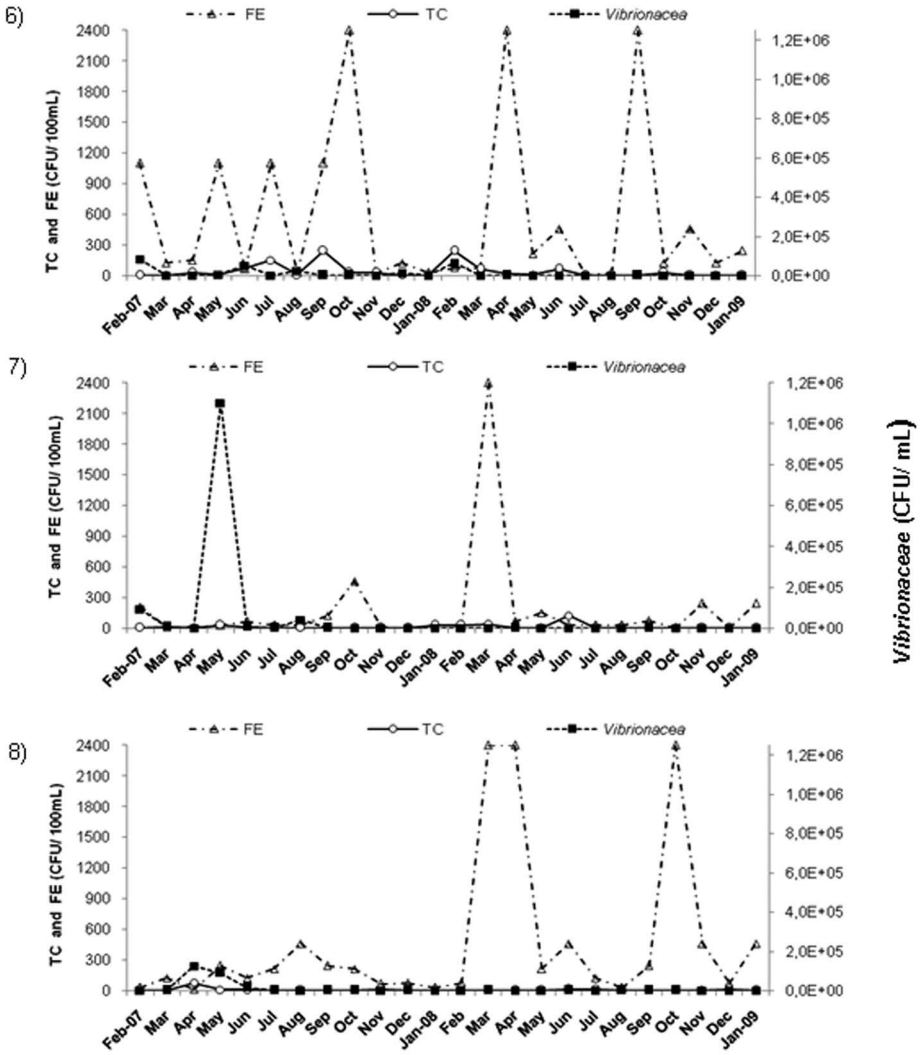
a = Mean values (± SD) were calculated on n=24 months. Df= Degree of freedom; F= Friedman's chi-squared test. The statistical significances are indicated with asterisks: ***p<0.001, **p<0.01, *p<0.05.

Fecal indicator bacteria and cultivable heterotrophic bacteria

Temporal fluctuations of the main fecal bacteria are shown in Figures (Figs 6-8), for each station, including the higher oscillation at S1, which was the most eutrophic site of the study. Vibrionaceae were detected at all stations, but not at all sampling dates. Their abundance was particularly high in spring 2007, with an additional peak in winter 2008 at S1 (Fig. 6). A high amplitude of the cultivable heterotrophic bacteria populations was observed throughout the 2-year survey, with an increased abundance from S1 ($3\ 10^5$ CFU mL^{-1}) to S3 ($8.8\ 10^5$ CFU mL^{-1}). Both these bacterial populations were moderately correlated to physical parameters (*i.e.* positively to T°, light and negatively to wind) and the main nutrients (*i.e.* DIN, NO_2-Cf. Table 2). In contrast, the Total Coliform (TC) abundances were low, ranging from 3 to 240 CFU per 100 mL, with a higher level at S3 (Fig. 8), which was moderately correlated to NO_2 (Table 2). While, the fecal enterococci (FE) displayed their highest abundances (up to 2400 CFU per 100 mL), after some high rainfall events (October 2007, March-May 2007, October 2008) and were significantly correlated to wind (Table 2).

Seasonal dynamics and diversity of phytoplankton

The overall phytoplankton growth, expressed by the chlorophyll *a* concentration, showed similar patterns throughout the investigated period at all three stations (*i.e.* $p > 0.05$ - Table1) with lowest values (2.1 to 2.6 $\mu g\ L^{-1}$) during the winters and highest values (10.5 to 19.3 $\mu g\ L^{-1}$) during the summers (Table S1 and Fig. 5). Not surprisingly, the chlorophyll *a* concentration was positively correlated



Figs 6-8. Seasonal dynamics of main fecal bacterial indicators, including the total coliforms (TC), fecal enterococci (FE) and Vibrionacea bacteria at the three stations station (**6**: st. a; **7**: st. b; **8**: st. c).

to the total radiation (Table 2) as higher irradiance is known to favor phytoplankton growth, was moderately correlated to air T°, pH and Si (OH), and negatively correlated to precipitation (which occurred mainly in winter 2008 - Cf. Table 2).

The total phytoplankton community in the Bir M'Cherga reservoir was composed of 62 taxa belonging to 51 genera within four taxonomic groups: the Cyanobacteria, *Bacillariophyceae*, *Chlorophyceae* and *Euglenophyceae* (Table S2). The *Bacillariophyceae* were the most diverse group (32 taxa), followed by the *Chlorophyceae* (15 taxa), the Cyanobacteria (12 taxa) and the *Euglenophyceae*, (3 taxa). *Bacillariophyceae* and Cyanobacteria biomasses were inversely correlated

Table 2. Spearman correlation coefficient values and significances between phytoplankton, bacteria and the main environmental variables

	Precip.	*Air T°*	*Wind*	*Light*	*pH*	*Water T°*	*Salinity*	*Si(OH)*	*NO2*	*NO3*	*DIN*	*TP*
Chl a	−0,328**	0,625***		0,645***	0,25*			0,49***				
Dominant species (%)												
Planktothrix agardhii							−0,46***					
Planktolyngbya sp.		0,25*					−0,27*		−0,38**	−0,26*		
Pseudanabaena limnetica					−0,26**				−0,25**	−0,28**	−0,24*	−0,18*
Pseudanabaenacatenata			0,60***				0,47**					
Cylindrospermopsis raciborski		0,46*				0,43*		0,44*				
Bacteria												
Total Bacteria (cells/ml)		0,34**	−0,40**			0,35**	−0,25*		0,30*		0,37**	0,29*
Vibrionaceae		0,43**	−0,25*	0,37**		0,35*						
Total coliforms									0,38**			
Fecal Enterococci			0,33*									

[a] Only the significant differences are noted in the table and indicated with asterisks: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$. The analysis was run from February 2007 to January 2009 at three stations (n=66).

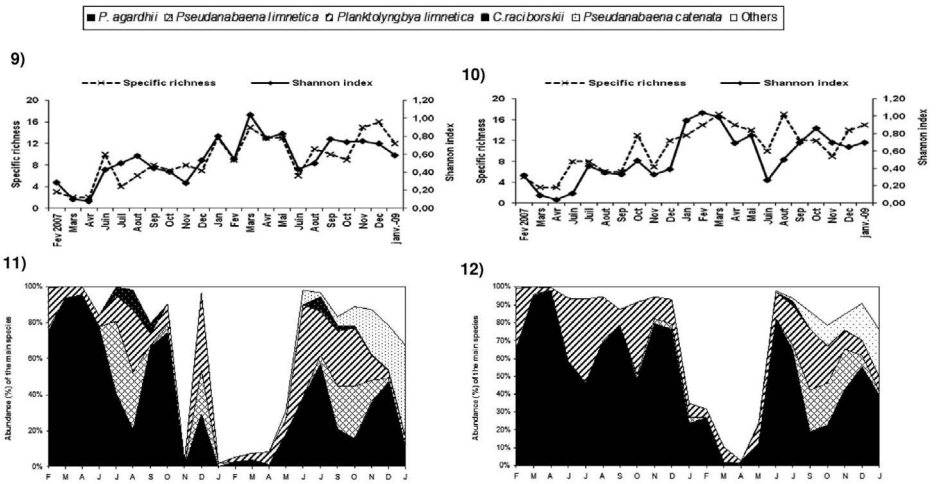
Table S1. Physical, chemical and biological characteristics of the Bir M'Cherga reservoir from February 2007 to January 2009.

	S1				S2				S3			
	Min	**Max**	**Mean**	**CV%**	**Min**	**Max**	**Mean**	**CV%**	**Min**	**Max**	**Mean**	**CV%**
Water temperature (°C)	11.1	30.0	**20.1**	32	10.5	30.0	**19.7**	30	10.6	29.1	**19.3**	30
pH	5.2	8.4	**7.6**	10	6.6	8.7	**7.9**	8	6.0	8.6	**7.7**	9
Salinity (mg·L^{-1})	1.4	4.9	**2.9**	31	1.4	2.9	**2.1**	25	1.4	2.9	**2.0**	24
O2 concentration (mg·L^{-1})	2.0	8.5	**5.0**	28	1.2	7.3	**4.4**	80	2.1	6.8	**4.3**	24
N-NH_4^+ (µM)	0.41	16.31	**5.80**	81	0.13	15.48	**5.41**	80	0.40	15.26	**5.27**	73
N-NO_3^- (µM)	0.30	92.35	**19.88**	119	0.70	24.22	**6.50**	81	0.45	57.52	**11.01**	126
N-NO_2^- (µM)	0.05	11.63	**2.31**	117	0.13	1.98	**0.69**	62	0.18	7.36	**1.15**	125
Total nitrogen (µM)	15.80	206.00	**49.50**	92	15.30	71.30	**27.70**	44	17.50	91.80	**33.60**	56
SRP (µM)	0.08	1.24	**0.48**	59	0.08	0.87	**0.40**	49	0.09	0.81	**0.46**	49
Total phosphorus (µM)	1.16	93.00	**7.58**	241	1.03	8.49	**3.45**	52	1.48	5.67	**3.48**	28
Silica (µM)	0.41	33.29	**10.16**	90	0.35	51.92	**9.35**	115	0.46	48.06	**10.38**	97
Secchi depth (m)	–	–	**–**	–	0.25	1.40	**0.71**	42	–	–	**–**	–
Chlorophyll a (µg L^{-1})	2.35	14.26	**6.24**	48	2.06	10.55	**6.30**	38	2.59	19.32	**7.70**	57
Phytoplankton biomass (mg·L^{-1})	0.25	36.40	**4.20**	244	0.27	14.80	**3.70**	91	0.24	23.93	**3.88**	134
Cyanobacteria biomass (mg·L^{-1})	0.00	3.10	**0.14**	466	0.00	14.80	**3.43**	38	0.00	23.93	**3.64**	146
Bacillariophyceae biomass (mg·L^{-1})	0.25	36.32	**3.09**	244	0.00	1.37	**0.21**	175	0.00	2.45	**0.20**	280
Chlorophyceae biomass (mg·L^{-1})	0.00	0.91	**0.07**	255	0.00	0.41	**0.05**	239	0.00	0.37	**0.04**	222
Euglenophyceae biomass (mg·L^{-1})	0.00	12.21	**0.88**	317	–	–	**–**	–	–	–	**–**	–
Shannon index	0.27	0.92	**0.63**	28	0.08	1.04	**0.55**	43	0.04	1.04	**0.53**	55
Total cultivable bacteria (CFU mL^{-1})	1.6 10^1	5.1. 10^6	**3.110^5**	336	1.5 10^1	1.0 10^7	**8.6 10^5**	280	1.7 10^2	1.0 10^7	**8.8 10^5**	273
Vibrionaceae (CFU mL^{-1})	0	81500	**9879**	227	0	11.10^5	**51879**	432	0	123500	**9577**	316
Total coliforms (CFU 100 mL^{-1})	3	240	**42**	165	3	120	**13**	188	3	75	**8**	183
Fecal enterococci (CFU 100 mL^{-1})	4	2400	**580**	168	3	2400	**178**	272	11	2400	**464**	164

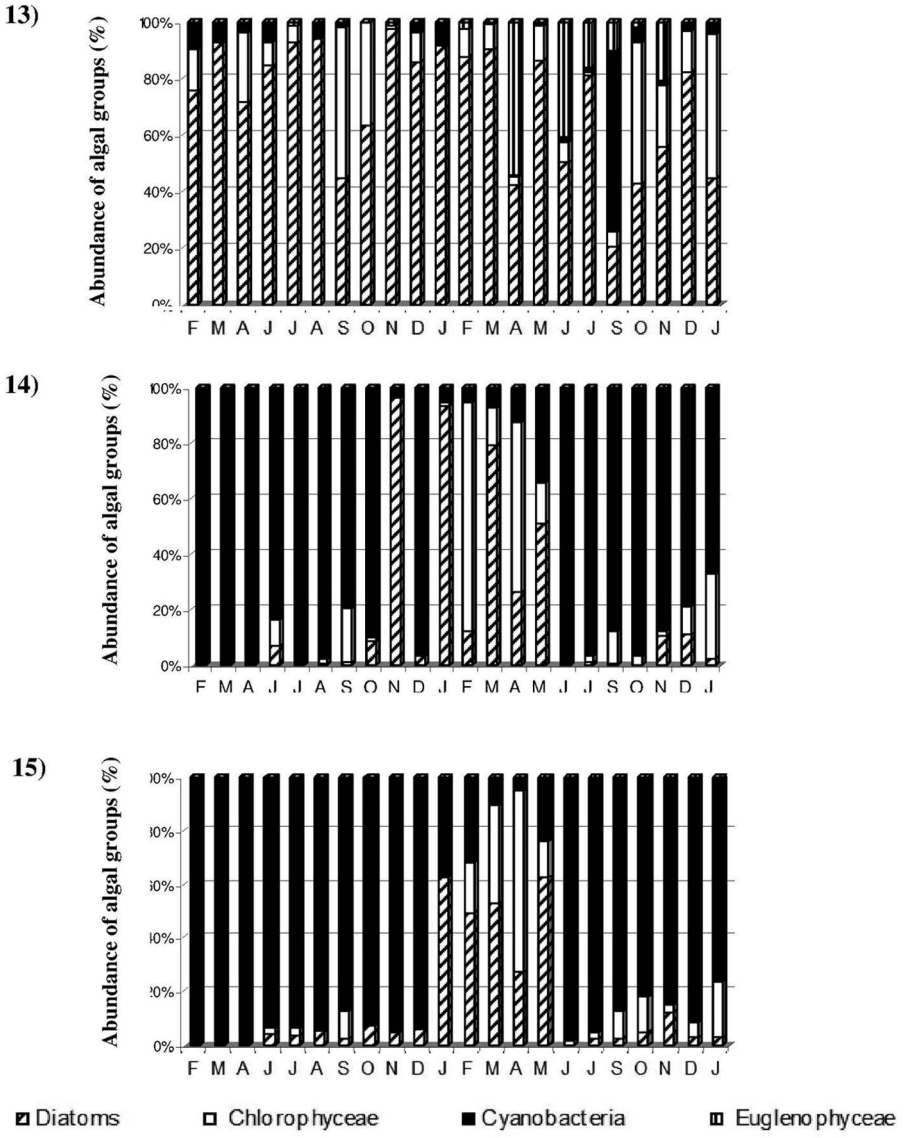
Table S2. List of phytoplanktonic taxa present, with the number of times (N) they occurred, at each study station in the M'Cherga Reservoir during the study period.

	Station 1		Station 2		Station 3	
Cyanobacteria (number of taxa)	7	N	10	N	12	N
Chroococcus sp	×	2	×	3	×	2
Coelomeron sp			×	2	×	2
Cylindrospermopsis raciborskii			×	9	×	5
Glaucospira sp					×	1
Limnothrix sp			×	1	×	1
Oscillatoria sp	×	1	×	1	×	2
Planktolyngbya limnitica	×	8	×	21	×	22
Planktothrix agardhii	×	18	×	21	×	22
Pseudanabaena catenata	×	3	×	8	×	8
Pseudanabaena limnitica	×	4	×	13	×	14
Raphidiopsis sp					×	1
Snowella sp	×	1	×	4	×	3
Euglenophyceae (number of taxa)	3		0		2	
Euglena sp	×	10			×	2
Lepocinclis sp	×	4			×	1
Phacus sp	×	1				
Chlorophyceae (number of taxa)	10		12		12	
Actinastrum sp					×	1
Actinotaenium sp			×	1		
Closteriopsis sp	×	5	×	12	×	12
Closterium navicula	×	1	×	1	×	2
Closterium sp1	×	14	×	3	×	6
Closterium sp2	×	2			×	3
Closterium sp3			×	2	×	2
Cosmarium sp	×	3	×	3	×	3
Goniochloris sp					×	1
Kirchneriella sp	×	7	×	10	×	10
Monoraphidium sp	×	2	×	6	×	7
Pediastrum sp			×	1		
Scenedesmus sp1	×	4	×	4	×	2
Scenedesmus sp2	×	2	×	5	×	5
Spirogyra sp	×	3	×	1		
Bacillariophyceae (number of taxa)	25		20		18	
Achnanthes sp	×	7	×	5	×	7
Actinastrum	×	1	×	1		
Amphipleura sp	×	18	×	4	×	7
Amphiprora sp	×	13	×	1	×	6
Ankistrodesmus sp	×	1				
Caloneis sp	×	4				
Chaetoceros sp	×	1				
Chaetoceros sp			×	1		

	Station 1		Station 2		Station 3	
Cyclotella sp	×	17	×	12	×	13
Cymatopleura sp	×	1				
Cymbella sp	×	1			×	1
Diploneis sp	×	2				
Gomphonema sp	×	13	×	5	×	2
Gyrosigma sp	×	4	×	1	×	6
Melosira sp	×	1				
Navicula renharditii	×	1				
Navicula sp1	×	16	×	12	×	13
Navicula sp2	×	11	×	1	×	7
Navicula sp3	×	2	×	2	×	1
Nitzschia sp1	×	19	×	8	×	10
Nitzschia sp2	×	8	×	5	×	6
Pinnularia sp	×	15	×	5	×	6
Planctonica sp			×	1		
Pleurosigma sp	×	1			×	1
Siderocytopsis sp			×	1		
Stenopterobia sp					×	1
Stephanodiscus sp			×	1		
Surirella sp	×	2	×	1	×	2
Taxa sp1			×	3	×	6
Thalassiosira sp	×	2			×	1
Trebouxia sp			×	1		
Vanheurckia sp	×	1				
Number of taxa per station	45		42		44	



Figs 9-12. Seasonal dynamics of the five main cyanobacterial species, including the Shannon index and seasonal fluctuations of specific richness (SR) (**9, 10**), within the total phytoplankton community (%) (**11, 12**) in the Bir M'Cherga reservoir (stations 2 and 3).



Figs 13-15. Seasonal distribution (%) of algal groups at each station (**13**: st. a; **14**: st. b; **15**: st. c) of Bir M'Cherga Reservoir

($r = -0.874$, $p < 0.001$), while diatoms were positively correlated to the *Chlorophyceae* ($r = 0.274$, $p < 0.001$) and *Euglenophyceae* ($r = 0.475$, $p < 0.01$).

However, when we looked at the number of times (N) each species was detected monthly, we found that very few species were present at all the sampling dates (Table S2). Only 2 cyanobacterial species were recorded in all samples at stations 2 and 3 (*P. agardhii* & *P. limnetica*). More than 70% of species were rarely

detected, being detected < 5 times, while a few species (10%) were found in the same range as the common taxa. Besides, the Shannon index (Figs 9-10) was rather low, averaging no more than 1.2 over the two-year period. The highest value (1.04) being found in early spring 2008, when *Bacillariophyceae* dominated the phytoplankton community, while the lowest H' values were obtained in March-April 2007, and coincided with the dominance of *Planktothrix agardhii* (Figs 11-12).

In fact, the algal community differed significantly at the three stations. While *Bacillariophyceae* ($biomass_{mean}$ = 3.1 $mg \cdot L^{-1}$), dominated at S1 all year round (except in Sept. 2008) and accounted for 85% of the total phytoplanktonic abundance (Fig. 13); Cyanobacteria were dominant at S2 ($biomass_{mean}$ = 3.4 $mg \cdot L^{-1}$) and St3 ($biomass_{mean}$ = 3.6 $\cdot mg \cdot L^{-1}$), reaching 74% and 81% of the total phytoplanktonic abundance (Figs 14-15 and Table S1), respectively. Five main cyanobacterial species were recurrent and often frequently detected; *Planktothrix agardhii* was the most abundant cyanobacterial species and constituted up to 99% of total phytoplankton abundance in March-April 2007 (Figs 11-12). A contrasting pattern of abundance of this species was nevertheless noted within the both annual cycles, from February to May in the pelagic part of the reservoir (Figs 11-12). Additionally, four filamentous species displayed a seasonal pattern, and co-occurred with successive peaks of *Cylindrospermopsis raciborskii* (11.8%) in August 2007, of *Planktolyngbya limnetica* (Lemmermann) Komárková-Legnerová et Cronberg, 1992 (100%) in April 2008, of *Pseudanabaena limnetica* (Lemmermann) Komárek 1974 (21.6%) occurring in October 2008, and of *Pseudanabaena catenata* Lauterborn 1915 (56.2%) in January 2009 (Figs 11-12). The correlation between the dynamics of the dominant species and environmental variations revealed that *C. raciborskii* was positively correlated to the air T° and water T° and appeared during the warmer months (Table 2). *Pseudanabaena catenata* was positively correlated to wind and salinity; *P. limnetica* was negatively correlated to pH and nutrients; while *Planktolyngbya limnetica* was positively correlated to air T° and negatively correlated to salinity and higher nutrient charges. Meanwhile, *P. agardhii* abundance was negatively correlated to salinity and NO_2, displaying low abundance at St1 which among other factors, displayed higher salinity throughout the year (Table S1).

Episodic fish die-offs

The three episodic fish die-offs, recorded in April and June 2007, and October 2008, were not season-specific. Indeed, no identifiable physicochemical nor biological (*i.e.* toxin, high biomass) variable could be linked specifically to any of these three events, except that for two of them, a strong decrease of O_2 was recorded at this time (Fig. 3). The O_2 value fell to 0.95 $mg \cdot L^{-1}$ during June 2008 and <1.5 $mg \cdot L^{-1}$ in October 2008, indicating active respiration processes and potential anoxia of the water column. Furthermore this marked reduction, (to below 2 $mg \cdot L^{-1}$) coincided with a significant decrease of *Planktothrix* biomass in June 2008 (Figs 3-5); which may reflect active degradation of organic matter and decomposition by bacterial activities linked to the bloom die-offs. However, the cell disruption of *Planktothrix* was not correlated to any positive detection of microcystins in the water by the PP2A method (data not shown).

Besides, no explanation can be provided for the first fish death event recorded in April 2007, on the basis of either physicochemical variables (no decrease in O_2) (Fig. 3) or toxin detection (the field samples screened for MC and CYN were always negative throughout the 2-year survey), which suggested that additional factors (not investigated here) may be involved in these sporadic phenomena.

DISCUSSION

In this study, cultivable heterotrophic bacteria, TC, FE and *Vibrionaceae*, all revealed marked seasonal fluctuations and irregular patterns of occurrence at the different stations, especially at S1, which had the highest nutrient loads of N and P. The low TC concentrations decreased from the reservoir inlet (S1) to its outlet (S3), indicating that fecal pollution originating from the main treated sewage effluent of El Fahs City (population 20,000), located 20 km upstream of the Bir M'Cherga reservoir, was limited as would be expected for a reservoir located in a sparsely-populated area with agricultural land in its vicinity (Mehaffey *et al.,* 2005). Generally, the concentrations of fecal indicators were in the lower range of values reported in the literature (Brugger *et al*., 2001; Wang *et al.,* 2010) and compared to other subtropical areas (Troussellier *et al*., 2004; Hong *et al.,* 2010). Consequently, the water of Bir M'Cherga cannot be considered to be severely contaminated by enteric bacteria. In contrast, *Vibrionaceae* abundances were high compared to the values reported in the literature for waterbodies (Blanch *et al.,* 2001; Eja *et al.,* 2008; Boukef *et al.,* 2010), but these high values have never coincided with fish kills. The *Vibrionaceae* abundances here, were moderately correlated to physical conditions (*i.e.* air T°, water T° and light), which corroborated the findings of Turner *et al.* (2009), who found that temperature and light intensity (Boulek *et al*., 2010) act as main significant seasonal controlling factors in *Vibrionaceae* dynamics.

The temporal dynamics of phytoplankton assemblages from 2007 to 2009, revealed three recurrent cyanobacteria: *Planktothrix agardhii, Planktolyngbya limnitica* and *Pseudanabaena limnetica*, with a "nearly-exclusive" dominance of *P. agardhii* from Feb. to May 2007. However, during this period, the total phytoplankton biomass expressed as chlorophyll *a* values never exceeded 19.32 µg/l, unlike the study of Ismael *et al.* (2010) who noted as high as 33 to 76 µg/l values in this reservoir from May to October 2009. Although previous studies had tended to focus on the presence/absence of phytoplankton species, rather than on the seasonal succession of algal species (cf Fathalli *et al.,* 2010, 2011; Ben Rejeb *et al.,* 2006, 2012), these data included *P. agardhii* as part of the flora present, but not as a dominant species in these waterbodies. In the last report of Ben Rejeb *et al.* (2012), the *Limnothrix* genus was dominant and accounted for 50 to 99% in these waters, whereas in our study, *Limnothrix* sp. occurred sporadically during the 2-year survey, as secondary or rare species (not exceeding 2.5% of total phytoplankton). Thus, the Oscillatoriales were dominant in both studies, but the blooming species present differed. Additionally, in Ben Rejeb's report, the cyanobacterial group accounted for 36% of the total phytoplankton, while our latest findings suggest an increased abundance of up to 99% of cyanobacteria, at least during winter-spring 2007 (cf. Results). Consequently, our data reveal some profound changes in the phytoplankton communities with a recent and successful colonization of *P. agardhii* during all the seasons except the winter of 2008, which allowed other genera to compete and to co-occur such as *Pseudanabaena* and *Planktolyngbya* and Diatoms (*i.e. Navicula sp*.). It was not possible by a statistical approach (*i.e.* correlation or multivariate analyses - data not shown) to distinguish any specific factors that were drivers of *P. agardhii* blooms, or any that were merely correlated to its rapid decline during a single annual cycle (in contrast to the second cycle), which highlighted the difficulty of predicting the sudden appearance of a cyanobacterial bloom in water, as many factors may have interactive effects on cyanobacterial growth and, consequently, on potential toxin production (N'Dong *et al.,* 2014).

The presence of *Cylindrospermopsis* in this reservoir was initially reported by Fathalli *et al.* (2011). Our findings revealed the prevalence of *C. raciborski* during the summer time, reaching 12% of total phytoplankton, which was not surprising in the warmest season, as this species was originally known as tropical species (Saker *et al.,* 2003), with an extended distribution into areas with a temperate climate (France, Briand *et al.,* 2002; Italy, Messino *et al*., 2009; Portugal, Saker *et al.,* 2003; Germany, Mischke, 2003).

However, despite the prevalence of *P. agardhii* and the occurrence of *C. raciborski* in the phytoplankton community, no microcystin nor cylindrospermopsin (=CYN) were detected in the field samples. Similar findings have already been reported for other African waterbodies (Guiers Lake, Senegal, Berger *et al.,* 2006; Tunisian reservoirs, Fathalli *et al.,* 2011), where no CYN was detected despite the presence of *C. raciborski* in the field samples. Furthermore, we have shown that other potentially toxic cyanobacteria, such as *C. raciborski* and *Microcystis aeruginosa,* occurred simultaneously in this reservoir and other species, such as *Pseudanabaena sp.* and *Planktolyngbya* sp., which have not been firmly confirmed to be toxic. So far, most worldwide research has focused on two major cyanotoxins (saxitoxins and MCs) and many other toxins are ignored (Merel *et al.,* 2013).

Overall, a great variability in succession and occurrence of phytoplankton species was observed since 2004, when the first fish deaths were reported. It seemed however, that neither the dominance of *Planktothrix*, nor the absence of cyanotoxins was the cause of these episodic flash deaths. Other factors may be responsible for fish deaths, such as low values of dissolved oxygen, and hypoxic water (defined less than 2 $mg \cdot L^{-1}$ O_2), which can lead to the asphyxia of organisms, especially fish, which have maximum O_2 requirements (Twomey *et al.,* 2002). In our study, hypoxic water was recorded during at least two fish death events out of three, with some O_2 levels of less than 1 $mg \cdot L^{-1}$ (lower than those mentioned in Ismael *et al.,* 2010). We have to keep in mind that all "*in situ*" field measurements were performed during the daytime, (*i.e.* when the photosynthesis process was at a maximum), meaning that during the night, we could have expected to find complete anoxia of the water due to two oxygen demanding processes (*i.e.* respiration and decomposition). A higher resolution forecast would be required (*i.e.* daily overnight) to confirm this hypothesis. Furthermore, one depletion of O2 (June 2007) coincided with a strong decrease in the *Planktothrix* biomass, which may have been explained by a massive degradation of this biomass by bacteria, leading to an increase demand for O2 as a result of microbial activities, as suggested by Twomey *et al.,* (2002). We cannot exclude the possibility that other factors, which were not studied in this study, may have different effects on fish development and survival, including gill clogging, pathogens, viruses, protozoan parasites and high pesticide levels. Further investigations would have to be carried out on tissues samples taken immediately after the fish deaths, to identify possible causes of death.

Finally, although not directly involved in the episodic fish kills, it is important regularly to check the temporal dynamics of these Oscillatoriales species, especially *P. agardhii,* in these waterbodies, as it is well known that this species is a bloom-forming one with potential toxicity (Keil *et al.,* 2002). Its fast-growing ability, which has been confirmed both by *in-vivo* studies (Ammar *et al.,* 2014) and *in-situ* investigations (Bonilla *et al.,* 2012), may lead to a worrying imbalance in the water system and the disruption of the functioning of the whole ecosystem, as a high cyanobacterial biomass can have harmful effects, *i.e.* markedly reduced transparency, reduced biodiversity, elevated primary production, and most importantly, offering a poor feeding resource for fish. Some studies have shown that for many fish, including

the common carp (*Cyprinus carpio*), which is the main species in this reservoir (Djemali, 2005) *Bacillariophyceae* and *Chlorophyceae* were primary dietary items, while Cyanobacteria were consumed to a lesser extent (Isanghedighi *et al.,* 2009).

Furthermore, the potential toxicity of *P. agardhii* cannot be ruled out, as one of the strains isolated (PMC 790.12) was an MC-producing strain (data not shown), suggesting that toxic clones (although rare at this sampling date) do exist in the Bir M'Cherga reservoir, which corroborated the previous findings of Fathalli *et al* (2011). It has been shown that in *P. agardhii* populations, both toxic and non-toxic genotypes co-exist simultaneously (Kurmayer *et al.,* 2004, 2005; Davis *et al.,* 2009), and in response to some unidentified changes in conditions, "flash episodic" toxic blooms may occur in water during which an unpredictable dominance of toxic clones over non-toxic ones occurs (Tonk *et al.,* 2005; Briand *et al.,* 2008). Several studies have reported that abiotic factors, such as light and higher T°, may have an indirect effect on the selection of MC-producing over the non-MC-producing strains (Briand *et al.,* 2008; Kurmayer *et al.,* 2004; Tran *et al.,* 2013), while recent studies tend to suggest that widespread eutrophication and increasing of T°, as a result of global warning, will further promote the proliferation and expansion of harmful cyanobacteria (*i.e.* the dominance of toxic clones) in water systems worldwide (O'Neil *et al.,* 2012; Paerl & Huisman, 2009). As a result, extensive and regular monitoring campaigns would be very necessary in dry and warm countries from Africa, and Asia, which have so far remained under-investigated in terms of potential toxic cyanobacterial developments and toxicological research (Merel *et al.,* 2013).

In conclusion, the Bir M'Cherga reservoir appeared to be a low-impact site in terms of bacterial fecal indicators, indicating negligible disturbance by the human populations living within its watershed. However, this report did highlight the fact that this reservoir does show signs of degradation of its ecological state, with the replacement of *Bacillariophyceae* by Cyanobacteria, and more recently, with a total modification of the phytoplankton structure. Although no cyanotoxin (MC or CYN) was detected during this period, the "nearly-exclusive" dominance of *P. agardhii* in waterbodies over several months, may seriously imbalance ecosystem functioning, and may affect all trophic levels in the food chain. Even if episodic fish mortality events cannot be directly attributed to microbial communities, the very low dissolved oxygen values, which may result from the decomposition of the cyanobacterial biomass for at least one event, may be a possible cause of fish mortality. However, other causes, which were not studied here, cannot be excluded as the possible cause of fish kills, and further investigations have to be carried out to identify direct causes of fish deaths. Regular water quality monitoring is essential, as several potentially toxic species are present at high abundances and may have detrimental effects in the light of the multiple uses of the reservoir, as we cannot exclude the possible development of toxic ecotypes, related to climatic change.

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