Toxicity of benthic *Phormidium* cyanobacteria: diversity and toxicity characterization

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Index

Index of tables

Index of figures

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Abbreviations

ABS: absorbance

ATX: Anatoxin-a

BLAST: Basic Local Alignment Search Tool

Chl: Chlorophyll-a

CYL: Cilindrospermopsin

HPLC: High-Performance Liquid Chromatography

LB: lysogeny broth

LC-MS: Liquid Chromatography-Mass Spectrometry

LIC: Lugar de Interés Comunitario

MYC: Microcystin

NCBI: National Centre of Biotechnology Information

OTU: Operational Taxonomic Unit

STX: Saxitoxin

SIDI: Servicio Interdepartamental De Investigación

UAM: Universidad Autónoma de Madrid

Abstract

Nowadays is demonstrated and is studied the toxicity that some cyanobacteria can create through a wide range of cyanotoxins affecting the ecosystem and the human healthy. One of the benthic cyanobacteria known as toxin producer is *Phormidium* which is described to produce the neurotoxin anatoxin-a.

Tn order to increase the knowledge of different species of *Phormidium*, this project has study toxicity of cultures and mats using different methodologies: diversity, toxin gene detection by PCR and toxin production by chromatographic and antibody binding procedures (ELISA test). 7 strains isolated from toxin benthic mats dominated by *Phormidium autumnale and, Phormidium corium* species were analyzed indicating that any those strains have the ability to produce toxins. 11 samples collected from river ecosystems were study and our results show that there is a high variability on the potential toxicity depending on the species composition and development of mats. An exhaustive study was carried out on mats forming blooms from Verde and Manzanares rivers which were toxic for anatoxin-a and also, saxitoxin in the case of Verde River. A proposal for a protocol to assess a protocol to detect and study potential toxic mats forming blooms is provided.

Keyword: *Phormidium*, river, benthic, anatoxin-a, saxitoxin, cylindrospermopsin, microcystin, PCR, ELISA, RBA, HPLC.

1. Introduction

1.1 Overview on cyanobacteria

Cyanobacteria are the most ancient photosynthetic organisms known, the most ancient fossils date from 3,500 million years, at the start of the Precambrian period. It is believed that were evolutionarily relevant because cyanobacteria are the precursors of the terrestrial atmosphere, because of this group were the first in use water molecules as electron donor, and this, give as a result liberation of oxygen that ended in the atmosphere (Whitton, 1992).

This group is compound by prokaryote organisms, Gram-negative, autotrophic and photosynthetic which were predecessors of the chloroplast of algae and other kinds of plants groups because they have the same photosynthetic apparatus, including chlorophyll-a and the two photosynthetic systems (Aguilera and Echenique, 2011).

This group is well known for its adaptation capacity to live in all kind of ecosystems, even at extreme environment because, between others, have the abilities of the production of a protective pigmentary sheath, formation of aikinites and some different adaptations.

Cyanobacteria are organism which, some of them can use atmospheric nitrogen (Aguilera and Echenique, 2011) as a source of N because they can synthetize a specific anaerobic enzyme, nitrogenase. Importance of this process resides in that nitrogen is a fundamental part of many structures of the living being, proteins, vitamins, amino acids, etc. and cyanobacteria also need it in the gas vesicles to buoyancy (Giannuzzi *et al.*, 2011).

Photosynthesis is a complex process which involve different cellular compartments and enzymatic mechanisms. Also, is a dependent process on the physicochemical variables of the environment (Mercado, 1999). Through this process plants, algae, photosynthetic bacteria, and some protist use the solar energy to synthesize organic compounds. All the photosynthetic organisms have one or more of the pigments able to absorb radiation, the most known pigment is chlorophyll, which has a maximum of absorption in 660 nm. The energy absorbed by the pigments is stored as chemistry energy (Pérez-Urria Carril, 2009).

Cyanobacteria are common in aquatic ecosystems, they can grow in the water column, planktonic, aggregated in the surface, metaphytic, attached to other algae, cyanobacteria or macrophyte, epiphytic or attached to the substrate, benthic (Paerl, 1988).

In these systems, cyanobacteria occur in low concentrations but, when the environment is favourable or for changes in the system's flow (Mitrovic *et al.*, 2010), global warming and water pollution (Lürling and Roessink, 2006), cells can multiply forming blooms.

These blooms can alter the aquatic ecosystem because of changes in the trophic structure and function, the mortality of fishes by the decay of the oxygen in the water and decreasing the water quality (Robarts *et al.*, 2005).

This increase in the number of cyanobacteria can also increase the number of toxins producers' cyanobacteria in the water column (Wu *et al.*, 2012).

1.1.1 Morphology of cyanobacteria

Cyanobacteria are a morphologically diverse group in which it can be found unicellular families and filamentous families, this group can have the following structures:

- **Vegetative cells**: Is a typical Gram-negative wall with small pores which break the structure (Castenholz, 1989), below this wall is found a periplasmatic membrane with a big variety of enzymatic systems because these cells are in charge of the photosynthesis process due to the presence of thylakoids and chlorophyll-a (Böhme, 1998) they are also in charge of the breathing process and therefore in these cells is where the production and consumption of energy occurs.
- **Sheath**: compound by polysaccharide acid secreted from the inside of the cells can wrap these cells, cyanobacteria use this sheath as an adaptation to dry seasons (Walsby, 1974). These cells are arranged along all the filament and nearby the sheath and form the trichome.
- **Heterocyst**: This structure present in some species of filamentous cyanobacteria that is a cell, separated from the vegetative ones, which contains big amounts of nitrogenase (Cardemil and Wolk, 1976), this enzyme confers to cyanobacteria the capacity of fix nit nitrogen from the Atmosphere. Heterocyst is vegetative cells which are irreversibly differentiated in combined nitrogen deficit conditions (Whitton, 1992).
- **Akinetes**: There are cells in which diverse reserve substances accumulate and confer resistance to inadequate environments because of the presence of very thick walls, there are resistance cells differentiated cells from vegetative cells after being in phosphorous or light limitation or carbohydrates conditions (Nichols and Adams, 1982).

- **Hormogonia**: There are little size reproductive filaments with a few cells which principal function is the dispersion because these cells can have some mobility (Whitton, 1992) and can adhere to solid surfaces (Herdman, 1982).

1.1.2 *Phormidium sp.*

Phormidium is a cyanobacterial belonging to *Oscillatoriales* order and *Oscillatoriaceae* family.

This species is a filamentous, non-branched cyanobacterium. Sheaths occur facultatively and strongly dependent on the environmental conditions; they are colourless and adherent to trichome, sometimes diffuse.

Trichomes are cylindrical from 2.5 to 11 μm wide, unconstricted. Cells are isodiametric or longer than wide. Apical cells are pointed, narrowed or rounded with or without calyptra. Reproduction occurs via trichome disintegration into motile hormogonia (Gomont, 1892).

Phormidium genus has 200 described species worldwide distributed, this cyanobacterium forms cohesive, gelatinous, mucilaginous, cartilaginous, membranaceous mats on very different ecosystems from wet soil to rocks and macrophytes in aquatic ecosystems (Prát, 1929).

This project is focused on the *Phormidium* species more common in Spain: *Phormidium autumnale, Phormidium corium, Phormidium aerogineo-caeruleum* and *Phormidium terebiforme* (Loza *et al.*, 2013).

- *Phormidium autumnale:* unbranched single trichomes, with an absence of constrictions at cross-walls and calyptra on mature filaments (Hašler *et al.*, 2012). According to the studies of Quiliber (2013) and some non-published studies performed in the UAM (Ramos, 2012; Kønig, 201*3*; Haya, 2016; Jimenez, 2018; Martín, 2018) some mats with this strain produce anatoxin-a.
- *Phormidium aerogineo-caeruleum*: unbranched single trichome with isodiametric cells, rounded apical cell and doesn't have calyptra *(Loza et al., 2013).*
- *Phormidium corium:* unbranched single trichome with cells longer than wider, without calyptra and the apical cell rounded (Loza *et al*., 2013). According to Quiblier *et al.* (2013) can produce microcystins.
- *Phormidium terebiforme: unbranched single trichome, with cells wider than longer, without calyptra and the apical cell hooked (Loza et al., 2013).*

In some cases, *Phormidium* species are also named as *Micocoleous* (Struncky *et al., 2013)*.

1.1.3 Distribution

Cyanobacteria are cosmopolitan organisms that can be found in very diverse habitats in the world: in intertidal marshes, alpine streams, thermal springs and Arctic and Antarctic lakes (Broady and Kibblewhite, 1991; Quesada *et al.*, 1999; Komárek and Anagnostidis, 2005) but also can in every terrestrial habitat, as desserts, rocks, acid soils and urban environments (Friedmann and Ocampo-Friedmann, 1984; Broady, 1996; Lukesová, 2001) .

Phormidium sp. is a benthic cyanobacteria widespread through riverine ecosystems worldwide (Aboal *et al*. 2002) and can be found in oligotrophic and eutrophic environments (Mez *et al.*, 1998; Komárek, 1999; Wood *et al*., 2012) forming extensive mats under optimal conditions.

The optimal environmental conditions for *Phormidium* are low flow rates, high nutrient concentrations and changes in the ratio TN:TP (Heath *et al*., 2015)

In Spain according with Perona *et al.* (2017) *Phormidium* mat can be found in at least 11 siliceous and calcareous rivers with a cover from 30 to less than 1% of the river basing (Table 1).

Table 1. Distribution of *Phormidium* mats in Spain. (Perona *et al*., 2017).

Site	Lithology	Trophic status	*Phormidium* cover (%)
Mediano	Siliceous	Oligo-mesotrophic	10-20
Manzanares	Siliceous	Oligo-mesotrophic	5-10
Lozoya	Siliceous	Mesotrophic	2
Eresma	Siliceous	Mesotrophic	2
Jarama	Siliceous	Mesotrophic	<1
Escabas	Calcareous	Eutrophic	2-5
Tajo	Calcareous	Mesotrophic	30
Guadiela	Calcareous	Mesotrophic	5
Brazato	Siliceous	Oligo-mesotrophic	20-25
Caldares	Siliceous	Oligotrophic	2-5
Mijares	Calcareous	Mesotrophic	5

1.2 Cyanotoxins

Cyanobacteria can produce different metabolites including alkaloids, lipopolysaccharides, polyketides, and peptides that may act as toxins on other organisms, this ability of toxin production is because of its intense and variate metabolism (Börner and Dittmann, 2005).

Until now only a few genes involved in the synthesis of toxins have been discovered, all belonging to the peptide/polyketides group (Börner and Dittmann, 2005).

The cyanotoxins can be divided according to the effect that causes to the organisms.

Hepatotoxins: the most common cyanotoxins, affects to the liber (Sivonen y Jones, 1999). Microcystins (MYC) and cylindrospermopsin (CYL) belongs to this group.

- Microcystins are cyclic heptapeptides (Figure 1.A), joined by the two terminal amino acids of the linear peptide. The general structure is cyclo-(D-alanine-X-D-erythro-β-methylas-partic acid-Z-Adda-D-glutamate-N-methyldehydroalanine) in which Z and X are variable amino acids (Bartram and Chorus, 1999; Duy, 2000).

  This toxin cannot penetrate the cell membranes so requires the uptake via ATP-dependant transporters; as result, toxicity of MYC is restricted to organs expressing this type of transporter as the liver (Hitzfeld *et al.*, 2000). MYC inhibits some protein serine/threonine phosphatases ending in hyperphosphorylation of cytoskeletal proteins and deforming hepatocytes. The liver doubles its side and leads to disruption of the sinusoidal epithelium (Rao *et al.*, 2002).

- Cylindrospermopsin: is a tricyclic alkaloid (Figure 1.B), is derived from a polyketide that uses an amino acid derived starter unit such as glycocyanamine acid (Bartram and Chorus, 1999; Duy, 2000).
  This toxin blocks the protein synthesis and makes that the kidney and liver fail (Bartram and Chorus, 1999). It also causes gastroenteritis, hepatitis, renal malfunction and haemorrhage (Duy, 2000)
- Nodularin: Is a cyclic pentapeptide synthesized by *Nodularia spumigena* in plankton and *Nodularia sphaerocarpa* in benthos (Beattie *et al*., 2000; Moffitt *et al*., 2001).
  This toxin act as a potent inhibitor of the protein phosphatases present in a cytosolic fraction of the liver.

Neurotoxins: there are three chemically different groups in cyanotoxins able to show the toxic effect at neuronal level or in the muscle-neuron interaction. These toxins are:

- Anatoxin-a (ATX): is an alkaloid of small size (Figure 1.C), the chemistry of this toxin is a bicycle secondary amine (2-acetyl-9-azabicyclo [4.2.1]-non-2,3-ene).

Inside this group exist another toxin who is a chemical variation of anatoxin-a, the homoanatoxin-a (Figure 1D), this toxin is chemically identical to anatoxin-a but has a propyl group instead of acetyl in the carbon-2 (Sivonen and Jones, 1999).

This toxin base its mechanism of action of in that ATX and HTX are cholinergic antagonist, so are able to join to the nicotinic receptors of acetylcholine, activating the nervous impulse and how the toxin has greater affinity than the acetylcholine, the toxin isn't released and moved to the tissue.

The result of this activation and the incapacitation of deactivation makes that the channels of Ca^{2+} and Na^{+} locally depolarize and subsequent impulses are inhibited, producing muscular paralysis and causing, in mammals, the death by pulmonary insufficiency after 30 minutes (Fawell *et al.*, 1999).

- Saxitoxin (STX): have a tricyclic structure with hydropurine rings (Figure 1.E). SXT is a group of carbonate alkaloid that can be no sulfated, singly sulfated or double sulfated. Exist over 20 isomers of this toxin depending on the of the substitutions in its molecular structure (dcSTX, neoSTX…)
  The mechanism of action of SXT is contrary to ATX, the toxin blinds the site 1 of the sodium channel blocking the nervous transmission, this causes muscular paralysis (Briand *et al.*, 2003).

Cytotoxins: They can cause damage un multiple organs and systems like liver, heart, kidney, stomach, adrenal glandules, vascular and lymphatic systems (Falconer and Humpage, 2006).

Dermatoxins: Associated with marine cyanobacteria. These toxins have inflammatory effects and are tumours promotors and activate the Kinase C protein (Sivonen and Jones, 1999).

- Aplysiatoxins: This toxin can cause severe ear irritation, inhibition of epidermal growth factor, activation of ornithine decarboxylase and has tumorigenic properties and are activators of the protein kinase-C (Fujiki *et al.*, 1982; Fujiki *et al.*, 1983; Horowitz *et al.*, 1983; Fujiki, 1990)
- Lyngbyatoxin: this toxin has a skin tumour promoting activity basing on the activation of the protein kinase-C because of replacing endogenous activator of this enzyme (Bartram and Chorus, 1999).

Lipopolysaccharides: these compounds are produced by all the cyanobacteria, are part of the cellular wall of the Gram-negative bacteria. The effect of these toxins is extremely low compared with other bacteria (Sivonen and Jones, 1999).

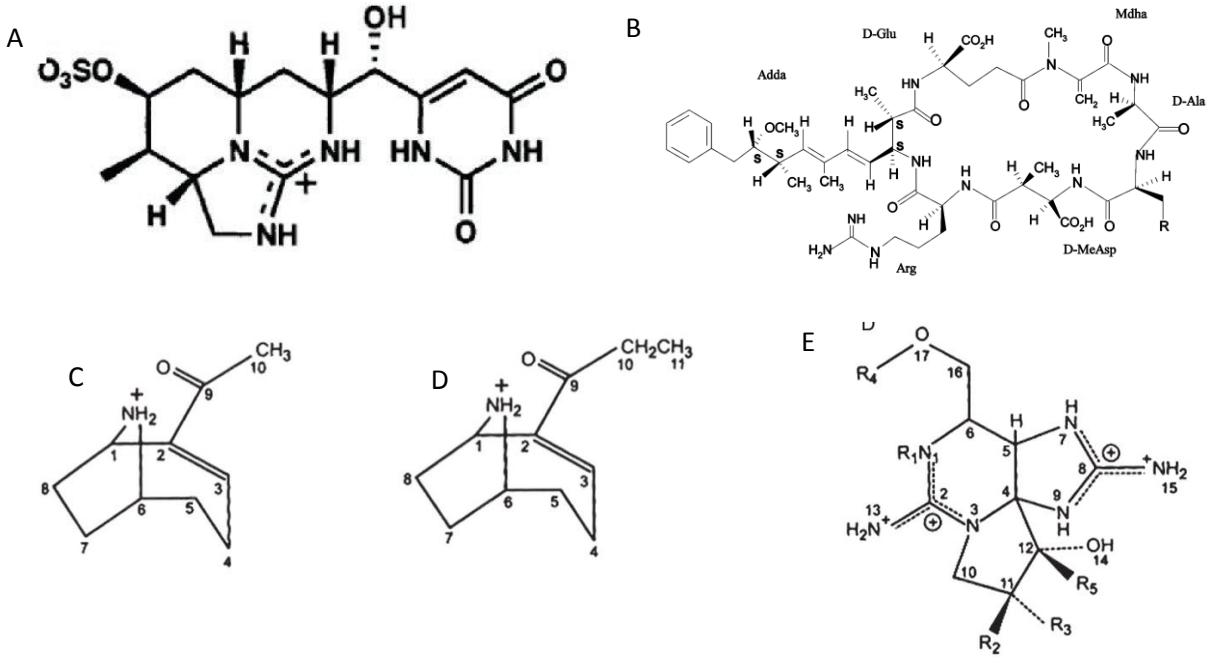

Figure 1. Chemical structure of (A) Cylindrospermopsin (B) anatoxin-a (C), homoanatoxin (D), Saxitoxin (E), Microcystins. Source: Duy. 2000; Molica and Azevedo, 2009; de Oliveira *et al*. 2011.

1.2.1 Toxins legislation

The general ignorance of these toxins and its effects in freshwater ecosystems, explains the lack of Spanish legislation about the toxin in recreational and drinkable waters.

Only MYC is nowadays regulated by the WHO (1998), in its guidelines for drinking-water quality established a provisional guideline value in drinkable water of 1µg/L, and by Spanish legislation (royal decree 140/2003) with a limit of 1µg/L but this measure only will take place is there is any suspicion of eutrophication in the water and after the exit of a drinking water treatment plant.

For the rest of toxins, even there are no limits in our legislation, by the moment, only New Zeeland has a provisional guidance value in drinkable waters for ATX, about 6 µg/L (Kouzminov *et al.*, 2007), there is no official guideline for SXT but Australia considers 3µg/L (Svrcek and Smith, 2004) and also, there is no official regulation for CYL, but there is a guidance value suggested in Canada of 1.5 µg/L (Svrcek and Smith, 2004)

1.3 *Toxic cyanobacteria*

The risk of toxins production was associated mainly to planktonic cyanobacteria and as a result the investigation in this group increased in the last years but there is a gap of information in relation to toxins production of benthic cyanobacteria(Oliver and Ganf, 2000; Gugger *et al.*, 2005; Briand *et al.*, 2009).

According to the data compiled by Quiblier *et al*. in 2013 (Table 2), there are some benthic cyanobacteria species in which toxins production was confirmed.

Table 2. Benthic cyanobacterial species in which toxin production was confirmed through strain isolation, culturing and toxin testing (Extracted from Quiblier, 2013).

Toxin	Species	Reference
Microcystin	*Planktothrix sp.*	Wood et al. 2010
	Anabaena subcylindrical	Mohamed *et al*., 2006
	Anabaena variables	Mohamed *et al*., 2006
	Nostoc spongiforme	Mohamed *et al*., 2006
	Plectonema boryanum	Mohamed *et al*., 2006
	Phormidium corium	Aboal *et al*., 2005
	Phormidium splendidum	Izaguirre *et al*., 2007
	Phormidium sp.	Aboal *et al*., 2005
	Rivularia biasolettiana	Aboal *et al*., 2005
	Rivularia haematites	Aboal *et al*., 2005
	Tolypothrix distorta	
Cylindrospermopsin	*Lyngbya wollei*	Seifert *et al*., 2007
	Oscillatoria sp.	Mazmouz *et al*., 2010
Nodularin	*Leptolyngbya frigida*	Hitzfeld *et al*., 2000
	Lyngbya wollei	Seifert *et al*., 2007
	Nodularia sphaerocarpa	Moffitt *et al*. 2001
	Planktothrix	Wood *et al*., 2012
	Phormidium sp.	Hitzfeld et al., 2000
	Phormidium murray	Wood *et al*., 2012
Saxitoxin	*Lyngbya wollei*	Yin *et al*., 1997
	Scytonema cf. crispum	Smith *et al*., 2011
Homo/anatoxin-a	*Oscillatoria sp.*	Aráoz *et al*., 2005
	Oscillatoria Formosa	Aráoz *et al*., 2005
	Phormidium autumnale	Heath *et al*., 2011
	Phormidium favosum	Gugger *et al*., 2005
Aplysiatoxins	*Lyngbya wollei*	Seifert *et al*., 2007
Lyngbyatoxin	*Anabaena sp.*	Videau *et al*., 2016
	Lyngbya wollei	Seifert *et al*., 2007

Phormidium genera is known, as previously mentioned, one of the cyanobacteria able to produce toxins (ATX and MYC), the knowledge about the toxin production of this family has been increasing through the years (Quiblier *et al., 2012)*.

The most important cases of toxins production of *Phormidium* are recorded by Gugger *et al.* (2005) and Wood *et al.* (2007), both articles point to *Phormidium* for being the responsibility of dog deaths by neurotoxicosis in New Zealand and France respectively.

The French case (Gugger *et al.,* 2005) it's reported to dogs deaths occur after drank water in the shoreline La Loue River, the results show that *Phormidium favosum* responsible for those deaths and HPLC analyses revealed that toxic mats produce an amount of 8 µg of toxin per Kg of mat, also ATX was found in the liver tissues of the animals shows an amount of 0.6 µg of toxin per Kg of tissue.

In the case of New Zealand (Wood *et al.,* 2007), at least five dogs died after the contact with *Phormidium* in Hutt River, the necropsy shows a big amount of algal material in the dog's stomach. Samples of *Phormidium* mats were analysed to find toxic strains and also tissue for the dog was analysed.

The results identify the *Phormidium* strains as *Phormidium autumnale* and show positive results for ATX and Homoanatoxin-a in both, the mat and dog's stomach, the toxin extraction samples were measured by Liquid chromatography—masses spectrometry (LC-MS) and shows that 27 µg of toxins were present for each Kg of *Phormidium autumnale* mat.

In Spain, there's no documented case of deaths of intoxication of animals or humans because of benthic cyanobacteria or *Phormidium* strains specifically, but some previous studies show that there is a toxic mat of *Phormidium* in Spanish rivers.

Ramos (2012) performed bioassays of toxicity of *Phormidium* strains, four from Mediano Steam and 2 from Manzanares River, the results show that *Phormidium autumnale* samples are toxic for *Artemia salina* because they produce MYC, mainly in spring and winter seasons.

Haya (2016) concludes in his studies that in Manzanares River, at least, the 70 % of the mats, in which *Phormidium* strains are the majority, are toxic for ATX., indicating that intoxication by toxins could be possible to succeed in Spain. However only those data are known making highly recommended to increase the knowledge about the frequency and the health risk that presence of Phormidium mats could produce.

1.4Objectives

The general aim is to increase the knowledge on the toxicity of *Phormidium* genera. To do this several specific objectives are established:

- To characterize, morphologically and toxicologically, isolated strains of cyanobacteria from the *Phormidium* genera.
- To identify and characterize in deep the potential toxicity of natural samples from Spain.
- To design a protocol for future analyses and set a procedure for the management of toxic samples.

1.5 Hypothesis

- Some Phormidium strain should be able to produce toxins because they were isolated from toxic mats.
- *Phormidium* mats are high varied depending on the procedure of the sample*Phormidium* mats in Spain are able to produce anatoxin-a.
- Samples from Phormidium mats, could be toxic if Phormidium autumnale is present, and those with episodes of death of dogs, seem to be a clear candidate to have anatoxin concentration and *Phormidium* species.

2. Materials and methods

2.1 Biological material and growth

The biological material used in this project were cultures of isolated strains of *Phormidium* from UAM (Universidad Autónoma de Madrid) collection from diverse Spanish rivers (Table 3)

Table 3. Culture samples of *Phormidium* used in the project

Sample	Code UAM	Sampling site	Culture media	Collector	Year
L2N	UAM483	Lozoya river	BG11	Perona, E.	2018
LE3	UAM484	León	BG11	Cirés, S.	-
Z52	UAM489	Mediano stream	BG11	KØng, S.	2012
MED1	UAM486	Mediano stream	BG11	KØng, S.	2012
MED2	UAM485	Mediano stream	BG11	KØng, S.	2012
M1	UAM487	Manzanares river	BG11	Haya, R.	2016
M2	UAM488	Manzanares river	BG11	Haya, R.	2016

Eleven natural samples from Verde river, Eresma river and Manzanares river were also used (table 4).

Table 4. Summary of natural samples (mats).

Name	Sampling site	Culture media	Collector	Year
P1A	Verde river	BG11	Lauren	2019
P2A	Verde river	BG11	Lauren	2019
PEA	Verde river	BG11	Lauren	2019
ERES4	Eresma river	BG11	Perona. E	2019
ERES6	Eresma river	BG11	Perona. E	2019
ERESF	Eresma river	BG11	Perona. E	2019
ERESO	Eresma river	BG11	Perona. E	2019
MANZPV	Manzanares river	BG11	Perona. E	2019
MANZPN	Manzanares river	BG11	Perona. E	2019
MANZPE	Manzanares river	BG11	Perona. E	2019
FLT1	Fuente de los Tilos	BG11	Amador. I	2019

2.1.1 Culture conditions and isolation.

From each sample site, using a binocular stereoscope, a single filament from each morphotype was separated and transfer to a multi-well plate with 5 mL of BG11 media.

The multi-well plates were incubated at 20ºC with a cycle light/darkness (16/8 hrs) for 1 month.

At the end, 48 single filaments from Verde river sample were isolated in multi-well plates, of which 16 growth enough to take biomass to proceed to the rest of the analysis.

From the other natural samples, 36 single filaments were isolated from Manzanares River samples, 18 from Eresma River and 6 from Fuente de Los Tilos.

At the moment of finishing the project none of these isolated filaments growth enough to perform further analysis.

A high number of filaments of different strains are involved in the isolation process at the end of this project, and, once the strains will be isolated, all of them will be included at UAM collection where they will be stored for future research.

2.2 Study area

There are eleven natural samples collected from four different sampling points: Eresma, Manzanares and Verde rivers and Fuente de los Tilos (Figure 2).

Figure 2: Situation of the sampling points in Spain. The withe stars mark the exact point of sampling. Own elaboration using ArcGIS. Photographys without author rights.

Thee samples were collected Verde river, located close to La Concepcion reservoir (Marbella, Spain) , this river belongs to the Andalusian Mediterranean hydrographic confederation. All the course of the river is a protected area, included as a LIC (Lugar de Importancia Comunitaria). This river has two different typologies being Rivers of humid Baetic mountains in the head and Mediterranean coastal rivers down water (Junta de Andalucía, 2015).

Seven samples were collected in two rivers at Sierra de Guadarrama (Madrid, Spain). Four mats were collected at Eresma River. This river belongs to Duero hydrographical confederation and is catalogued as Mediterranean siliceous mountain river (Confederación Hidrográfica del Duero, 2015).

Other three samples were collected at Manzanares river (Madrid, Spain) which is a river of Mediterranean siliceous mountain with a length of 10,3 km with and good ecological status (Confederación Hidrográfica del Tajo, 2015).

Along the river, four protection figures are present: National park, regional park, biosphere reserve, and LIC. The sampling point was in the high part of the river were the human alterations are low or non-existing called Cantocochino.

The last natural sample was collected in Fuente de Los Tilos spring (Beteta, Cuenca, Spain) in this point the water comes out from groundwater, 50 meters apart of the source of the spring.

The physic-chemical parameters of the four sampling sites are shown in Table 5

Table 5. Physic-chemical parameters in Verde river, Manzanares river, Eresma river and Fuente de los Tilos steam. Nitrate, nitrite, ammonia and phosphorus are show in mg/L(Data from Verde river extract from Junta de Andalucía, 2018)

Parameter	Verde river	Manzanares river	Eresma river	Fuente de los Tilos
pH	7.52	7.00	6.85	7.57
Conductivity	937 µS (at ºC)	22 µS (at ºC)	204 µS (at ºC)	987 µS (at 23ºC)
Hardness	20.1 mg $CaCO_3$/L	3.5 mg $CaCO_3$/L	12.8 mg $CaCO_3$/L	284 mg $CaCO_3$/L
Nitrate	0.05 N-NO_3	0.14 N-NO_3	0.11 N-NO_3	0.04 N-NO_3^-
Nitrite	0.01 N-NO_2^-	0.084 N-NO_2^-	0.031 N-NO_2^-	0.002 N-NO_2^-
Ammonia	0.03 N-NH_4^+	0.06 N-NH_4^+	0.00 N-NH_4^+	0.05 N-NH_4^+
Phosphorus	0.07 P-PO_4^{3-}/	0.02 P-PO_4^{3-}/L	0.01 P-PO_4^{3-}/L	0.00 P-PO_4^{3-}/L

2.3 Morphological analyses

To identify the species, present at the natural samples and to confirm the previous identification of the isolated strains, the morphology was studied carrying out microscopical observations using a binocular stereoscope and an optical microscope (Olympus CX41 provided with a camera DP20 CDD). The morphological characterization was made following the method described by Loza *et al.* (2013), using as reference the length and wide of the cells, using a micrometric ocular scale coupled to one ocular of the microscope, composition of the trichome, the presence or absence of calyptra and the apical cell, between other characters. Using this data, the species assignation of each strain was given using the study of Komárek and Anagnostidis (2005)

Finally, to verify that the deaths of dog in Verde River are related with the *Phormidium* bloom stomach and intestine content were diluted in water and observed using microscope.

2.4 Genetic analyses

DNA extraction of each natural sample was performed using the Power Biofilm kit from Quiagen Laboratories, Inc., and following the procedure described by the manufacturer with some small modification.

Between 0.05 to 0.20 g of biomass (with no liquid media) were measured in an Eppendorf tube and resuspended in 350 ml of Solution BF1. The samples were frozen using liquid nitrogen and thawed using a drill. this procedure was repeated three times to ensure that all the cells were broken. Instructions provided by the kit was followed, but after the use of the solution BF6 the sample was allowed to dry the ethanol in it for 5 minutes to avoid problems in the measurement in the nanodrop and PCR, after letting them dry the following steps indicated by the kit were followed using 50 µL of solution BF7 instead of 100 µL, this process was repeated by duplicate.

To obtained DNA from cultured samples same procedure was made but using Ultraclean microbial DNA isolation kit from Quiagen Laboratories, Inc.

In this case, the samples were also breaking by a thermal crash, using liquid nitrogen, and mechanically using a drill with a pistil, all this process with the MD1 solution as media.

Once the DNA extraction was obtained the DNA was measured through electrophoresis in agarose gel (0.8%) and using a Nanodrop(ng/µL). DNA samples were stored into a freezer (-20ºC) to preserve them till they were used.

To study diversity using metagenomics analyses, on DNA samples from Verde river, samples were tested performing a PCR for variable region V3–V4 from 16S rRNA gen using primers showed at table 5. Samples were sent to an external laboratory (Parque Científico, Madrid) to perform metagenomic analyses. All DNA samples (from mats and cultures) were used to carried out a screening of toxins genes performing specific PCR for ATX, MYC, SXT, and CYL using the primers in Table 6 and the PCR conditions described in the literature

PCR product was check using agarose gel (1,5%) and read through UV light.The PCR product was preserved at -20ºC.Samples with positive results of PCR for toxic genes will be cloned, this process was performed in three steps:

- Linkage: the amplified fragment was linked with ampicillin resistance vector using 2x Ligation buffer pGEM-T easy vector and T4 ligase from Promega.

Table 6. Primers used to perform specific PCR for taxonomy and screening for toxin genes.

Target gen	Primer	Sequence	Reference
Ra	CYA359F	5′-GGGGAATYTTCCGCAATGGG-3′	Nübelet *et al*., 1997
	781Ra	5′-GACTACTGGGGTATCTAATCCCATT-3′	
	/781Rb	5′-GACTACAGGGGTATCTAATCCCTTT-3′	
anaF	atxoaF	5′-TCGGAAGCGCGATC GCAAATCG-3′	Ballot *et al*., 2010)
	atxoaR	5′-GCTTCCTGAGAAGGTCCGCTAG-3′	
mycE	HEPF	5′-TTTGGGGTTAACTTTTTTGGGCATAGTC-3′	Jungblut and Neilan (2006)
	HEPR	5′-AATTCTTGAGGCTGTAAATCGGGTTT-3′	
sxtA	sxtAF	5′-GCGTACATCCAAGCTGGACTCG-3′	Casero *et al*. (2014)
	sxtAR	5′-GTAGTCCAGCTAAGGCACTTGC-3′	
cylJ	cylsulF	5′-ACTTCTCTCCTTTCCCTATC-3′	Ballot *et al* (2011)
	cylnamR	5′-GAGTGAAAATGCGTAGAACTTG-3′	

- Transformation: using E. coli (DH5α) competent cells in which the plasmid with the target gen was introduced using thermal shock (20 min at 4ºC, 2 min at 42ºC and 10 min at 4ºC). The transformation product was incubated in liquid Lysogen broth media (LB) described by Bertani (1951) at 37ºC and with string by 1 hour. After the time of incubation, the mix was seeded in a petri dish with solid LB media with ampicillin and X-Gal and was incubated at 37º overnight
- Selection of positive clones: positive clones will show white colour meanwhile negative will show up blue colour, the positive clones will be passed to another dish and a PCR for the plasmid will be performed using T7 and SP6 primers (Table 7).

Table 7. Primers used for PCR to confirm positive clones.

Primer	Sequence	Reference
T7	5′-TAATACGACTCACTATAGGGAGA-3′	Dunn *et al.*, 1983
SP6	5′-ATTTAGGTGACACTATAGAAG-G-3′	Brown *et al*., 1986

Positive clones (with the target gene) were grown overnight using liquid LB media and the plasmidic RNA was purified using Plasmid DNA Miniprep Kit (ThermoFisher) and were sent to sequency by Sanger protocol at Parque Cientifico de Madrid.

Finally, treatment of the data will be performed using BioGen software and the sequence will be compared with the NCBI database using the BLAST tool.

2.5 Metagenomics analyses

To analyze the metagenomics and the structure of the community, DNA templates from the natural samples were sent to the genomic unit in Fundación Parque Científico.

The procedure used was PCR amplification of 16S ribosomal RNA (rRNA) gene and Illumina MiSeq sequencing.

The variable region V3-V4 from 16S rRNA gene was amplified with CYA359F and 781Ra /781Rb performing separated reaction for each reverse primer (Boutte *et al.,* 2006)

Once the results were obtained the samples were prepared using qiime and qiime 2 to identify the OTUs (Operational taxonomic units).

- The first step is a quality filtrate to avoid the use of sequences with a high probability of error.
- Derreplication: the sequences are grouped in to get equal unique lectures and grouped in a single representative lecture.
- Lectures that only appears one time will be erased because it is considered that are sequencing errors.
- Erased of chimaeras: this step erases all the lectures that are a mixture of lectures.
- Aggrupation in OTUs: it is an aggrupation of similar sequences that may belong to the same species.

Taxonomical assignations of each OTUs were, performed using greengenes, SILVA 128 and SILVA 138 databases, the genetical library of genes sequences of UAM. Specific BLAST (Basic Local Alignment Search Tool) were performed using the webpage of NCBI (National Centre of Biotechnology Information), the final assignations will be given according to those of the UAM collection with over 97% of similarity, in this case the identification will be given till species level in the final sequences, if the level of similarity was lower than that percentage the assignation will be till family level using the more similar assignation from the other databases used.

2.6 Toxicity analyses

The same toxins that in genetical analyses were measured. Each sample was divided into three small pellets/aliquots previously weighted (similar weights were prepared).

To quantify the amount of each toxin, sample an extraction of cellular content was prepared following protocol described by Cirés *et al.* (2012), broking mats in 5 mL methanol (90%),

friction with a pestlesonication by 10 minutes. Extracts were stores in darkens and cold for 1 hour. A clean extraction was obtained by centrifugation at 5000 rpm for 5 minutes, and the same procedure was repeated but with 3 ml of methanol instead of 5 (The supernatant of both extractions was mixed and chlorophyll-*a* (chl) concentration was measured spectrophotometrically, measuring absorbance (ABS) at 665 and 750 (for turbidity) nm to know the amount of in all samples. The extract was preserved at -20 ºC and dark till its use.

Separated evaporation of 2 ml were prepared for each toxin to analyse because solvent for each analysis are different depending of the toxin assay. Evaporations were made multiple evaporator at 43 ºC and shaking. Evaporated samples were resuspended in 2 ml of miliQ water and shaking it a few seconds with a vortex.an aliquot of 1 mL was sent to SIDI (Servicio Interdepartamental De Investigación) and Chemical Engineering Department (UAM) to measure the toxins with HPLC (High-Performance Liquid Chromatography). Other 1 mL aliquot was used to perform the other quantitative toxin assays.

ELISA (Enzyme-Linked Immunosorbent Assay for the Determination of toxins in water samples) assays for ATX, SXT, MYC, and CYL were performed using an ELISA, Microtiter plate kit (Abraxis) following the procedure indicated by the reaction of this kit turned at the end the samples to intense yellow in an inverse proportion to toxin concentration. The colour showed at the end of the ELISA-ATX text (Figure 3) was measured through ABS at 450 nm using a Biotek-Synergy HT.

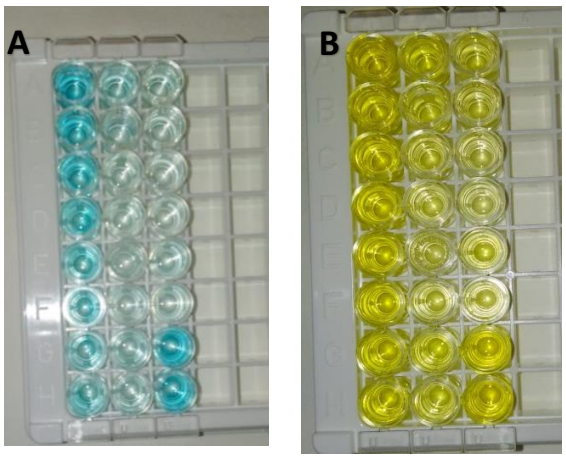

Figure 3: Photography of the colouration process in ELISA assays.
(A) without a stop solution, (B) with a stop solution. Own elaboration.

To quantify anatoxin-a RBA (Receptor-Binding Assay) for Determination of Anatoxin-a in Fresh Water kit (Abraxis) was also used and performed according to the instructions procedure

included into the kit. Again, yellow colour intensity was measured through ABS at 450 nm, using the same machine.

To calculate the amount of each toxin by biomass is present, analysis dry weight and chlorophyll-a contents of each samples were analyzed. Dry weigh was measured adding a known fresh biomass into an empty Eppendorf tube (previously weighed) and introducing those tubes an oven at 60 ºC for 24 hours. Dry tubes were weighed again after 1 hour and 24 hours. The results were expressed as g/sample

Chlorophyll-a content was extracted on a pellet (with 0.04 g, adding 1 mL of acetone (90%). The sample was broken using a pestle and helped by sonication for 10 min. Extraction was obtained after maintaining 24 h into, he fridge, and cleaned by centrifugation for 15 min at 5000 rpm, ABS of the supernatant was measured at 665 nm and 750 nm of wavelength. This process was repeated two times to obtain the total chlorophyll-a extraction of the sample. The amount of chl was expressed as mg chl/sample using the following formula:

$$Chlorophyll\ \ (mg\ chl/sample) = \frac{(ABS\ 665\ nm - ABS\ 750\ nm) * 11.19}{volumen\ of\ the\ extract\ (ml)}$$

3. Results

3.1 Characterization of *Phormidium* strains

3.1.1 Morphological analyses

First studies were to characterize cultures strains using morphological analyses, which were identify as *Phormidium* strains. (Table 8). The colour of the mats *Phormidium* also varies from one sample to another, from greenish to brownish.

Most of the cultures (LE3, L2N, MED2, M1 and M2) were identify as *Phormidium autumnale* sharing the same morphology, the cells that compound the trichome are more or less isodiametric, non-constricted, with calyptra (Fig 4b) and the apical cell is rounded or conical. The morphotype of *Phormidium autumnale* found in the culture LE3 was also seen some cells with granules that are suspicious to be aerotopes (figure 4A.) due to this is the only sample that procced from a reservoir instead of a river.

One of the cultures (MED1) has been identify as *Phormidium corium* (figure 4F) it was found that the cells are wider than long and had a tiny sheath very close to the trichome but without calyptra. The last *Phormidium* was identified at culture Z52 as *Phormidium terebiforme* (figure 4G). It has isodiametric cells similar to *Phormidium autumnale* but smallest.

Table 8. *Morphological characterization of the cultured samples*

Code	Sampling site	UAM code	Strain	Cell length (µm)	Cell wide (µm)	Apical cell	Calyptra	Sheath	Colour	Figure
LE3	Leon	*UAM484*	*Phormidium autumnale*	4	3	Rounded	Yes	No	Dark green	4 A
L2N	Lozoya river	*UAM483*	*Phormidium autumnale*	5	5	Rounded	Yes	No	Brownish	4 B
MED2	Mediano stream	*UAM485*	*Phormidium autumnale*	-*	3	Conical	Yes	No	Green	4 C
M1	Manzanares river	*UAM487*	*Phormidium autumnale*	4	5.25	Rounded	Yes	No	Dark green	4 D
M2	Manzanares river	*UAM488*	*Phormidium autumnale*	3.5	3.25	Rounded	Yes	No	Green	4 E
MED1	Mediano stream	*UAM486*	*Phormidium corium*	-*	6.5	Rounded	No	Thin	Brownish	4 F
Z52	Mediano stream	*UAM489*	*Phormidium terebiforme*	1	1	-	No	-	Green	4 G

* " - " means that the measure can be performed because of the resolution of the microscope or by the morphology of the filament.

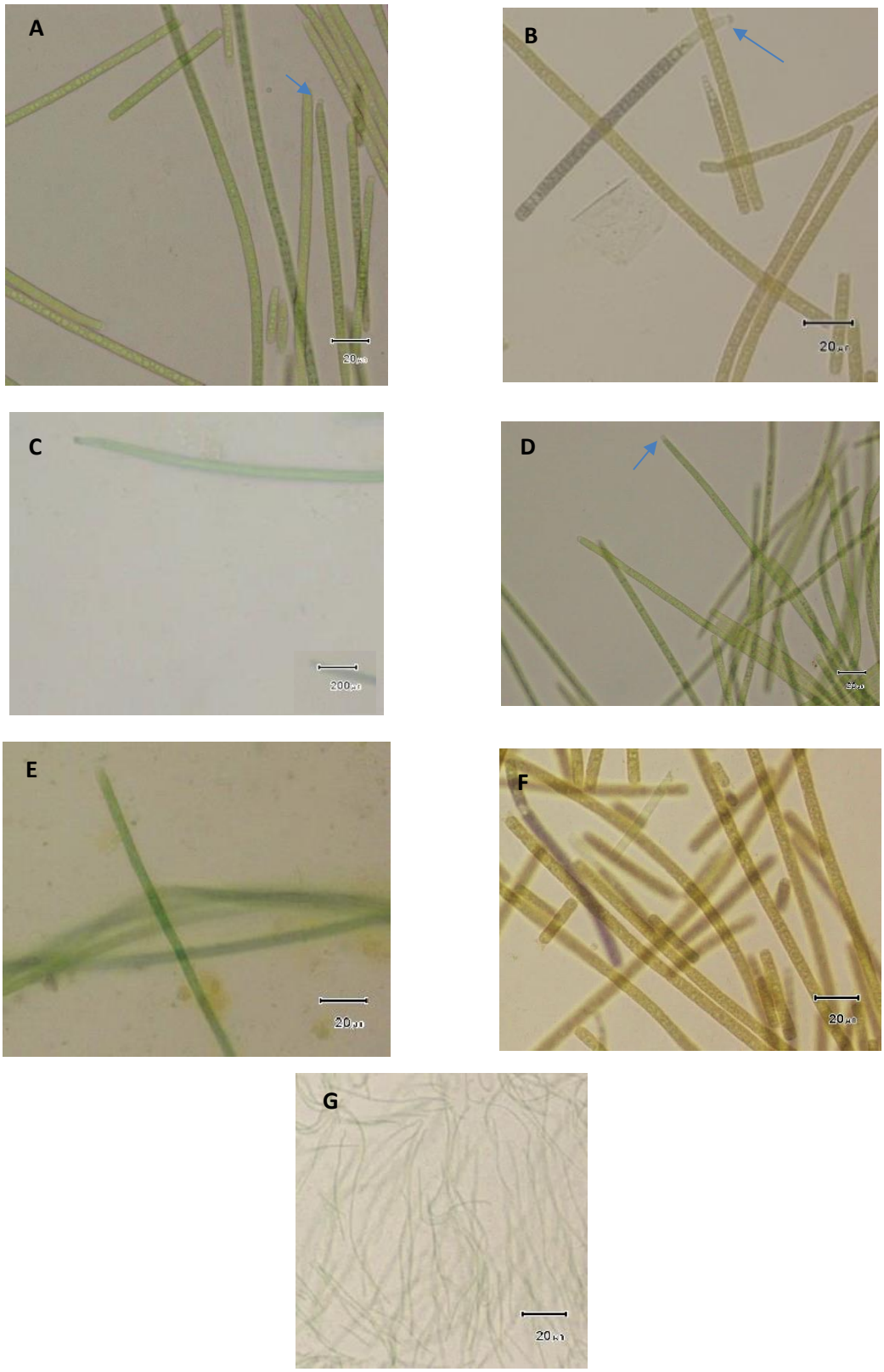

Figure 4. Microphotographs of the different species and morphotypes of *Phormidium* in the cultured samples. (A) LE3, *Phormidium autumnale*, (B) L2N, *Phormidium autumnale*, (C) MED2, *Phormidium autumnale*, (D) M1, *Phormidium autumnale*, (E) M2, *Phormidium autumnale*, (F) MED1 *Phormidium corium*, (G) Z52, *Phormidium terebiforme (*Scale = 20 µm*)*. ↑ shows calyptras.

3.1.2 Screening of potential toxicity

Specific PCR reactions were performed to detect potential toxicity in all the cultures of *Phormidium*. Results of the specific PCR obtained (Table 9) shows that only one strain of *Phormidium autumnale* (LE3) is suspicious to have the toxicity gen *anaF* which codify the creation of ATX but not for the rest of the genes. Any positive PCR reaction were obtained to the rest of the cultures showing that apparently these cultures are not toxin producers strains gen.

Table 9. Results of the screening for toxin production genes by specific PCR.

Name	Strain	*anaF*	*sxtA*	*cylJ*	*mycE*
LE3	*Phormidium autumnale*	+	-	-	-
L2N	*Phormidium autumnale*	-	-	-	-
MED2	*Phormidium autumnale*	-	-	-	-
M1	*Phormidium autumnale*	-	-	-	-
M2	*Phormidium autumnale*	-	-	-	-
MED1	*Phormidium corium*	-	-	-	-
Z52	*Phormidium terebiforme*	-	-	-	-

3.2 Characterization of potentially toxic mats of Spanish rivers

3.2.1 Verde river natural samples

Three samples (P1A, P2A and PEA) with apparent toxicity from Verde River were received from the owner of a dog that died in the area and a complete analysis to characterize them, not only in morphology and also their potential toxicity was studied carried out.

3.2.1.1 Morphological analyses

The morphological resume of the microscopic characterization of this samples from Verde River are showed in Table 10 More than one species was present at the samples.

The community of sample P1A is dominated by two *Phormidium* species identified as *Phormidium autumnale,* the most representative species, and *Phormidium corium,* in this sample also appear *Pseudanabaena mucicola* but in a low percentage.

Phormidium autumnale (figure 5A) morphotype was a big bright greenish/brown filament able to be seen using a binocular stereoscope, with isodiametric cells, with calyptra, without sheath and the apical cell rounded or conical.

The second most abundant species was *Phormidium corium* (figure 5D), this morphotype was characterized in the sample as greenish filaments with isodiametric cells in its trichome but smaller than the cells of *Phormidium autumnale*, without calyptra but with a tiny sheath beneath the trichome, the apical cell was conical.

The last species found in the sample, the less abundant, was *Pseudanabaena mucicola* (5B), this species is for the family *Pseudanabaenaceae*, it has brown/withe filaments with isodiametric cells or slightly longer than wider, a visible non-coloured sheath and without calyptra, the apical cell was squared.

The morphology of the P2A community were similar to P1A because *Phormidium* species dominate the sample. In this case, there are four different species of *Phormidium* in the sample; *Phormidium autumnale, Phormidium corium*, the only variation with sample P1A for these two species is the percentage of apparition in the community, *Phormidium aerogineo-caeruleum*, with dark green filaments and cells wider than longer, without sheath and calyptra and the apical cell conical or hemispheric. The last *Phormidium* species is *Phormidium terebiforme* (5E), only a few filaments of this species were found in the sample, with isodiametric cells, the apical cell rounded with sheath and without calyptra (table 10).

In addition to *Phormidium* species, also appear *Oscillatoria limosaa* (5C), which has single green trichomes and the cells significatively wider than longer.

An Extra sample (PEA) collected at other stretch in Verde River, which showed similar mats were sent to compare this sample with those where dogs were died. Under microscope, two *Phormidium* species were found in it: *Phormidium autumnale* and *Phormidium terebiforme*, being this last one in less proportion in the sample. The morphotypes found for this species in the sample are the same morphotypes that the found in the previous samples.

In all the samples the community seems to be dominated by *Phormidium* species and from those the major representant in the community is *Phormidium autumnale*, which have similar morphotypes in all the samples.

Table 10: Morphological characterization of the species present at samples from Verde River.

Sample	Specie	Cell length (µm)	Cell wide (µm)	Apical cell	Calyptra	Sheath	Mat colour	Filament colour	Figure
P1A	*Phormdium autumnale*	7.5	7	Rounded/conical	Yes	No	Dark green	Green/brown	5A
	Phormidium corium	5	5	Conical	No	Tiny		Green	-
	Pseudanabaena mucicola	5	5	Squared	No	Yes		Black	5B
P2A	*Phormidium autumnale*	7	7	Rounded	Yes	No	Brownish	Green/brown	-
	Phormidium terebiforme	3	3	Conical	No	Tiny		Green	-
	Phormidium aerogineo-caeruleum	3	7	Conical	No	No		Dark green	-
	Oscillatoria limosa	1	5	Rounded	No	No		Green	5C
	Phormidium corium	5	5	Conical	No	No		Green	5D
PEA	*Phormidium autumnale*	7	7.5	Rounded/conical	Yes	No	Brownish	Green	-
	Phormidium terebiforme	5	5	Conical	No	Tiny		Green	5E

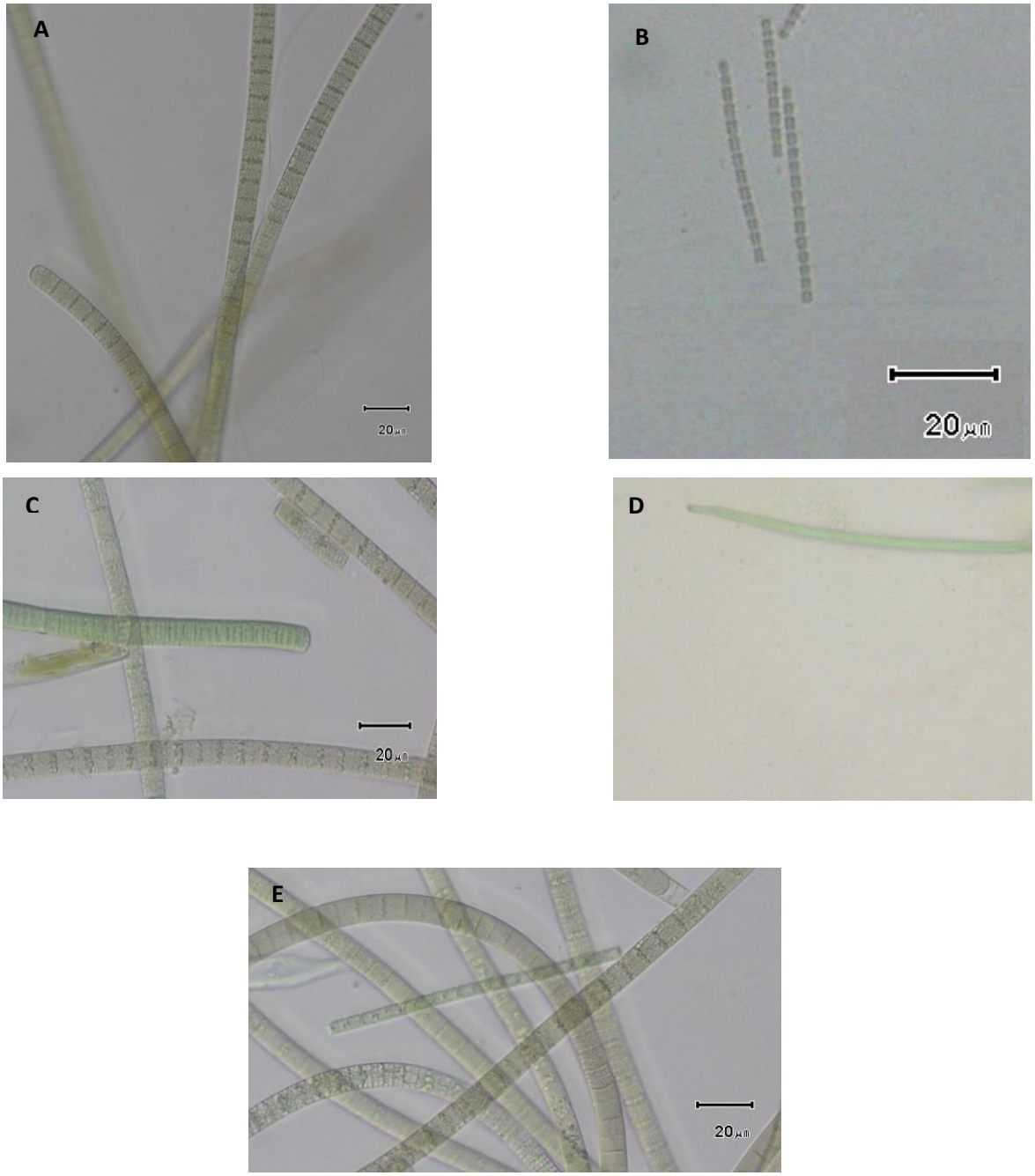

Figure 5. Microphotography of the different species of *Phormidium* in Verde River samples. (A) *Phormidium autumnale,* (B) *Pseudanabaena mucicola*, (C) *Oscillatoria limosa,* (D) *Phormidium corium* and (E) *Phormidum terebiform.*

3.2.1.2 Community structure

A study on community structure of the samples were done using two techniques.

According to the morphological and microscopic results the communities of Verde River are compose by at least two genera of cyanobacteria (Figure 6).

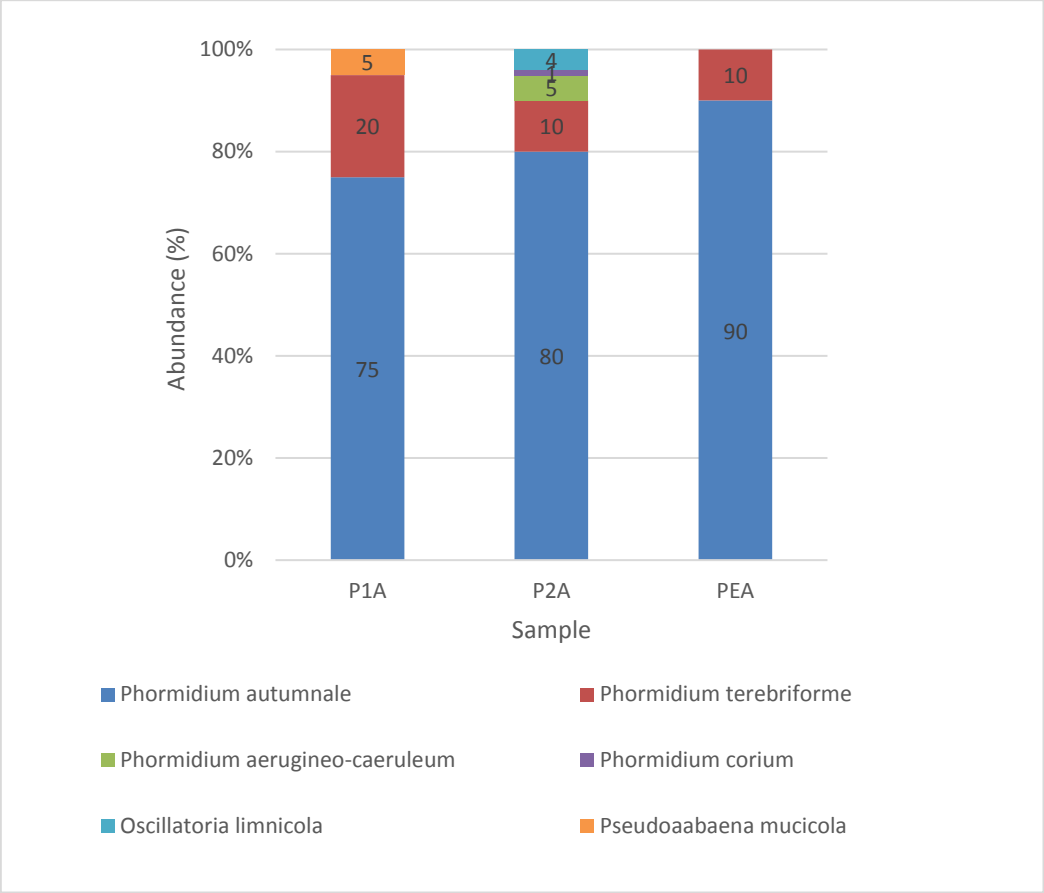

Figure 6. Relative abundance (%) of cyanobacteria in the samples from Verde River.

All the samples from Verde River are dominated by *Phormidium autumnale*, with an abundance higher than 75% of the community, follow by *Phormidium terebiforme* which represent the 10% of the community.

Other species were also found in the samples as *Pseudanabaena mucicola*, *Oscillatoria limosa*, *Phormidium corium* and *Phormidium aerogineo-caeruleum* but together they do not represent more than the 5% of the community.

A parallel study was performed using metagenomics analysis to deeply know and compare the structure of the community using a partial sequence of 16S rRNA (V3-V4, see Material and methods). Very good results were obtained and high number of sequences (DNA lectures) were obtained: 92810 (P1A sample), 61327 (P2A sample) and 61457 (PEA sample).

Rarefaction curves (Figure 7) for each sample indicate the robust results indicating that it is representative. In all the samples the number of DNA reads are enough to reach the total of the biodiversity of the samples, with only 30000 lectures the plateau of the curve is reached.

It is important to highlight that the samples P2A and PEA have a low number of lectures in comparison with the sample P1A.

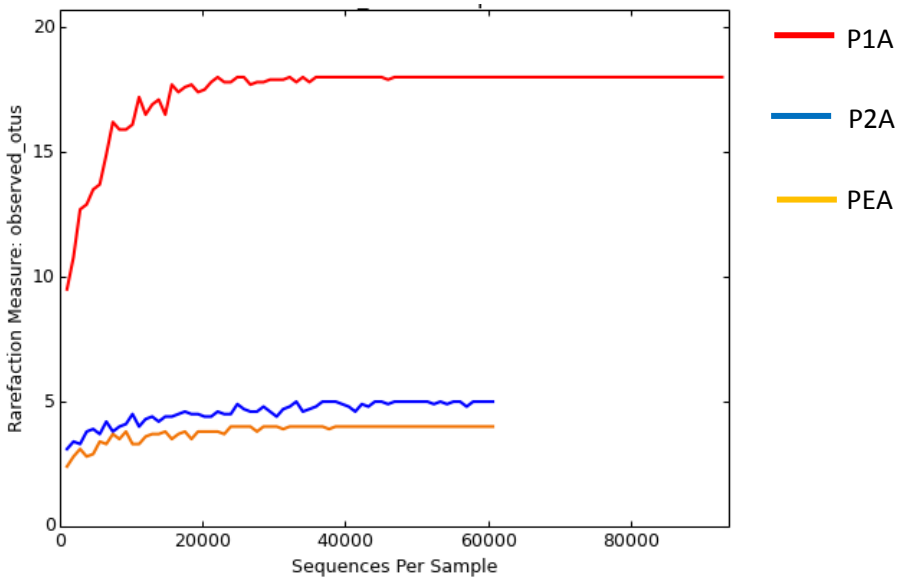

Figure 7: Rarefaction curve of natural samples from Verde River.

A total of eighteen different OTUs are present to describe community structure d using metagenomic analysis in all the samples. There are differences in the number of Otus, present at each sample, so, all OTUs are present in the sample P1A, but only four OTUs were present in P2A and PEA samples.

For taxonomy assignation only those OTUs with more than the 1% of representation in the sample were used.

Table 11. Taxonomical assignation of each OTUs obtained at Verde river samples, using NCBI, greengenes and SILVA 128 databases.

OTU	Taxonomy	Similarity
OTU 1	*Phormidium autumnale CAWBG646 (BLAST)*	100
OTU 2	*Phormidiaceae* (SILVA 128)	100
OTU 3	*Phormidium animale* (Greengenes)	100
OTU 4	*Nostocales* (SILVA 128)	100

The metagenomics analysis showed up that only one OUT (OTU1 assigned as *Phormidium autumnale* represent most than the 90% of the cyanobacteria present in the sample, representing 94.21% in P1A, 99.67% in P2A and 99,55% in PEA (Figure 8)

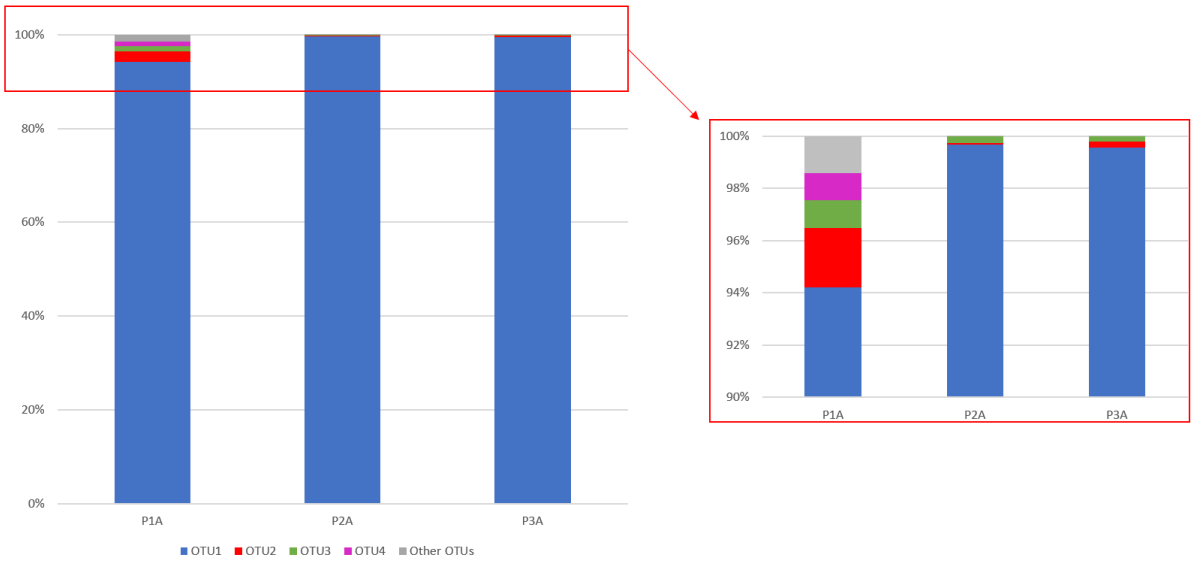

Figure 8. Relative abundance (%) of the different OTUs assigned at the Verde river samples.

Once the taxonomy analysis was performed, the OTU1, the most abundant, was identified as *Phormidium autumnale* according to NCBI database. The OUT 2 after the comparations performed with the three databases used has been impossible to assign any family or species of which belongs, further analyses should be done in a next future.

As it can be seeing at Figures 6 and 8, the abundance of cyanobacteria using both techniques (metagenomics and microscopy indicates that the genera *Phormidium* (or OTUs 1 and 3) is the most representative in all the samples, representing 95.29%, 99.92% and 99.75 % of the community at sample P1A, P2A, and PEA respectively.

Other genera composing the community the samples are less relevant contribution to the diversity per se.

3.2.1.3 Morphological characterization of isolated Phormidium strains of the samples from Verde River

As the tree samples from Verde river (close to La Concepción) are apparently highly toxic (according previous information), Isolation of strains catching single filaments were intensely carried out trying to obtained toxic strains. A total of 48 singles filaments were prepared which only 16 are truly isolated and they growth well to be use for further analyses. At least one filament of each species found at the different communities/samples was isolated and grow (Table 12).

As it was expected, most of the strains isolated correspond to *Phormidium autumnale* species because of this is the dominant species in all the samples, but Isolated strains showed some differences comparing with natural shape, , the most clear is the cell size and the wide of the trichome, where, the cells decreases in all the cases in culture conditions (Table 12) .

3.2.1.4 Potential toxicity analyses

3.2.1.4.1 Potential toxicity of natural samples of Verde River

A screening for the toxic genes was carried out by PCR on thee samples and it are showing that according to the PCR results (table 13) the three communities from Verde river are positive for *anaF* gene. It indicates that at least one morphotype of one species could be able to produce anatoxin-a, but no other toxins (negative results were obtained), except P1A sample which the procedure reveals that this sample can be toxic also for saxitoxin.

The natural samples from Verde River were suspicious from the beginning to be toxic, and apparently by the screening that anatoxin could be the toxin produce. For that reason, specific analyses to detect the presence of toxins and their concentrations were carried out for the samples P1A and P2A. ELISA and RBA test were performed for all the samples and in parallel toxins' extracts were sent to SIDI and chemical engineer to perform analysis using mass spectrometry and HPLC. As the sample PEA was not representative of the toxic community, quantification test of toxins was not performed.

Results of toxin concentration from HPLC, ELISA and RBA are shown in table 13. According to these results, there are high concentration for anatoxin-a showing similar values with the ELISA and RBA tests.

Table 12: Morphological characteristic of the strain isolated from each natural sample from Verde river.

Sample	Origen	UAM code	Specie	Cell length (µm)	Cell wide (µm)	Apical cell	Calyptra	Sheath	Colour
1.1.B	P1A	UAM490	Pseudanabaena *mucicola*	2.5	2.5	Squared	No	Yes	Black
1.6.G	P1A	UAM491	*Phormidium autumnale*	6	6	Rounded	Yes	No	Green
2.1.B	P2A	-	*Phormidium autumnale*	5.25	5	Rounded	Yes	No	Dark green
2.2.B	P2A	-	*Phormidium autumnale*	7.5	5	Rounded	Yes	No	Dark green
2.3.B	P2A	-	*Phormidium corium*	5	6.5	Rounded	No	No	Dark green
2.3.G	P2A	-	*Phormidium terebriforme**	-	-	Conical	No	No	Green
2.4.G	P2A	UAM492	*Phormidium autumnale*	6	6	Rounded	Yes	No	Green
2.6.G	P2A	-	*Phormidium autumnale*	5	6	Rounded	Yes	Tiny	Green
2.7.G	P2A	-	*Phormidium corium*	5	5	Rounded	No	Tiny	Green
2.8.G	P2A	-	*Oscillatoria limosa*	2	5	Rounded	No	Tiny	Green
2.9.G	P2A	-	*Oscillatoria limosa*	2	5.5	Rounded	No	Tiny	Green
2.10.G	P2A	UAM493	*Phormidium autumnale*	5	5	Conical	Yes	No	Green
2.11.G	P2A	-	*Phormidium autumnale*	5	6	Conical/rounded	Yes	No	Green
2.12.G	P2A	-	*Phormidium autumnale*	5	5	Rounded	Yes	Tiny	Green
3.1.G	PEA	-	*Phormidium terebriforme**	-	-	Rounded	Yes	No	Green
3.3.G	PEA	-	*Phormidium corium*	5.25	5	Rounded	No	No	Green

*In the samples of *Phormidium* terebiforme the cells are so small that it's impossible to measure the length and wide with the microscope in 40X.

Table 13: Summary of PCR result for the presence of toxin genes at Verde River samples.

Sample	*anaF*	*sxtA*	*cylJ*	*mycE*
P1A	+	-	-	-
P2A	+	+	-	-
PEA	+	-	-	-

Table 14 Toxin concentration of ATX, MYC, STX, and CYL in antibody assays (ELISA and RBA) and HPLC for P1A and P2A from Verde river. Results of toxins expressed as µg of toxin/g of sample (dry weight). n.a means not analysed.

Test	Sample	ATX (µg/g)		CYL (µg/g)		SXT (µg/g)		MYC (µg/g)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
HPLC	P1A	244.51	106.73	0	0	n.a	n.a	32.081	35.30
	P2A	217.12	82.10	0	0	n.a	n.a	9.80	4.40
ELISA	P1A	5689.98	5667.53	n.a	n.a	0,073	0.0021	1.14	0.094
	P2A	3656.79	2264.05	n.a	n.a	0,12	0.011	1.83	0.055
RBA	P1A	4982.05	6106.90						
	P2A	3651.09	2259.59						

However, results obtained by HPLC analyses showed a dissimilarity between the results from both analyses, being the results from antibody assays one or two magnitude orders bigger for ATX or MIC concentration measured.

Our results also indicate that high variability between samples, because the results represent the mean, and the values between one to the other are very different, which give us high standard deviations, the reason of this variation can be the variability per se of the toxic morphotype inside the community.

In a general view, it is clear that the mat P1A is the most toxic because the amount of toxins measured by the different methods is higher comparing with P2A. In both cases, the presence, even in low concentrations, of toxins as MYC and SXT and the amount of ATX are worrying and should be taken into consideration to perform measures in the water's treatment source.

Once the toxicity of the natural samples was confirmed by PCR, antibody assays and HPLC indicating us that the samples are toxic and it is clear that they have the possibility of produce toxins, we tried to identify the potential producer (species or morphotype) using the isolated strains. For that reason, another PCR screening for all toxin genes was performed and the results are shown at Table 15.

Only four strains (from 16 tested) can be potentially toxic producers because they have shown positive results for gene presence., three of them codified for anatoxin gene (*anaF*), two from

the sample P1A (1.1.B and 1.6.G strains) and one (2.10.G strain) from P2A sample. Also, culture 2.4.G strain, from P2A was found potentially producer for STX (stxA gene presence).

The positive potential producers of anatoxin 1.6.G, 2.4.G, and 2.10.G have been identified as different *Phormidium autumnale* morphotypes, while 1.1.B, identified as *Pseudanabaena mucicola* seems to be saxitoxin producer.

Table 15. Results of PCR screening for toxin genes in each isolated strains from Verde river mats.

Specie	Sample	*anaF*	*sxtA*	*mycE*
Pseudanabaena mucicola	1.1.B	+	-	-
Phormidium autumnale	1.6.G	+	-	-
Phormidium autumnale	2.10.G	+	-	-
Phormidium autumnale	2.4.G	-	+	-
Phormidium autumnale	1.3.B	-	-	-
Phormidium autumnale	1.5.G	-	-	-
Phormidium autumnale	2.6.G	-	-	-
Phormidium autumnale	2.7.G	-	-	-
Phormidium autumnale	3.1.G	-	-	-
Phormidium corium	1.1.G	-	-	-
Phormidium corium	2.2.B	-	-	-
Phormidium corium	3.3.G	-	-	-
Phormidium terebriforme	1.2.G	-	-	-
Phormidium terebriforme	3.2.G	-	-	-
Oscillatoria limosa	2.3.B	-	-	-
Oscillatoria limosa	2.3.G	-	-	-

Positive PCR products from anatoxin potential producers were cleaned and sent to Servicio de Genómica in UCM for sequencing. Sequences obtained were treated and BLAST performed showing that the gen amplified for the three cultures present high similitudes with other *anaF* genes (Table 16) so, all the three strains could be producing the toxin.

In the case of 2.10.G, the gen found was Ks2 which is a gene that also codifies to polyketide synthase in the gens of ATX and homo-ATX in *Oscillatoria sp.* In the case of 1.1.B and 1.6.G is clear that the gene if for ATX and homo-ATX production. In 1.6.G and 2.10.G genes are from a *Phormidium* strain (*Phormidium uncinatum* and *Phormidium autumnale respectively)*.

Table 16. Blast similarity of sequences from positive PCR for anaF genes from isolated strains.

Sample	Blast	Similarity
1.1.B	Uncultured cyanobacterium clone 227 anatoxin-a synthetase F (*anaF*) gen, partial cds	99.49%
1.6.G	*Phormidium cf. uncinatum* CYN103 polyketide synthase (*anaF*) gen, partial cds	100%
2.10.G	*Phormidium autumnale* CYN53 ks2-like gen, partial sequence	99.68%

To know if the strains are able to produce toxins, specific analyses to detect it were run to know the amount of toxin that these cultured strains are producing. ELISA test where performed for those samples that are confirmed to have anaF gene (Table 17). The results shown that all the samples positive for the toxic gen (*anaF*) are able to produce ATX and they are producing it in low concentrations under laboratory conditions.

Table 17. Anatoxin concentration of cultures strains measure by ELISA. Data are expressed as µg of ATX/ µg chl-a.

Sample	Procedure	Specie	ATX (ATX/ µg chl-*a)*
1.1.B	P1A	*Pseudanabaena mucicola*	0.0011
1.6.G	P1A	*Phormidium autumnale*	0.0072
2.4.G	P2A	*Phormidium autumnale*	0.00067
2.10.G	P2A	*Phormidium autumnale*	0.00045

3.2.1.4.2 Stomach and intestine content of dogs

Under microscopy analyses of the stomach and intestine of the dog that presently dead after the ingestion of cyanobacteria from Verde River (Table 18 and Annexe III) some filaments of *Phomidium, Oscillatoria* and *Pseudanabaena* appeared in those samples, also appeared another biological content as diatoms and pollen.

Table 18. Review of the stomach and intestine content of a dog presently dead by neurotoxicosis.++ = abundant, + = present, -= no present

Specie	Stomach		Intestine	
	Presence	Observations	Presence	Observations
Phormidium autumnale *Phormidium corium* *Phormidium terebiforme* *Phormidium aerogineo-caeruleum*	++	Filaments are starting to be degrades making difficult the identification, *Phormidium autumnale* was identificated due to the presence of calyptra	++	All the filaments of *Phormidium* are highly degraded making difficult the identification.
Oscillatoria limosa	+	Filaments don't show any type of degradation	+	Most of the filaments are complete and without sings of degradation
Pseudanabaena mucicola	-	Filaments don't appear in the sample	-	Filaments don't appear in the sample
Other	++	Diatoms, green algae, rest of macrophytes and pollen	++	Diatoms, green algae, rest of macrophytes and pollen

3.2.2 Characterization of natral samples from Eresma and Manzanares rivers and Fuente de Los Tilos.

3.2.3.1 Morphological analaysis

Similar analyses than those for the samples of Verde River were run using other mats collected in Madrid (Eresma and Manzanares River) and Cuenca (Fuente de los Tilos) (see table 4 at material and methods). Figure 10 shows the results of this characterization.

Eresma mats (ERESG, ERESO. ERES$ and ERES6) are mainly composed by *Phormidium* species (Figure 10B and C) as dominant. Only at ERESG mat seems to be another cyanobacterium with cells like *Pseudanabaena*, more quadratic cells and with aerotopes between cell separation in the trichome (Fig 9A).

In which *Phormidium* strains are referred, all the mats in the river are composed in a high percentage of *Phormidium autumnale* which can be recognized by the calyptra that has at the terminal cell in the trichome (Fig 9B and D). The morphotypes observed at the 4 mats studied are similar from one sample to another in Eresma River, only the colour is change between mats from greenish to brownish (visible also under microscope). *Phormidium autumnale* filaments have longer than wider cells, almost without sheath and with blurred intercellular separation, as is common in this specie.

Manzanares samples (MANZPG, MANZPB and MANZPSH) are formed by various species of *Phormidium*. Ecologically, the three samples are different, the sample MANZPG was a *Phormidium*, green mat it was growing attach to the stone into the river, and it is mainly composed by *Phormidium corium* (Figure 9E). The other two samples MANZPB and MANZPSH were mat mainly composed by *Phormidium autumnale* (Figure 9F and 9G respectively). In both cases, they are black and brownish mats, where MANZPB was growing on the rocks and MANZPSH were found floating at the Rivershore forming small balls.

The natural sample from Fuente de Los Tilos (FDLT) in Cuenca was a spherical floating mat formed by a single strain of *Phormidium sp*. This strain has big isodiametric cells (7.5 x 7.5 μm), with very thin sheath, and with the apical cell rounded, this strain was identified as *Phormidium corium* (Figure 9H).

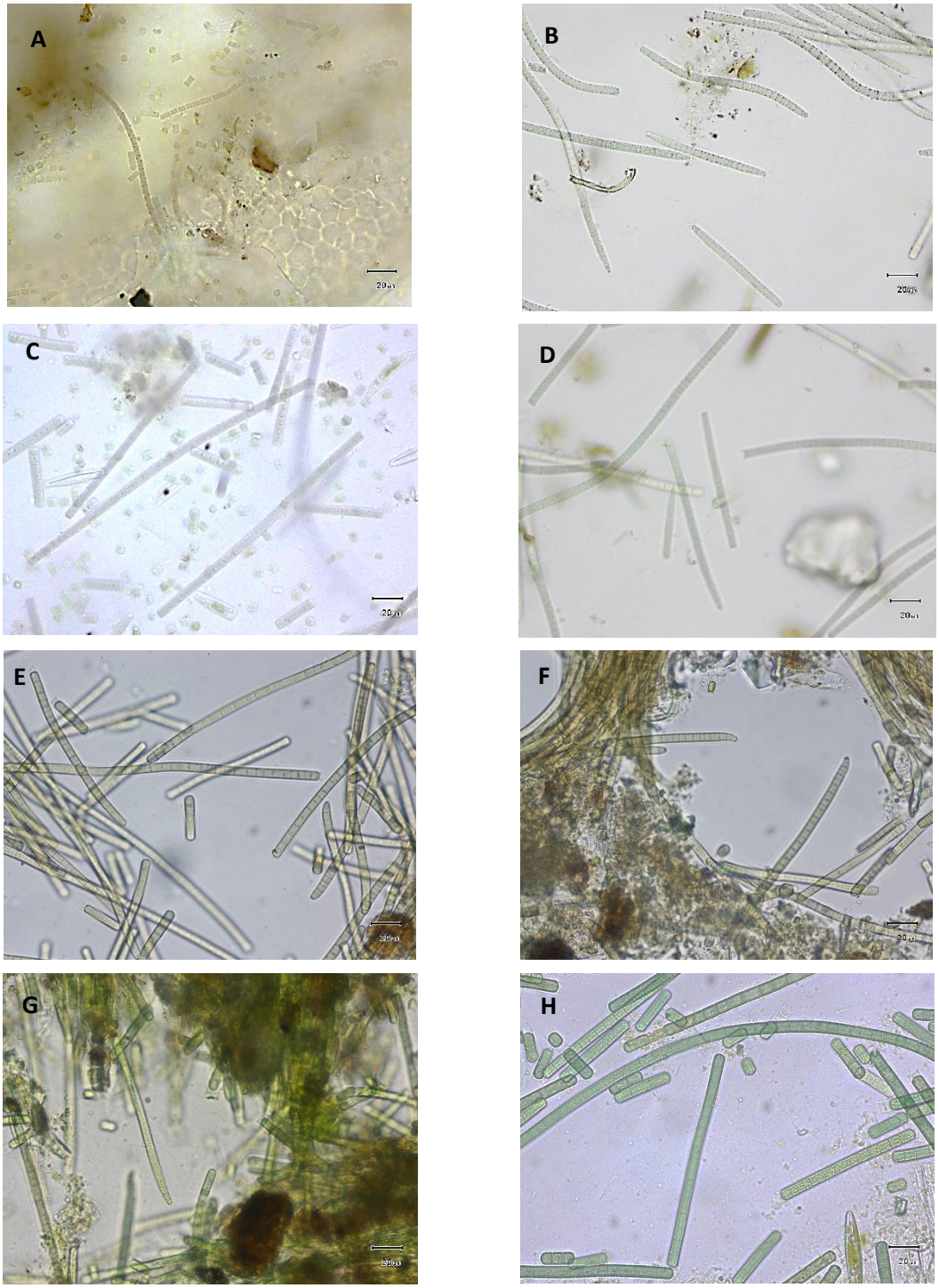

Figure 9. Microphotography of the natural samples from Manzanares River, Eresma River, and Fuente de Los Tilos. (A) ERESG, (B) ERESB, (C) ERESF, (D) ERESO, (E) MANZPG, (F) MANZPB, (G) MANZPSH, (H) FDLT.

3.2.3.2 Potential toxicity

A screening to detect potential toxicity of *anaF* gene, and ELISA test were carried out on the eight *Phormidium* mats collected from different rivers of Madrid community and Cuenca. Results showed at table 19 indicates that only one sample MANZPB (from Manzanares river) shows a positive result under PCR for *anaF* indicating that at least one species found within the mat, potentially, have a set of genes that could codify the synthesis of ATX.

Table 19. Screening of presence of anaF gene (by PCR), concentration of ATX (by ELISA) and biomass of mats collected at Eresma and Manzanares rivers and Fuente de los Tilos spring.

Sample	Sampling point	*anaF*	Chlorophyll (mg chl)	Dry weight (g)	ELISA (µg /g dry weight)
ERESG	Eresma River	-	n.a	n.a	n.a
ERESB	Eresma River	-	n.a	n.a	n.a
ERESF	Eresma River	-	n.a	n.a	n.a
ERESO	Eresma River	-	n.a	n.a	n.a
MANZPG	Manzanares River	-	0.059	0.052	0.016
MANZPB	Manzanares River	+	0.067	0.053	0.017
MANZSH	Manzanares River	-	0.060	0.049	0.014
FDLT	Fuente de los Tilos	-	n.a	n.a	n.a

An ELISA test was performed only to those samples with positive *anaF* gene (MANZPB) and the other samples of the Manzanares river. Even the gene didn't appear at the PCR results for *anaF* toxins, ELISA test (table 19) has quantified ATX at all the samples with similar concentrations, For MANZPB ATX was found in 0.017 µg ATX/g of the sample; 0.016 µg ATX/g for MANZPG and 0.014 µg ATX/g in MANZPSH, indicating the toxicity of those mats.

3.3 Protocol purpose for sampling and study of potentially toxic cyanobacterial mats

To study the potential toxicity of cyanobacterial mats, involves not only the ecosystem behavior but also the human health risks. So, it is important to have the major information possible of data about the community, the biota and also the chemical features of the sample point/site to know where the toxic strains are appeared and try to know why these species are grown-forming a bloom, to be able to check, control and remediate the situation.

For this reason, the first important step is to go to sampling sites (rivers) in which toxic cyanobacterial mats are suspicious to be found.

To collect a potentially toxic cyanobacteria the most important factor is to consider the sample size. Due to the variability of toxin production within the mat, it is recommended to collect a high biomass of sample to be sure that at least one toxic sample has been collected in the toxic community. The second step suring sampling is the identification of environmental parameter of each mat found at the river (macro and microhabitats.

Once the mats are identified at the river, samples will be taken to analyses the different "visu" morphotypes, different textures, colours, habitats… It's important to take more of one sample of each mat, always taking care of not spread filaments which can create downstream a mat and also collecting in an appropriate way to preserve the environment (maintaining diversity)

Once the sample is at the laboratory, they should be process as follow:

1. Prepare different types of samples from the mats for further analyses:
    a. Keep a small piece of the biomass fixed with soft algae fixer. this biomass fixed can be useful to see under microscope the community structure without changes at the moment that the sample arrived.
    b. Freeze small aliquots from the sample, at least, 2 for genetic analysis and another 2 as stock to further analysis.
    c. Another sample of biomass to perform, by triplicate, dry weigh analyses.
    d. Finally, another samples, also to freeze to perform toxicity assays.
2. 100 mL of filtrated water for toxicity analyses (to know if the toxins have been released to the environment)
3. Finally culture the sample, as a community and also, trying to separate single filaments from the sample to culture single strains of the cyanobacteria present in the natural sample (To perform further analysis).

To proceed with the toxicity analysis is recommended to perform a screening of each toxin through PCR procedures to know which of the toxins are present on the sample.

To quantify the amount of toxins in the samples is important to perform ELISA, RBA and HPLC analysis (at least two of them to confirm results) the test related to ATX right after the toxin extraction because this toxin is the most labile and it degrades faster. A faster screening of the potential toxicity should be run using specific PCR for each toxin genes (PON AQUI LOS GENES). If there is any previous hypothesis regarding the community, a selection of genes could be done e.g. only study *anaF* gene if it is a *Phormidium* mat.

Always, the extract with all the toxins should be kept in dark and under 20 ºC to avoid degradation of the rest of the toxins if the use will be instantaneous or freeze to use it later.

Based on the degradation rates and trying to lose the smallest amount of toxins as possible after the assays of ATX the assays of CYL and STX should be performed and in the last step the analysis of MYC because this last toxin is the most stable.

If the samples offered us positive results to any of the toxins an advice to the corresponding authorities, of the water body should be done. They need to be warned about the problematic and the risk that these toxins are involving.

Due to that there are high possibilities that we are working with toxin samples, all the wastes generate during the analysis performed should be correspondingly treated and marked as toxic waste and remove in an appropriate way.

Because of lack of time the sampling protocol couldn't be tested, but this protocol has been used in this project and our results on toxicity has been satisfactorily measured. Moreover, our intense work isolation refers also good results, and at least 4 single strains cultures were obtained following the protocol described and they are growing satisfactorily which allows to other researchers to perform further studies in which toxins production refers.

4. Discussion

The morphological study developed on the mats from Manzanares, Eresma and Verde Rivers and Fuente de los Tilos (springs) shows that two kinds of *Phormidium* mats exists. that can be differentiated in our rivers. The main variation is the colour of the mat per se, which can be brownish/black or green, both can be found in the same substrate, rocky substrate, and mainly in areas with a low flow. Similar results were found by Ramos (2012) in her study on microhabitat preferences of *Phormidium* mats in rivers.

After the analysis under microscope, despite of the colour, in both mats appeared the same species of *Phormidium* and, more or less, in the same abundance, these species were *Phormidium autumnale* as the dominant specie and others as *Phormidium corium and Phormidium terebiforme*. These similarities between both mats, and also, the fact that the isolated cultures from those samples showed greenish colours, from dark green to bright green, lead to two theories. The first theory about the colouration of the mats is that they vary depending on where the mat location into the river: if it is at and open sunny areas of the river or on the contrary in the shadow areas (under trees or similar) most of the day. So, in this last

situation, *Phormidium* strains would change the proportion of the different pygmies that it has, lowering the amount of chlorophyll and creating pigments more reddish/brownish which let them absorb lower wavelengths in shadow places, granting darker and more brownish colours. This theory seems to be validated by the study of Palinska *et al.* (2011) that shows that *Phormidium autumnale* strains, which is the most representative in the natural samples, can change the amount of some different pigments depending on the different light conditions in which they are growing.

The second theory is that this change could be related with the level of mat development, in this case, the newest mats would have bright green colours meanwhile the most ancient mats would have darker and brownish colours. This theory seems to be sustained with the fact that the brownish mats from Manzanares river, which were taken for this project, were thicker, denser and easier to detach from the rock than those with greenish colour. Also, under the surface of the brownish mats were found bright green filaments forming a mat or biofilm under the brownish filament, in these green parts of the mat is where the higher amount of hormogonia were found meanwhile in the greenish mats exist a higher amount of hormogonia in all the mat.

On the project of Ramos (2012) the morphology results show that only the mats dominated by *Phormidium autumnale* showed a variation in colour from brownish to green, this could explain the variation of colour in the mats used in this project because all of them are dominated by this species.

In a close-up approach, despite the cultures and isolations became from different mats and rivers, can be seen that the cultures from the same species are similar ones to another even they become from brownish or green mats, this data shows that there is not much variability in the species of *Phormidium* found Spanish rivers or in its morphotypes.

In all the mats the same four species of *Phormidium* have found (*Phormidium autumnale, Phormidium corium, Phormidium terebiforme* and *Phormidium aerogineo-caeruleum*). Those species can be differentiated ones to another depending on the cell length and wide and in the absence or presence of calyptra, being *Phormidium autumnale* the only one who has calyptra, but, within the same species exist different morphotypes with changes in the apical cell. The same composition of different *Phormidium* mats were studied by Loza *et al*. (2013) analyzing the morphological and genotypical differences between those species and also find clear relations with the environment properties. These variations can be found indiscriminately in all the different samples and all the different rivers.

Previous studies developed at the biology department (plant physiology) from UAM also showed that those four species are the ones that appear in other Spanish rivers as Mediano steam, Guadalix River and also in Manzanares River in previous years (Haya, 2016; Martín, 2018).

Because of in the samples analyzed of the genera *Phormidium* the apical cells are present in different forms (round or conical) for the same species and also in some samples, both types of cells are present for the same species, suggesting that there are not a clear differentiation between mats of different colours nor mats to different rivers and it is necessary to identify them at least by microscopy.

The unique differences found between samples was that MANZPG sample was formed mainly by *Phormidium corium* instead of *Phormidium autumnale*. However, at samples from Verde river, the community is formed by more species than *Phormidium* as *Pseudanabaena mucicola* and *Oscillatoria limosa* this two species appeared in low percentage, but both are closely related with *Phormidium*. According to algaebase (Guiry, 2019) *Pseudanabaena mucicola* is actually known as a synonym of *Phormidium mucicola*.

Results obtained with the metagenomic analysis are partially confirming this variability at Verde River samples, in which only four species (OTUs) are found at the total community with an abundance bigger than 1%, and from those, only one species compound more than the 90% of the community is *Phormidium autumnale*. The rest of the species found are included in the *Oscillatoriales* order in which *Oscillatoria limosa* is, or in the *Phormidiaceae* family which can be referred to other *Phormidium* species found under the microscope (*Phormidium corium, Phormidium terebiforme* or *Phormidium aerogineo-caeruleum*) or even to *Pseudanabaena mucicola* which can be also named as *Phormidium* (as mentioned before) .

But there are still differences between microscopy and metagenomic technics. It is probably due to only one DNA sample from Verde River communities was sent to perform the analyses. As those species are in lower abundance, may not appear in this analysis but appear in the samples under microscopy. Finally, another problem maybe with the data management because we have used Qiime 1.9.0 version which is demonstrated by researchers in the department that at the time to form the OTUs sometimes this pairing is wrong because compare the date with other databases and not with the data itself, this problem can be solved using Qiime 2.0.

Regarding toxicity, only the samples mats from Verde and Manzanares Rivers have showed to be toxic, but all the samples analyzed in this project are suspected of being toxic because of the previous information about the dominant species in they, showing that mats mainly composed by *Phormidium autumnale* have high probability of being toxic (Gugger *et al.,* 2005; Wood *et al.,* 2007; Faassen et al., 2012; Ramos, 2012; Kønig, 2013, Haya, 2016; Martín, 2018). Even the rest of the samples did not show toxicity doesn't it mean that *Phormidium* mats in these water bodies aren't toxic, because is studied that there is a high variability of toxin and non-toxin-producing genotypes whiting a *Phormidium* mat and as a result in the stretch in which the samples are taken. Mats that are apart one from the other only a few meters are found that one is toxic meanwhile the other is not (Wood *et al.,* 2012). Is important, when toxic production have to be analyzed to get a big sample (high biomass) to find toxicity mats in the sample.

The samples from Verde River were suspected to present toxicity due to dogs deaths occurred in the area. In our study filaments of cyanobacteria were found in different grades of decomposition in the stomach and intestine content and the deaths were described with the typical symptoms of neurotoxicosis (according with the veterinarian autopsy) produced by the poisoning with ATX.

The first assays performed to these samples were genetic assays by screening specific PCR. They showed positive results for the gens *anaF* and *sxtA*, which means that some strains in this community could be produce at least ATX and SXT. These results were subsequently confirmed by the test performed to quantify the amount of toxins in the samples with antibody assays (ELISA, RBA) and alternatively with HPLC, this assays not only prove that the samples are toxic for ATX and SXT but also confirm other toxins, MYC and CYL, and they could be producing the death of dogs.

There seems to be a variability between the results of the different assays of quantification and the genetic analysis, this can be explained with the study of Wood *et al.* (2012) and Haya (2016) in which the toxicity of *Phormidium* mats of the same stretch of a river was measured and found that exist a huge variability between the toxicity of samples less than 1 m apart, and also, between samples from the same mat.

Based on this variability and knowing that the procedure of DNA and toxins extraction are destructive procedures, seems to be clear that *mycE* and *cylJ* genes did not appear in the genetic analysis because of the biomass used in this process was different and much less than for toxins extractions and filaments with those genes maybe were not included.

Another explanation for this discordance is than in PCR performed in this project only one gen of each toxin was search but there are much more genes involved in the production of toxins that may be appeared on the sample.

Studies as Schembri *et al*. (2001), Fewer *et al.* (2007), Rantala-Ylein *et al*. (2011) Misson *et al.* (2012) and Casero *et al.* (2014) showed that exist at least two genes that codify anatoxin-a production (*anaF* and *anaC*), two for cylindrospermopsin (*cylJ* and *cylC)*, three for microcystin (*mycE, mycD* and *mycB*) and five for saxitoxin (*sxtA, sxtG, sxtH, sxtI* and *sxtX*). Further analyses should be run with the other genes not tested here

Similar problem happened with the samples from Manzanares River, in which, only one sample had positive results in the PCR assays, but the three samples showed positive results in toxin quantification by ELISA, indicating than PCR analyses should be positive. Haya (2016) previously indicated that to confirm negative results using PCR text analyses should be repeated

In the case of the Verde river samples there also can be found a high variability between the results of the quantification assays and between subsamples (triplicates) used to perform those tests. The range of toxicity using antibody assays were high for all toxins measured confirming the high variability on toxicity of the mats

On one hand, in which the difference between methods is referred, the study of Metcalf *et al.* (2006), for MYC, or Humpage *et al.* (2010) which show a clear difference between HPLC and ELISA technics, this variation is due to HPLC methods are more sensitive and selective. In the case of this project only one toxin was measured at the time with this method, but using ELISA kits (and RBA for ATX) over one toxin from the same family are measured e.g. ELISA for ATX measure also Homoanatoxin-a, ELISA for MYC measure also nodularin (Fischer *et al.*, 2001), etc. Also, according to the test instruction, the presence of other toxins can produce cross-reactivity and the results can be altered, in order to avoid this alteration could better use specific technics and methods of toxin extraction, and in this way have only one toxin per extract.

On the other hand, the source of the variation between the triplicates from the same sample (which make the standard deviation higher than expected) is the already mentioned high variation of toxicity in the same mat or community (Wood *et al.,* 2012) because the sample used is a natural sample and therefore is not expected to be homogeneous in terms of toxicity.

Our results are the first time that toxic *Phormidium* was found and deeply studied in Verde River and it is important to control the development of this cyanobacteria in the stretch as well as upstream, in the reservoir and downstream.

Regarding the toxicity of *Phormidium* mats from Manzanares River, for 6 years ago, according to the studies of the department of Biology of UAM (Kønig, 2013; Haya, 2016, Asens 2017), *Phormidium* mats are appearing in the river and approximately 40 % of those mats were toxic (Haya, 2016 but he coverage of *Phormidium* mats were less than 5 %. However, this year the problem has increased because the mats and their toxicity were more worrisome because *Phormidium* mats occupies more than the 25 % of the river channel.

Of the four rivers analyzed in this project only found toxicity at Manzanares and Verde Rivers. According to the previous data from the hydrographical confederations (Confederación Hidrográfica del Tajo, 2015; Junta de Andalucía, 2015) of those rives, the mean of the temperature is increased at both rivers and also the river flow has decreased, possibly caused by climatic change and that can explain the increase of this cyanobacterium in the rivers.

Moreover, it Is also important to mention that the toxicity in Verde River is much higher than the toxicity found in Manzanares river. The theory is that the samples from Verde river were taken in from the part of the river in which a reservoir starts, which means that the flow at this stretch is lower than the rest of the river. In this case the conditions of being close to the reservoir in the mixing area the function of the riverine ecosystem can be altered and make easier the growth of *Phormidium* and the production of toxins in this case, meanwhile in Manzanares River the samples were taken in a stretch that is far from any reservoir or place that can alter those conditions.

This difference between both types of environment can be also found in some other studies on the topic. Gugger *et al.* (2005) and Wood *et al.* (2007) in rivers from France (8 µg of ATX/kg of sample) and New Zealand (9.4-27 µg of ATX/kg of sample) who found similar values to those found in Manzanares river (15-17 µg of ATX/kg of sample). Faassen *et al.*(2012), at Lake igmeer (272 µg of ATX/kg of sample) who found similar values to those from Verde River in the proximity to La Concepcion reservoir (136-368 µg of ATX/kg of sample, according with HPLC analysis) with these dates is expected, as a general rule, to found higher values of toxins in reservoir and its vicinities than in rivers.

Both in Verde River (Ayuntamiento de Istán, 2019) and in Manzanares River (Parque Nacional de la Sierra de Guadarrama, 2019) once the toxicity was confirmed, the authorities created in

situ alerts and also in internet warnings about the status of this problem. In order to decrease the risk in the area, cyanobacterial growth should be monitored similarly that were studied at New Zealand (Wood and Williamson, 2012) creating three different kinds of alerts according to the percentage of coverage of the sediment with potentially toxic benthic cyanobacteria.

When a potential toxic mat is found it is important to isolate strains for all the potentially toxic species and morphotypes present in the sample. The purpose of it is to be able to know and characterize in detail the toxic species and morphotypes, and also to perform several studies in order to know under which specific circumstances the toxin will be produced and released. For that reason, seven isolated cultured strains from previous toxic mats (Kønig, 2013; Haya, 2016) and sixteen new isolations from Verde River were studied in order to characterize its morphology and toxicity.

From 23 isolated culture strains, over 50 % are *Phormidium autumnale*. This can be because of, in the cultures from UAM, the mats of which the sample came were dominated by *Phormidium autumnale* and *Phormidium aerogineo-caeruleum* (Kønig, 2013, Haya, 2016) because that are the strains more present in Madrid river, where the samples came (Ramos, 2012; Kønig, 2013; Haya, 2016; Jimenez, 2018; Martín, 2018).

The wide and length of the filaments of *Phormidium autumnale*, that makes possible to see the filaments only using a binocular stereoscope, is, also, a reason for the easy isolation process and therefore most of the cultures are from Phormidium autumnale.

The same happens with the isolated filaments from Verde River-The samples from this river are dominated by *Phormidium autumnale* (95-99 % according to metagenomics and microscopy analyses) and in this case the filaments were bigger than in the other sample, that makes easier to isolate this species and also the abundance, which makes difficult to take another filament, not belonging to *Phormidium autumnale* group, along. Also, *Phormidium autumnale* where the aim of the study because is known that produce ATX and MYC (Quiblier *et al.*, 2013).

But even most of the cultures and isolated samples are *Phormidium autumnale* there is still high variability within the samples because some different morphotypes of *Phormidium autumnale* can be found in the samples, more than 10 isolation were done to be sure that some toxic strain was isolated. Filaments showing different colours and morphotypes (with different terminal cells) were collected

The size of the filament is difficult to compare from one sample to another because the size depends on the stage on which the cyanobacteria are and changes dramatically from the natural samples to the isolated, cultured samples. Only one characteristic is common in all the morphotypes of *Phormidium autumnale*, the presence of calyptra.

There are also cultures of other species as *Phormidium corium, Phormidium aerogineo-caeruleum, Phormidium terebiforme, Pseudanabaena mucicola* and *Oscillatoria limosa*.

The isolated culture strains used in this project can also show another data related with the theory about changes on colours of the mats depending on the age. We have observed that, even brown and green filaments were isolated, they change colour after a short period of growth-., All the new filaments forming the biofilm in the culture became green or dark green, also, this change at the filament's colour can be due to all the isolations were growth in the same light regime, which, also supports the theory about the changes depends on the amount of sunlight that they receive. In order to know the reason for the change in the colour of the mats more research should be performed, following the grow for a biofilm of *Phormidium* to a complete mat, and study the changes in the colouration of the mats depending on the light regimens as was commented by Palinska *et al.* (2011).

In the case of this project, and more concretely, the samples of Verde River from all the filaments isolated only 16 were truly isolated at this time and they have enough biomass grow to perform analysis. Only 25 % of the cultures analyzed were toxic, 3 samples are toxic for ATX and only 1 sample for STX, this low percentage of toxic isolations makes clear the importance of increasing the sample size in order to have more opportunities to isolate toxic filaments.

For the 4 strains that were toxic 3 of them were *Phormidium autumn*ale and the other one was *Pseudanabaena mucicola*, but that doesn't mean that the other species found in the samples are not toxic, because of several reasons: as is said before is needed to take in account the variability in the mat toxicity, in this project only one gen per toxin were studied but exists more, and also some species that appear in the samples as *Phormidium corium* and *Oscillatoria sp.* are described to be able to produce toxins (Aboal *et al.,* 2005; Aráoz *et al.,* 2005; Quiblier *et al.,* 2013). For these reasons the negative samples should be revised in future research trying to confirm or refute that they are not toxic.

From the cultures of UAM collection, previously isolated from Manzanares and other rivers, none of the benthic samples shows toxicity for the genes that were searched for in the project, but, the sample LE3 (*Phormidium autumnale*) that proceeds for a planktonic sample from a

reservoir show toxicity for ATX. In this project, *Phormidium autumnale* is confirmed to be able to produce ATX, supported by previous studies (Ramos, 2012; Wood, 2012; Quiblier *et al.*, 2013; Kønig, 2013; Haya, 2016; Martín, 2018), and due to this strain has the gen *anaF*, also, this gene is present also in *Pseudanabaena mucicola* as in the study of Quiblier *et al.* (2013). If those previous results are compared with the results of this project, almost all the positive PCR for this gen are from *Phormidium autumnale* cultures, and, the mats in which this gen appear, according to metagenomic analyses, are dominated by this species in amounts bigger than the 70% of the total composition.

In these studies, is very important and highly recommended to perform sequencing analyses for the toxic genes were PCR are positives, because the results may not be conclusive, because even these assays did not show results for toxicity, the strain can have the toxic gen but not producing the toxin under the ideal culture conditions for the growth. As can be seen in the results for ELISA test performed for the isolated cultured from Verde River, they are producing toxins but, at low concentrations, that a method of quantification with low sensitivity could have missed the date and the samples will be classified as non-toxic even they really are toxic.

The main problem of these types of projects resides in the difficulty of isolating toxic strains with the inherent variability described because, even a mat was toxic doesn't mean that all the filaments that are into the mat have these genes. This is the principal problem at the time to continue with these kinds of studies. This was the case of Martín (2018) and Jiménez (2018) which even using 11 *Phormidium* strains and 7 *Nostocales* strains were studied respectively, from toxic mats, they could not find any toxic strain in UAM cultures. In this project, only 21% of the total of isolated cultures ended up being toxic even all of them became from toxic mats.

Taking in account the toxicity and morphology results toxic *Phormidium* species can't be distinguished from non-toxic ones, in this study in which the sample size is low and only 4 cultures shows toxicity, these cultures belong to a different morphotype and in those morphotypes there are cultures who are not toxic supporting the results of Wood *et al.* (2012). This project opens new paths for research on toxins that using the toxic cultures obtained, because they are the first one from benthic rivers at UAM culture collection.

It should be highly interesting to increase the effort to find toxic cultures from the single strains with isolation unfinished of those previously initiated or they didn't grow enough yet, and, to perform PCR for different toxins genes from those who were studied in this project and from other kind of toxins that are not studied here.

Secondly, it is also important to continue with the characterization of the isolations that according with this project are toxic, on the one hand is important to perform analysis in order to know if these strains have different toxic genes than those that were analyzed, performing sequencing of the genes that are not already done. Also, to sequence 16S rRNA gene in order to have a genetic taxonomical approach should be interesting to characterize the strains in detail. Finally, to perform ELISA and HPLC analysis to these samples in order to know if they are producing toxins, in which amounts, and in which conditions to find relations on toxin production and some parameters like light, temperature between others.

Finally, now that now that the isolation of toxic strains is achieved is important to perform several experiments to know under which kind of conditions the strains will produce and release the toxins, for example exposing the samples to different temperatures or different media with deficit or abundance of some nutrients and after a time of exposure measuring the amount of toxin in the media and in the cells, and also the morphological differences that can give us a way to know were a mat will be more toxic.

Moreover, it could be also interesting, also, to perform metagenomic analysis of the V3-V4 region on other natural mats, as in the analysis used in this project for Verde river samples, in order to be able to compare the composition and structure of different mats with others Spanish rivers.

5. Conclusions

- The main specie that can be found in the isolated cultured samples (L2N, LE3, MED2, M1 and M2) is *Phormidium autumnale,* also another species appeared as *Phormidium corium* (MED1) and *Phormidium terebiforme* (Z52).
- There is a clear variation from *Phormidium* mats to cultures, in the mats the size of the cells is, in all the cases, bigger than in the culture, and in some cases the colour change, mainly from brownish to dark green but this is not depending on the procedure of the sample
- Mats from Verde River are dominated by *Phormidium* strains, mainly *Phormidium autumnale* in a (95-99%) but also appear another species that can be responsible for the toxicity as *Pseudanabaena mucicola* and *Oscillatoria limosa*.
- mats from Manzanares and Eresma Rivers and Fuente de los Tilos spring were also dominated by *Phormidium autumnale*, and *Phormidium corium* in some cases (MANZPG mat)

- Most of the *Phormidium* mats from Manzanares and Verde River, where *Phormidium autumnale* is dominant, are able to produce ATX.
- All benthic cultured strains of *Phormidium sp.* were characterize as potential anatoxin producer, do not show any potential toxicity except the culture LE3 isolated from a reservoir at León what was positive for *anaF* gene.
- 11 mats of *Phormidium* from different rivers were studied, the 36 % have *anaF* gene and the 54 % shows toxins detected with an ELISA test. *Phormidium* mats from Verde River are also potential producers of SXT, MYC and CYL due to the positive results by specific PCRs. samples from Eresma River and Fuente de los Tilos are not toxic according to PCR analysis.
- Anatoxin toxicity of mats from Verde River has high level indicating probably the causes of dog deaths the area, join with the presence of filaments into the sto ach and intestine of one died dog. Low concentration of other toxins MYC, CYL, and SXT were obtained-
- *Phormidium* mats found in Manzanares River are toxic for ATX as is confirmed by PCR and ELISA test but those
- Some of the new isolations strain from Verde River have *anaF* gene (less than 17% of the filaments isolated) all of them are *Phormidium autumnale* strains and only 6 % has *sxtA* gene, characterize as *Pseudanabaena mucicola*.
- To assess and detect health risks events it is important to normalize a protocol of action and analysis of potentially toxic samples in order to have the more accurate results.
- More research is needed in order to know about the environmental conditions that increase the presence of these toxic mats and also when toxins are producing at different environmental parameters.

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Annexe I

Table 20. Comparation of the taxonomical assignation using NCBI, greengenes and SILVA 128 databases.

OUT	NCBI (BLAST)	Similarity (%)	Greengenes	Similarity (%)	SILVA128	Similarity (%)
OUT 1	*Phormidium autumnale CAWBG646*	100	Phormidium	100	Cynabium PCC-6307	97
OUT 2	*Uncultured Phormidiaceae cyanobacterium clone 239 16S ribosomal RNA gene, partial sequence*	99.51	Oscillatoriophycideae	85	Phormidiaceae	100
OUT 3	*Phormidium formosum P07 16S ribosomal RNA gene, partial sequence*	99.45	Phormidium animale	100	Kamptonema PCC-6407	95
OUT 4	*Uncultured cyanobacterium clone LV20_356 16S ribosomal RNA gene, partial sequence*	100	Cyanobacterium	97	Nostocales	100

Annexe II.

Table 21. Raw data of ELISA, RBA and HPLC measurements of each toxin for Verde River samples in µg of toxin/g of dry weight.

Method	Sample	ATX	STX	MYC	CYL
HPLC	1.1	188,82	0	18,18	n.a*
	1.2	367,56	0	8,17	n.a
	1.3	177,14	0	5,85	0
	2.1	215,08	0	6,44	n.a
	2.2	300,22	0	72,22	n.a
	2.3	136,06	0	14,78	0
ELISA	1.1	12053,91	0,072	1,24	n.a
	1.2	3829,43	0,072	1,05	n.a
	1.3	1186,59	0,076	1,15	n.a
	2.1	3835,60	0,13	1,85	n.a
	2.2	1308,64	0,11	1,77	n.a
	2.3	5826,13	0,11	1,87	n.a
RBA	1.1	12026,57			
	1.2	1734,35			
	1.3	1185,25			
	2.1	3829,93			
	2.2	1307,39			
	2.3	5815,95			

*N.A= Not Analyzed

Annexe III.

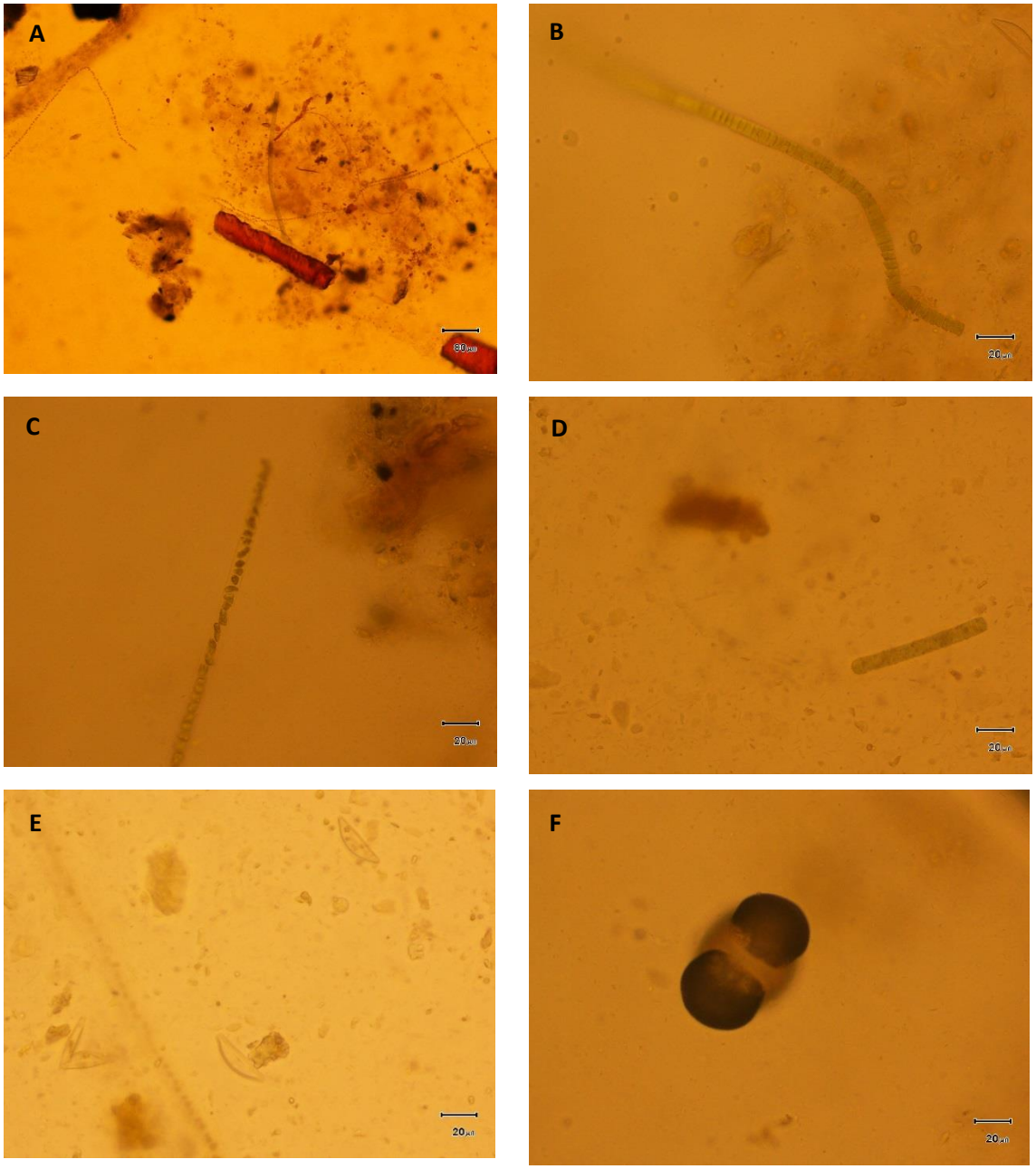

Figure 10. Microphotographies of the stomach and intestine content of dog dead by cyanobacteria ingestion. (A) stomach content, (B) Filament of *Oscillatoria limosa*, (C) Filament of *Phormidium,* (D) Hormogonia of *Phormidium*, (E) Diatoms in the intestine, (F) Pine tree pollen.