DISTRIBUTION AND ECOLOGY OF CYANOBACTERIA AND CYANOTOXINS IN SPANISH WATER RESERVOIRS

Miguel Medina Cobo

Universidad Autónoma

de Madrid

2022

Doctoral Dissertation

Tesis Doctoral

MIGUEL MEDINA COBO

Madrid, 2022

Distribution and ecology of cyanobacteria and cyanotoxins

Distribución y ecología de cianobacterias y cianotoxinas

Memoria presentada para optar al Grado de Doctor en

Microbiología

Miguel Medina Cobo

Licenciado en Biología

Director:

Dr. Antonio Quesada del Corral

Catedrático de Universidad

Departamento de Biología

Universidad Autónoma de Madrid

Codirectora:

Dra. Caridad de Hoyos Alonso

Doctora en Biología por la Universidad de Salamanca

Directora de Programa en el Centro de Estudios Hidrográficos

del CEDEX (2010-2016)

A mi familia: presente y futura

Table of contents

Summary ..... **i**

Resumen ..... **iii**

Abbreviations ..... **v**

Figure index ..... **vii**

Table index ..... **x**

Supplementary data index ..... **xi**

General introduction **1**

Cyanotoxin production 3

Invasive cyanobacteria species 6

Cyanobacteria and remote sensing 9

Thesis objective **12**

Chapter 1. Estimation of cyanobacteria biovolume in water reservoirs by MERIS sensor **14**

1.1 Abstract 15

1.2 Introduction 16

1.3 Available data 19

1.4 Field sampling and analysis methodology 19

1.5 MERIS image methodology 20

1.6 Data selection and area of study 23

1.7 Results validation and statistical analysis 25

1.8 Results and discussion 25

1.9 Conclusions 34

Chapter 2. Ecology of invasive cyanobacteria from two contrasting climates: Nordic and Mediterranean habitats 36

2.1 Abstract 37

2.2 Introduction 38

2.3 Database comparison 43

2.4 Results and discussion 47

2.5 Species autecology 55

2.6 Conclusions 59

Chapter 3. The untapped potential of cyanotoxin production. Laboratory vs Natural conditions 61

3.1 Abstract 62

3.2 Introduction 63

3.3 Methodology 66

3.4 Results and discussion 71

3.5 Conclusions 77

General Discussion 81

The power of remote sensing 81

The dangers of invasiveness 83

The dangerous peak of cyanotoxin production 84

Conclusions and further work 86

Bibliography 93

URL's 101

Supplementary material 102

Summary

Cyanobacteria are photosynthetic microorganisms that inhabit all kinds of aquatic environments. Freshwater planktonic cyanobacteria usually inhabit lentic waters such as reservoirs and lakes. Cyanobacteria represent a serious hazard for human health, not only because of the negative effects of massive growth of phytoplankton can cause, but also because cyanobacteria can produce toxic metabolites called cyanotoxins. Apart from the potential toxicity, blooms can also be harmful because they distort the aquatic environment due to blocking sunlight, exhausting the nutrients and/or strongly decreasing and even depleting the oxygen of the body of water in which they are located.

Due to these risks it is essential to study innovative monitoring methods, understand the ecology that organizes the distribution of cyanobacteria, especially those that can be invasive and therefore have the potential to damage any new niche they try to colonize and finally understand the mechanisms that limit cyanotoxicity to understand what the real toxic potential of a toxin-producing cyanobacterium is.

To achieve this in this thesis we will divide these objectives into 3 chapters: In Chapter 1, we will study the estimation of the biovolume of cyanobacteria by remote sensing with the MERIS sensor. To do this, we will rely on the possible measurement of phycocyanin, a pigment practically exclusive to cyanobacteria and compare it with

biovolume measurements in the field. A significantly accurate estimation was achieved, as these estimated values of biovolume were significantly similar to biovolume samples measured from field samples from the same day

In Chapter 2, we will see in what ecological conditions several invasive and native species are found in two very different climates: the Nordic and the Mediterranean. The objective of this study is to identify ecological traits that promote the distribution of 5 cyanobacteria taxa on a global scale, specific ecological traits were found for each taxa, and our results for invasive species is that thy seem to have a more niche ecological needs (certain temperature, level of eutrophication, etc…), meanwhile ubiquitous cyanobacteria thrive in a very wide range of environments.

In Chapter 3, with the aim of studying the toxic potential of certain species of cyanobacteria, we will study how much toxin some species of cyanobacteria are to produce in the field and compare it with how much they produce the same isolated strains and under optimal laboratory conditions. The result was that, depending of the toxin, the cyanobacteria has the potential to produce between 100 to 750 times more cyanotoxins per unit biomass that they are producing in the field, a scenario that is worrying when this could happen in water bodies linked to human health.

Resumen

Las cianobacterias son microorganismos fotosintéticos que habitan en todo tipo de medios acuáticos. Las cianobacterias planctónicas de agua dulce suelen habitar en aguas lénticas como embalses y lagos. Las cianobacterias representan un serio para la salud humana, no solo por los efectos negativos que puede provocar la eutrofización típica del fitoplancton, sino porque las cianobacterias pueden producir metabolitos tóxicos llamado cianotoxinas. La producción de cianotoxinas se eleva en periodos de elevada eutrofización, causada por la aportación natural o artificial de nutrientes y en casos extremos pueden provocar la aparición de blooms, formaciones masivas de cianobacterias visibles a simple vista. Aparte de la potencial toxicidad los blooms también pueden ser dañinos debido a que distorsionan el medio acuático debido a bloquear la luz solar y/o fuertemente disminuir e incluso agotar el oxígeno de la masa de agua en que se encuentran.

Debido a estos riesgos es esencial estudiar métodos de monitoreo innovadores, entender la ecología que organiza distribución de las cianobacterias, especialmente aquellas que puedan ser invasivas y por lo tanto tengan el potencial de dañar cualquier nuevo nicho que intenten colonizar y por último entender los mecanismos que limitan la cianotoxicidad para entender cuál es el real potencial tóxico de una cianobacteria productora de toxinas.

Para conseguir esto en esta tesis dividiremos estos objetivos en 3 capítulos: En el **Capítulo 1**, estudiaremos la estimación del biovolumen de cianobacterias por teledetección con el sensor MERIS. Para ello nos basaremos en la posible medida de ficocianina, un pigmento prácticamente exclusivo de las cianobacterias y lo compararemos con medidas de biovolumen en campo; En el **Capítulo 2**, veremos en qué condiciones ecológicas se encuentran varias especies invasoras y autóctonas en dos climas muy diferentes: el Nórdico y el Mediterráneo. El objetivo de este estudio es abordar es identificar rasgos ecológicos que promuevan la distribución de ciertos taxones de cianobacterias a escala global; En el **Capítulo 3**, con el objetivo de estudiar el potencial tóxico de ciertas especies de cianobacterias, estudiaremos cuanta toxina son algunas especies de cianobacteria de producir en campo y lo compararemos con cuanto producen las mismas cepas aisladas y en condiciones óptimas de laboratorio.

Abbreviations

ATX: Anatoxins

B2: Fluorescence of the first absorption peak of Chl-a, approximately 442 nm (MERIS band 2)

B5: The maximum solar radiation that penetrates into the water at 560 nm, corresponding to the green band (MERIS band 5)

B6: Absorption peak of PC, centred at 620 nm (MERIS band 6)

B7: Second absorption peak of chlorophyll-*a*, approximately 665 nm (MERIS band 7)

B9: Fluorescence of Chl-a, approximately 705 nm (MERIS band 9)

Chl-*a:* Chlorophyll-*a*

CYN: Cylindrospermopsin

EOLi: Earthnet On-Line Interactive

$E_{dir}\mu_{il}$: Direct flux arriving at the surface

E_{dif}: Diffuse flux arriving at the surface

ESA: European Space Agency

FR L1b: Level 1b full resolution products

HPLC: High Performance Liquid Chromatography

L_0*:* Atmospheric path radiance

L_{TOA}*:* Surface reflectance images derived from the Top-of-atmosphere radiance

MC: Microcystins

n.d: not detected or under detection levels

NGS: Next Generation Sequencing

OTUs: Operational Taxonomy Units

PC: Phycocyanin concentration

PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

RMSE : mean square error

S: Atmospheric spherical albedo, which represents the reflectance of the atmosphere for isotropic light entering from the surface

STX: Saxitoxin

$T_{\uparrow}$: Total atmospheric transmittance (for diffuse plus direct radiation) in the observation direction

TPR: Toxin Production Ratio

WHO: World Health Organization

θ_{il},: Measurement between the solar ray and the surface normal

ρ_s: Surface reflectance.

μg: micrograms

μ_{il}: Cosine of the illumination zenith angle

<L.C: Positive result but under reliable quantification levels

Figure index

Figure 1. Map of the Spanish watersheds and the water reservoirs passing the inclusion criteria. (1) Arcos; (2) Castro de las Cogotas; (3) Cuerda del Pozo; (4) Pontón Alto; (5) El Burguillo; (6) Santa Teresa; (7) Cíjara; (8) La Colada; (9) Alange; (10) Vega del Jabalón; (11) Huesna; (12) Maria Cristina; (13) Bellus; (14) Borbollón; (15) Guajaraz; (16) Navalcán; (17) Portaje; (18) Rivera de Gata; (19) Rosarito; (20) Salor; (21) Valdecañas; (22) Valmayor; (23) Cazalegas. 23

Figure 2. Chlorophyll-a concentration comparisons between both methodologies. Both linear regressions were forced to begin in the origin (0,0) for validation purposes. Broken red line represents y=x (RMSE = mean square error). 26

a) Chlorophyll-a concentration measuring methodologies comparison. n=52; p<0.001; RMSE=0.024..... 26

b) Normalized chlorophyll-a concentration measuring methodologies comparison. n=52; p<0.001; RMSE=0.031. 26

Figure 3. Correlation between phycocyanin concentration estimated by remote sensing and total cyanobacterial biovolume. n=65; p<0.001; RMSE=0.06. (RMSE = mean square error) 28

Figure 4. Correlation between phycocyanin concentration estimated by remote sensing and number of cells per millilitre. n=65; p<0.001; RMSE=0.19. (RMSE = mean square error) 29

Figure 5. Correlation between phycocyanin concentration and total cyanobacterial biovolume depending on the dominant order (RMSE = mean square error). (Nostocales: n=30, p<0.001, RMSE=0.08; Oscillatoriales: n=30, p<0.001, RMSE=0.07; Chroococcales: n=13, p<0.05, RMSE=0.08) 30

Figure 6. Blue line (left y axis): Determination coefficients (R2) of the correlation between estimated cyanobacterial biovolume with field sampled cyanobacterial biovolume in Rosarito reservoir depending on the days of difference between both data. Red broken line (right y axis): Number of samples for each correlation. 33

Figure 7. Timeline comparing estimated cyanobacterial biovolume (calculated from phycocyanin estimated by MERIS) and field sample cyanobacterial biovolume. 34

Figure 8. Selected cyanobacteria of interest: a) *Cylindrospermopsis raciborskii*; b) Chrysosporum ovalisporum; c) D. lemmermannii; d) Cuspidothrix issatschenkoi; e) Planktothrix agardhii 40

Figure 9. Sampled lakes from Denmark and localization of P. agardhii, D. lemmermannii and C. issatschenkoi. Only data were biovolume was quantified for these species was considered. 42

Figure 10. Sampled water reservoirs from Spain and localization of Ch. Ovalisporum, C. issatschenkoi, *C. raciborskii* and P. agardhii. Only data where biovolume was quantified was considered. 43

Figure 11. Distribution of the selected cyanobacteria along the year grouped by country. Average biovolume per month from every sample with a quantified measured biovolume of each cyanobacterium 49

Figure 12. Box plot of: A) Chlorophyll-a; B) Total phosphorus; C) Secchi Depth; D) Ammonia; E) Nitrate+Nitrite. Only for locations with the cyanobacteria in question. Letters on top of the box plots (a, b, c, and combinations significantly represent groupings ($p<0.05$). (*): Analysis of variance did not find significant differences between any dataset. 50

Figure 13. Box plot of: A) Alkalinity; B) Conductivity; C) Oxygen saturation; D) pH; E) Max depth of water bodies. Only for locations with the cyanobacteria in question. Letters on top of the box plots (a, b, c, and combinations significantly represent groupings ($p<0.05$). (*): Analysis of variance did not find significant differences between any dataset. 51

Figure 14. Box plot of: A) surface Water Temperature; B) Minimum Air Temperature (what height); C) Sum of accumulated precipitation during growing season (June – November) in log scale; D) Mean air Temperature; E) Sum of Hours of bright sunshine precipitation during growing season (June – November); F) Maximum air Temperature. Only for locations with the cyanobacteria in

question. Letters on top of the box plots (a, b, c, and combinations significantly represent groupings ($p<0.05$). (*): Analysis of variance did not find significant differences between any dataset. 52

Figure 15. Principal Component Analysis (PCA) for each country dataset 53

Figure 16. Aerial photo of Rosarito reservoir 66

Figure 17. Proportion of phytoplankton in the samples of Rosarito (only showed the species representing above 5% of the total phytoplankton biomass) 71

Table index

Table 1 Minimum, maximum, mean, median and number of samples (n) of physical and biological parameters of the reservoirs studied. 24

Table 2. Summary of time differences between time both methodologies (n=65) 27

Table 3. Methodology for biological and physical and chemical analyses..... 44

Table 4. Water bodies abiotic and biological parameters from samples with identification(s) of any cyanobacteria of interest..... 47

Table 5. Biovolume Data summary 48

Table 6. Rosarito's reservoir characteristics 67

Table 7 Results of phytoplankton identification and quantification. Biovolume measured in mm3/m3. 72

Table 8. Content in μg/L in the field samples measured with ELISA (Casero, et al. 2019) 73

Table 9. Content in cultures isolates. Toxins are measured in μg/L; n.d: not detected or under detection levels; <L.C: Positive result but under reliable quantification levels 74

Table 10. Toxin quantity and cyanobacteria biovolume ratio (TPR) in field samples and isolates. (*) This ratio was calculated with *Limnothrix redekei* biovolume..... 75

Table 11 NCI BLAST identification of the cyanotoxin producers strains and comparison with related strain analysed in Casero et al (2019). Similitude cover is a percentage that describes how much of the sequence of the isolate's DNA is covered by the target sequence (strains from the NGS). Percent identity is how many in each sequence are identical in percentage (the higher the percent identity is, the more significant the match)..... 78

Supplementary data index

Table A1. List of parameters used in the PCA 103

Table A2 .NCI BLAST identification of relevant OUT's from Casero et al (2019) 104

General introduction

Cyanobacteria are photosynthesizing microorganisms whose evolutionary importance is crucial on a global scale. Signs of their presence on the Earth's surface date back 3.5 billion years (Cavalier-Smith 2006). They played a fundamental role in Earth's history by enabling the rise of atmospheric oxygen in the atmosphere, known as the Great Oxidation Event, 2.3 billion years ago (Sánchez-Baracaldo and Cardona 2020). Their evolution into oxygenic photosynthesis gave rise to the most significant bioenergetic process on our planet, making possible the oxygenation of Earth's atmosphere and oceans and the diversification of complex life (Bohunicka 2015). Throughout their evolution, cyanobacteria became one of the most diverse and widely distributed prokaryotes. Nowadays, cyanobacteria are a phylum distributed worldwide (Whitton and Potts 2002), occupying many niches within benthic, planktonic and terrestrial habitats (Walter, *et al.* 2017).

Cyanobacterial development is significantly stimulated by eutrophication and nutrient excess in water (Vasconcelos 2006; Njagi, *et al.* 2022). In limnological studies, eutrophication levels are assessed by quantifying phytoplankton biomass (Willen 1997) and chlorophyll-*a* (Chl-*a*) (Hart 1984). The Water Framework Directive[1] and official regulations at country level (Carvalho, *et al.* 2013) specify

that the ecological status based on phytoplankton should be defined by measuring biomass, composition and blooming events in the phytoplanktonic community. Chl-*a* concentration is a good parameter to evaluate the biomass of the whole phytoplanktonic community. However, it does not provide information on the composition of the phytoplankton community, which needs to be assessed through biovolume estimations. Recent studies show that planktonic cyanobacterial biovolume can be used as a suitable metric for the assessment of the ecological status of lakes and reservoirs (Carvalho, *et al.* 2013). These planktonic cyanobacteria thrive mainly in lentic water bodies, including lakes and water reservoirs, and in some cases, toxin-producing cyanobacterial population can dominate the phytoplankton (Chorus, *et al.* 2021). This could affect humans as consumers of water for different purposes, including direct water consumption and its use for recreational purposes or crop irrigation (Weber, *et al.* 2020).

As a result of increasing eutrophication, cyanobacterial blooms are spreading in frequency and intensity in many inland water bodies at global scale (Paerl and Barnard 2020). These blooms are of growing concern for public environmental entities and agencies, water authorities and international health organizations, due to the fact that cyanobacterial blooms severely disrupt ecosystem functioning and that many cyanobacterial species produce a variety of toxins harmful to both humans and animals (Agha, *et al.* 2012). As lakes and reservoirs worldwide are affected by these blooms, regardless of underlying

natural or human causes, the resulting eutrophication process favours toxic cyanobacterial bloom formation over other types of true algae (Merel, *et al.* 2013). The spread of cyanobacterial blooms has become a thriving concern associated to numerous health problems of increasing scientific interest and public awareness (Harke, *et al.* 2016; Huisman, *et al.* 2018).

Cyanotoxin production

In terms of human health risk assessment, cyanobacteria can pose hazards when producing cyanotoxins, secondary metabolites affecting human organs and systems (liver, kidneys, skin, nervous system, cells, etc.) (Chorus, *et al.* 2021). Harmful consequences of cyanobacterial toxins have been extensively reported in the scientific literature. Their harmful effect is reported not only in laboratory bioassays, but also in clinical and epidemiological studies, confirming the effects of toxic cyanobacteria in humans (Lee, *et al.* 2017; Kaloudis, *et al.* 2022) among the most toxic natural- compounds (Chorus, *et al.* 2021).

The production of cyanotoxins is closely related to larger quantities of cyanobacterial biomass, especially when large blooms are formed (Spoerke and Rumack 1985). In this regard, world-renowned organizations such as the World Health Organization (WHO), have established criteria specifying cyanobacteria abundances (cell numbers) and biovolumes corresponding to different levels of

threats to human health (Chorus, *et al.* 2021). To promptly respond to dangerous cyanotoxin-producers blooms, it is necessary to continuously monitor the accumulation of cyanobacterial biomass in water bodies. Worldwide, national governments have shown increased concern on this matter, and have developed tailored regulations and guidelines to monitor and control cyanobacteria blooms in lakes, reservoirs and rivers, especially if related to bathing zones, by quantifying cyanobacterial biovolume, cell number, Chl-*a* or cyanotoxin (i.e. microcystins) concentration (MAGRAMA 2013; Chorus, *et al.* 2021).

The efficient management of water resources from harmful cyanobacterial blooms is of fundamental importance to protect human health (Merel, *et al.* 2013). Rising concerns regarding the monitoring of cyanobacteria proliferation and their potential toxicity have led to the identification of most widespread cyanotoxins and their occurrence: microcystins (MCs), anatoxins (ATX), saxitoxins (STXs) and cylindrospermopsin (CYN) (Merel, *et al.* 2013). A wide variety of planktonic cyanobacteria can produce cyanotoxins, which are also broadly classified into hepatotoxins, neurotoxins, dermatoxins and cytotoxins (Chorus and Bartram 1999; Meriluoto, *et al.* 2017).

Despite the risk they have been shown to cause, very few cyanotoxins are regulated for water monitoring, including those affecting water for human consumption (Chorus, *et al.* 2021). Some cyanotoxins can quickly deteriorate in

aquatic environments, leading to a diluted toxic effect with a non-immediate threat (Lezcano, *et al.* 2017). However, processes such as the formation of cyanobacterial blooms, usually due to excess of nutrients (mostly phosphorus and nitrogen) (Conley, *et al.* 2009; Li, *et al.* 2022) can increase the production and accumulation of large amounts of toxins into the environment (Boopathi and Ki 2014). A high significant increase in the production of cyanotoxins can cause them not to safely degrade in the environment itself. Also, the high mortality of cyanobacteria reported in cyanobacterial blooms adds toxicity to the environment. In these formations, cell breaks are highly remarkable, leading to the release of significant quantities of toxins to the environment (Lee, *et al.* 2017; Vilar and Molica 2020).

Climate change further aggravates this problem (Jöhnk, *et al.* 2016).The frequency and magnitude of cyanobacterial blooms are expected to worsen in the near future due to increased surface water temperatures and vertical stratification (Mishra, *et al.* 2019). The combination of water scarcity, high temperatures and eutrophication, aggravated by human activities including excess of nutrients from different human sources (like industry, agriculture, untreated sewer water, etc.) leads to a future scenario of increasing cyanotoxicity (Paerl and Huisman 2008), more noticeable in lentic water bodies such as lakes and reservoirs (Jöhnk, *et al.* 2016). Numerous studies report which cyanobacteria species produce toxic

metabolites, but few focus on the amount of cyanotoxins each specie produce per unit volume in certain controlled conditions (Akcaalan, *et al.* 2006). It is still unknown if in nature the production threshold of cyanotoxins has been already reached or if, on the contrary, it is limited. This is a significant source of concern due to climate change uncertainties, since current environmental conditions may change, adding a potential multiplier to cyanotoxins production (Merel, *et al.* 2013). Further research on the quantity of cyanotoxins produced is not only crucial at field level but under controlled laboratory conditions (e.g., light intensity or nutrient availability) to further understand the potential capacity for toxicity of cyanobacteria in changing climate environments.

Invasive cyanobacteria species

Future predicted scenarios of increasing cyanotoxicity (Paerl and Huisman 2008) are specially remarkable for cyanobacteria species capable of adapting to extreme ecosystems, especially toxin-producers invasive species (Zong, *et al.* 2019). Nowadays, in the course of global warming, many species could expand their range from lower to higher latitudes (Cottingham, *et al.* 2020; Olofsson, *et al.* 2020). It is well established that not only cosmopolitan cyanobacterial species, but also species with holarctic or pantropical distributions exist (Komárek 1995; Hoffmann 1996). Cylindrospermopsis raciborskii is a prime example of a cyanobacterial species that has spread from tropical and subtropical to temperate latitudes of the northern and southern hemisphere over the last decades (Wiedner, *et al.* 2007).

The term *invasive* or alien cyanobacteria commonly appears in the literature, where invasive means that the species has been transported by humans, while alien means that the species has been established outside of its natural range (Mehnert, et al. 2010; Al-Awadhi, et al. 2017; Kim, et al. 2021). These terms can be objectively understood as a biogeographical phenomenon, meaning that any species that invades another non-native habitat and colonizes it, affecting native inhabitants (and even disrupting the ecosystem equilibrium), is defined as invasive (Colautti and MacIsaac 2004; Sukenik, et al. 2015; Sagoff 2018). The invasive species threat is so relevant for the protection of the environment that specific legislation has entered into force to control their populations to avoid harming the environment (EU 2014). This concern grows when it is considered that invasive cyanobacteria, when growing massively, may lead to the accumulation of a variety of secondary metabolites that are toxic to both humans and animals (Meriluoto, et al. 2017; Chorus, et al. 2021). For management purposes, it is essential to understand the ecological traits of cyanobacterial communities in lentic water bodies and to pay special attention to these potentially toxic invasive species new to the invaded ecosystem (Boopathi and Ki 2014; Mehnert, et al. 2014).

Invasive species could aggressively colonize their new environment elevating in high proportion the risk of massive toxin production. This risk is

mainly due to the invasive cyanobacteria producing, among other toxins, non-monitored cyanotoxins (e.g., cylindrospermopsin), more potent cyanotoxins or cyanotoxins from which the authorities lack reliable countermeasures. In this thesis, the definition of invasive is the accepted term by the International Union for Conservation of Nature, that defines invasive as the widespread of non-native species that have adverse effects on the invaded habitat (Colautti and MacIsaac 2004; Sukenik, *et al.* 2015). Therefore, invasive cyanobacteria will be treated herein as species clearly mentioned in the literature under such definition.

Although most remarkable legislation and monitoring programmes are devoted to macroscopic organisms, more recent studies have analysed the biogeographical and ecological preferences of invasive microorganisms (e.g., Wilk-Woźniak and Najberek 2013; Sukenik, *et al.* 2015). Such analyses conclude that anthropogenic disturbances such as climate change and eutrophication, in combination with specific preferential factors affect the distribution of such organisms (Kokociński and Soininen 2012; Wilk-Woźniak, *et al.* 2015). These disruptions may have played an important role in the invasive nature of cyanobacteria over the last decades as they could offer new opportunities to colonize non-native ecosystems in which invasive cyanobacteria could not have developed before (Paerl and Huisman 2009).

Common abiotic parameters have long been shown to influence selection and growth rates of potentially toxic species (Pitois, *et al.* 2014). Further research on climate influence on cyanobacteria is crucial to have more insights of the potential future situation regarding cyanobacteria (species composition, toxin occurrences) worldwide, identifying the ecological characteristics that promote the distribution of certain invasive cyanobacteria taxa at global scale.

In this regard, recreational and drinking water bodies are monitored by relevant authorities to prevent and control animal and human intoxications related to cyanobacteria blooms and their toxins (Lee, *et al.* 2017). Recommended monitoring programmes include assessing total phytoplankton biomass estimated from chlorophyll-*a* concentrations, identifying the genera or species of cyanobacteria present, and measuring cyanotoxins concentrations in situ (Chorus 2005; Weber, *et al.* 2020).

Cyanobacteria and remote sensing

Source-based water monitoring programmes require extensive financial resources including staff time to complete sampling, laboratory analyses, and technical expertise to interpret data (Almuhtaram, et al. 2021). Using traditional field sampling methods are costly, time-consuming, and often not feasible to carry out in multiple waterbodies or across multiple states (Mishra, *et al.* 2019). Monitoring cyanobacteria promptly and accurately in numerous larger water

bodies on a routine basis and to retrospectively assess the historical status of these water bodies requires complementary advanced techniques such as satellite remote sensing (Dörnhöfer and Oppelt 2016; Yan, *et al.* 2018).

Remote sensing offers thriving advantages in the detection of aquatic pigments (Meroni, *et al.* 2009; Almuhtaram, *et al.* 2021). Earth Observation methods cover a range of procedures focused on monitoring the Earth by means of electromagnetic radiation sensors located on spaceborne or airborne platforms (Donlon, *et al.* 2012). The information provided allows for spatial and temporal scale-information unfeasible to be achieved by traditional ground measurements (Guo, *et al.* 2022) . Particularly, optical passive remote sensing methods are based on the study of the Earth's surface by measuring the reflection of solar radiation by the observed target, transmitted through the atmosphere to the sensor[1]. Many advances in instrumental design and processing algorithms have been achieved in the last decades, enabling the launch of international satellite missions for Earth observation, including the European Space Agency's (ESA) Envisat mission (Ali, *et al.* 2013).

Different Earth Observation instruments were placed on board the satellite Envisat. Among them, the Medium Resolution Imaging Spectrometer (MERIS) [2], a programmable spectrometer with high spectral and radiometric resolution that captured images of the entire world from March 2002 until April 2012. MERIS

had a within a global mission covering open ocean and coastal zone waters (Rast, *et al.* 1999). In particular, MERIS sensor narrow bandwidth allows for an accurate estimation of photosynthetic pigments in inland waters, although its spatial resolution (300m) is not suitable for some freshwater studies (Kutser, *et al.* 2006). However, its periodicity, which captures images of the whole Iberian Peninsula every three days permits the collection of an extensive record of images to analyse the variation of the phytoplanktonic community in large water bodies located in regions with scarce cloud cover days (Rast, *et al.* 2010).

Therefore, large water bodies and water reservoirs are tailored targets for MERIS's sensor studies, especially considering that most species of inland phytoplanktonic cyanobacteria usually live in lentic water bodies (Koponen, *et al.* 2001). However, although MERIS sensor has proven to accurately measure Chl-*a* in surface waters (Härmä, *et al.* 2001; Ali, *et al.* 2013), Chl-*a* is a pigment present in many different phylum of planktonic algae in addition to cyanobacteria (Whitton 2012).

Using remote sensing techniques in particular through information collected by MERIS can give reliable complementary results to monitor water quality frequently and efficiently, but in-situ measurements are needed to improve the measurement accuracy of using remote sensing imagery to cyanobacteria monitoring (Cook 2021).

Thesis objective

The objective of this PhD is to study the distribution and toxicity of cyanobacteria in a holistic and comparative way including different climatic regions worldwide, and a multi-factor methodology ranging from satellite-based, in-situ and laboratory measurements to understand the patterns of cyanobacterial presence in inland waters and its associated toxicity. In particular, Chapter 1 focuses on the estimation of cyanobacteria biovolume using traditional in-situ measurement against beyond state-of-the-art methods based on remote sensing technologies to monitor cyanobacteria blooms occurrence in diverse large water bodies, also on a retrospectively historical manner (Mishra, *et al.* 2019).

In particular, Chapter 1 determines cyanobacterial biomass as biovolume using satellite-imagery derived from MERIS for a set of 23 water reservoirs located in various climatic regions of the Iberian Peninsula, a complex pattern of environmental zones like Mediterranean South, Mediterranean North and Mediterranean Mountains (Ergon, *et al.* 2018). In Chapter 2, we explore the distribution of invasive and ubiquitous cyanobacteria biovolume in wide-spread climatic conditions ranging from Mediterranean ecosystems and Nordic ecosystems, which represent the opposite ends of a gradient of changes in temperature and precipitation patterns, ranging from increasingly warmer and drier summers in the south versus increasingly warmer and wetter winters in the north (Ergon, *et al.* 2018). In

particular, we assess cyanobacteria biovolume occurrence of toxin-producing cyanobacteria in both regions by looking at climatic and bio-physical-chemical parameters inherent to each region. Lastly, in Chapter 3, we assess cyanobacteria biovolume from the toxicity-production perspective, comparing the ratio of cyanotoxin produced per cyanobacteria biovolumes under laboratory and natural conditions.

Chapter 1: A study of cyanobacteria detection by using remote sensing for the development of a tool to detect large concentrations of cyanobacteria by satellite imagery automatically.

Chapter 2: A study of the invasiveness of cyanobacteria by comparing different ecologies of the most contrasting regions of Europe in terms of climate: the Nordic versus the Mediterranean.

Chapter 3: A study on the production of cyanobacteria cyanotoxins comparing laboratory cyanotoxins-production versus cyanotoxins production in the field.

Chapter 1. Estimation of cyanobacteria biovolume in water reservoirs by MERIS sensor

Chapter 1. Estimation of cyanobacteria biovolume in water reservoirs by MERIS sensor

1.1 Abstract

Planktonic cyanobacteria primarily develop in lentic water bodies including lakes and water reservoirs. In some instances, toxin-producing cyanobacterial population might dominate the phytoplankton community. Satellite remote sensing has been found useful as a tool for large spatial scale monitoring of cyanobacteria. MERIS sensor, from the Envisat satellite, took worldwide images at high frequency for 10 years. This short lapse for imaging permits the collection of an extensive record of images to analyse the variation of the cyanobacterial communities in water reservoirs for management and scientific purposes. The objective of this work is to find a relationship between measured cyanobacterial biomass as biovolume, and the estimations derived from MERIS imagery. This thesis encompasses two independent studies relying on data from 23 water reservoirs. First, a long-term global limnological research with field data collection, which, among other variables, includes cyanobacterial biovolume. Second, a survey applying processed images derived from Envisat MERIS sensor. Chlorophyll-a (Chl-a) and phycocyanin concentration (PC) were estimated from the MERIS images. PC estimated by remote sensing and total cyanobacterial biovolume measured in field samples were found to

be significantly correlated (R^2 = 0.6219; $p<0.001$). No relevant differences were found between taxonomical groups, indicating that this tool allows a good estimation irrespective of the cyanobacterial group. For validation, the algorithm derived from the entire dataset was applied to the MERIS image dataset of Rosarito reservoir. An estimated cyanobacterial biovolume time series was performed and compared to biovolume data collected in an extensive sampling schedule spanning 4 years. Results Indicated a strong correlation (R^2=0.72; $p<0.001$) for measured and estimated data acquired the same day.

1.2 Introduction

C-phycocyanin, a photosynthetic pigment mostly produced by cyanobacteria, is the most representative cyanobacterial compound. Other types of phycocyanins can be found in rhodophyte species (R-phycocyanin), but they are easily differentiated from C-phycocyanin because they have different absorption peaks (Jiang, *et al.* 2001). There are also trace concentrations of C-phycocyanin in some species of cryptophyte (Rowan 1989). In some previously published works, field sampling methodologies were used to show that Chl-*a* and phycocyanin concentration (*PC*) are very closely related to cyanobacterial biovolume and cell count (Brient, *et al.* 2008).

As stablished before, MERIS sensor (Rast, *et al.* 1999) has a narrow bandwidth which permitted an accurate estimation of photosynthetic pigments in inland waters, although its spatial resolution (300m) is not suitable for some freshwater studies. If there is no cloud cover, Envisat MERIS sensor presents the advantage of gathering images of a large region, such as the Iberian Peninsula, every three days. This high frequency permits the collection of an extensive record of images to analyse the variation of the phytoplanktonic community in water bodies located in regions with scarce cloud cover days, such as the countries of the Mediterranean coast (Rast, *et al.* 1999).

With the development of accurate algorithms, successful estimates of *PC* with MERIS were achieved when compared to *PC* from field samples (Simis, *et al.* 2007; Ruiz-Verdú, *et al.* 2008; Guanter, *et al.* 2010; Domínguez, *et al.* 2011). MERIS sensor produced a large collection of images that allows to recreate a time series of the cyanobacterial community seasonal distribution in water using either Chl-*a* (Binding, *et al.* 2011) or *PC* (Agha, *et al.* 2012). Although good correlation between *PC* and cyanobacterial cell count or biovolume has been found by sampling and measuring (Brient, *et al.* 2008; Randolph, *et al.* 2008), few researchers have directly compared *PC* values measured by remote sensing to cell abundances or cyanobacterial biovolume, which are much more useful variables for water quality monitoring (Willen 1997; Chorus 2012; Carvalho, *et al.* 2013). Although previous studies have compared the *PC*

estimated by airborne remote sensing to the cyanobacterial cell count with good results (Hunter, *et al.* 2010), airborne remote sensing would be too expensive for monitoring purposes, especially in countries with a large number of inland water bodies. Some algorithms have also been developed for the MERIS sensor relating Chl-*a* levels to the cyanobacteria biovolume (Matthews, *et al.* 2012). However, until now, there have been no studies that relate *PC* estimated by the MERIS sensor to cyanobacterial biovolume or cell counts. The few studies in inland waters that compared *PC* values with cyanobacterial cell numbers or compared Chl-*a* with cyanobacterial biovolume only covered four or less water bodies (Hunter, *et al.* 2010; Matthews, *et al.* 2012). In this article, we use data from a large number of water bodies with different trophic statuses and with different chemical characteristics distributed over a wide geographical scale.

In Spain, there are more than 1,500 water reservoirs, in some of them cyanobacteria are frequently dominant, especially in the southwest region of the Iberian Peninsula (De Hoyos, *et al.* 2004; Quesada, *et al.* 2004). Spanish water reservoirs have also been analysed by remote sensing for water quality purposes and to analyse cyanobacterial parameters such as cyanobacterial biovolume or cell count (Ruiz-Verdú, *et al.* 2008; Agha, *et al.* 2012).

In this work, water reservoirs from all over Spain from 2004 to 2010 were sampled, resulting in a large database of field samples that can be compared to the MERIS images. The objective of this research is to find the relationship between cyanobacterial parameters obtained by both methods and to investigate whether this relationship depends on the taxonomic classification of the dominant biomass. This relationship can be used to estimate the cyanobacterial biovolume of a water reservoir from the images taken by the MERIS sensor.

1.3 Available data

Data were obtained from two independent studies: a limnological survey in which numerous water reservoirs from all over Spain were sampled several times between 2004 and 2010 and another study in which all available MERIS sensor images from the Iberian Peninsula taken close to the time of the limnological research were retrieved.

1.4 Field sampling and analysis methodology

Field sampling took place during the summer months (usually from June to September) over one or more years in many water reservoirs. The sampling points were located 100 meters from the dam, and samples were taken from a single point located at the maximum Chl-*a* concentration depth (as defined by the profile obtained with the probe YSI6600 V2). This was always within the euphotic zone

(defined as 2.5 times the Secchi disc depth). No scum episodes were detected during the samplings.

Phytoplankton was counted in the laboratory according to the Utermöhl sedimentation method (Sournia 1978). Phytoplanktonic organisms were identified at the finest taxonomic level possible. Biovolume was calculated by giving each species an approximate simple or compound geometrical shape (Hillebrand, *et al.* 1999). Chl-*a* was extracted with 90% acetone and quantified with a spectrophotometer using the trichromatic equations (Parsons and Strickland 1968).

1.5 MERIS image methodology

The MERIS images used in this report are level 1b full resolution products (FR L1b). Suitable FR L1b images from the water reservoirs sampled were acquired from Earthnet On-Line Interactive (EOLi). Atmospheric correction (SCAPE-M_B2), Geometrical Correction (Georeferencing) and mosaicking methodologies were applied to the images. SCAPE-M_B2 is an improved version of SCAPE-M, with a correction in band 2 (Domínguez, *et al.* 2011)(see below). SCAPE-M was developed and validated in several European water bodies (Guanter, *et al.* 2010). The SCAPE-M equation is the following:

$$L_{\mathrm{TOA}} = L_0 + \frac{1}{\pi}\frac{\rho_s(E_{\mathrm{dir}}\mu_{\mathrm{il}} + E_{\mathrm{dif}})T_{\uparrow}}{1 - S\rho_s} \qquad (1)$$

Where L_{TOA} represents the surface reflectance images derived from the Top-of-atmosphere radiance; L_0 is the atmospheric path radiance; μ_{il} is the cosine of the illumination zenith angle θ_{il}, measured between the solar ray and the surface normal; $E_{dir}\mu_{il}$ and E_{dif} are the direct and diffuse fluxes arriving at the surface, respectively; S is the atmospheric spherical albedo, which represents the reflectance of the atmosphere for isotropic light entering from the surface; $T_{\uparrow}$ is the total atmospheric transmittance (for diffuse plus direct radiation) in the observation direction; and ρ_s is the surface reflectance.

SCAPE-M was corrected in MERIS band 2 by means of an interpolation between the values of band 1 and band 3 because if the original value is used, a maximum in vegetation and in water is obtained, not a minimum, because that value corresponds to the absorption of Chl-*a*. Thus, the correction with SCAPE-M is performed twice, and the assigned value of band 2 corresponds to the interpolated value of band 2 minus the value obtained without interpolation (Domínguez, *et al.* 2011). The result of this improvement is the atmospheric correction algorithm SCAPE-M_B2.

A water mask was used in the images to separate water zones from land areas, including the dam. Water/land pixels were discriminated with a simple threshold in the near-infrared band (Agha, *et al.* 2012).

In water pixels Chl-*a* and *PC* were estimated with the following algorithms (Domínguez, *et al.* 2011) and MERIS thematic mapping were generated:

When Chl-*a* ≤ 19.34 mg/m^3: Chl-*a* = $19.34e^{6.1257}$ (2)

When Chl-*a* ≥19.34 mg/m^3: Chl-*a* = $19.34e^{5.2044[(B9-B7)/(B9+B7)]}$ (3)

PC = $46.\ 478e^{5.186[(B9-B6)/(B9+B6)]}$ (4)

Where B2 is the fluorescence of the first absorption peak of Chl-a, approximately 442 nm (MERIS band 2); B5 is the maximum solar radiation that penetrates into the water at 560 nm, corresponding to the green band (MERIS band 5); B6 corresponds to the absorption peak of PC, centred at 620 nm (MERIS band 6); B7 is the second absorption peak of chlorophyll-*a*, approximately 665 nm (MERIS band 7); and B9 is the fluorescence of Chl-a, approximately 705 nm (MERIS band 9). Chl-*a* algorithms were calibrated with HPLC field data and the *PC* algorithm was calibrated with calibrate fluorometer (Domínguez, *et al.* 2011).

The Chl-*a* and *PC* values retrieved from the MERIS thematic mapping correspond to the water pixels located in a midpoint near the dam, covering the points where field samples were obtained. The MERIS sensor captures the first optic thickness, which is equivalent to 0.6 times the Secchi disc depth.

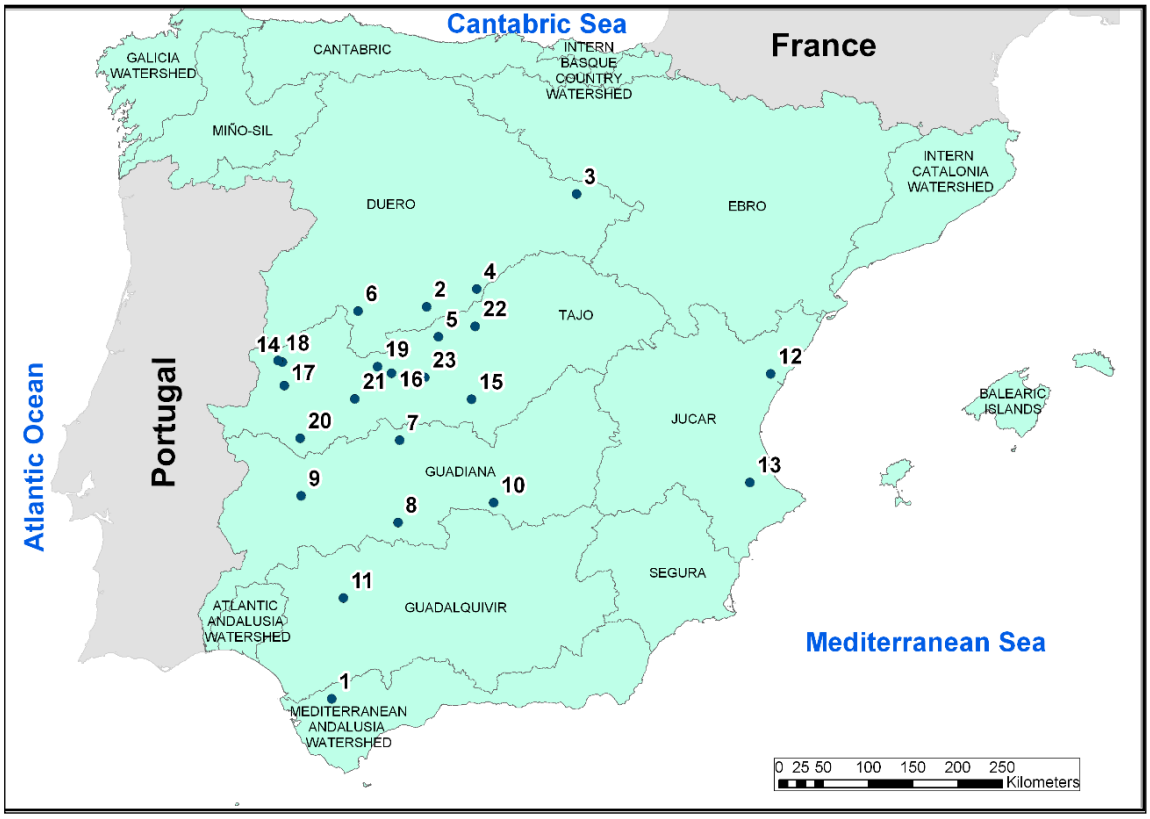


Figure 1. Map of the Spanish watersheds and the water reservoirs passing the inclusion criteria. (1) Arcos; (2) Castro de las Cogotas; (3) Cuerda del Pozo; (4) Pontón Alto; (5) El Burguillo; (6) Santa Teresa; (7) Cíjara; (8) La Colada; (9) Alange; (10) Vega del Jabalón; (11) Huesna; (12) Maria Cristina; (13) Bellus; (14) Borbollón; (15) Guajaraz; (16) Navalcán; (17) Portaje; (18) Rivera de Gata; (19) Rosarito; (20) Salor; (21) Valdecañas; (22) Valmayor; (23) Cazalegas.

1.6 Data selection and area of study

Within the database, two conditions for image inclusion were established:

1) The MERIS image must have a clear pixel that covers the field sampling point. Consequently, water reservoirs with narrow dams or small surfaces were rejected.

2) Only field sampling data with cyanobacterial biovolume greater than 0.2 mm^3/l are used. This threshold was chosen because it corresponds to the first alert level for a cyanobacterial hazard.

Water Reservoirs Physical Parameters					
	Minimum	**Maximum**	**Mean**	**Median**	**n**
Dam height (m)	16	98	49.93	47	23
Surface (ha)	88	7300	1462.89	692	23
Volume (hm^3)	7.8	1670	250.87	58.65	23
Biological Parameters					
	Minimum	**Maximum**	**Mean**	**Median**	**n**
Phycocyanin (µg/l)	17.1	456.51	87.4	69.64	65
Chlorophyl-a (µg/l) (Laboratory analysis)	0.9	290.82	56.51	25.95	65
Chlorophyl-a (Remote Sensing) (µg/l)	3.43	315.73	114.52	23.31	65
PC: Chl-*a* (MERIS)	0.28	19.61	2.58	2.34	65
Cyanobacterial cell count (cells/ml)	4716	9933031	1018974	142516	65
Total Cyanobacterial Biovolume (mm^3/l)	0.2	49.91	9.13	4.67	65
% Cyanobacteria of total phytoplankton	3.09	99.529	61.6	66.57	65

Table 1 Minimum, maximum, mean, median and number of samples (n) of physical and biological parameters of the reservoirs studied.

Using these premises, we selected a total of 23 different Spanish water reservoirs and a total of 65 field samples (Figure 1, Table1). Twelve of these water reservoirs are in the Tajo River watershed, and 21 of them are in the west-central region of Spain, which is characterised by a hot-summer Mediterranean climate.

Regions with this climate typically experience hot, dry summers and mild, wet winters. Precipitation is higher during the colder months. Comparing the MERIS sensor images to the field data is especially useful in regions with climates characterised by infrequent cloud cover days, because there would be more MERIS images matching the field sampling date.

1.7 Results validation and statistical analysis

We validated the empirical results in the complete dataset using an external dataset from a hypertrophic water reservoir, the Rosarito reservoir. Rosarito reservoir belongs to the Tietar River located in the Tajo watershed, the dam height is 38 m, has a water surface of approximately 12 km^2 and has a capacity of 92 hm^3. Rosarito reservoir was chosen because it is the reservoir with the densest data because has the most consecutive summer field samples available in the database (from 2007 to 2010).

1.8 Results and discussion

1.8.1 Chlorophyll-a comparison

Lab-measured and remote-sensing estimated Chl-a concentrations (Figure 2a) were compared and a strong correlation between them was found (R^2 = 0.967; p<0.001). Even when data are normalized the correlation is still very solid (R^2 = 0.947; p<0.001) (Figure 2b). Therefore, we can conclude that outliers are not giving a strong leverage to the R^2 value.

It is well known that distribution of cyanobacteria in a water body is not perfectly homogeneous. In fact, some papers have reported that patchy distribution of scums can produce unreliable pigment estimations by MERIS sensor (Kutser 2004). However, in the present work no scum episodes were detected during the samplings. Therefore, it was expected no impact in the correlation due to scums heterogeneous distribution.

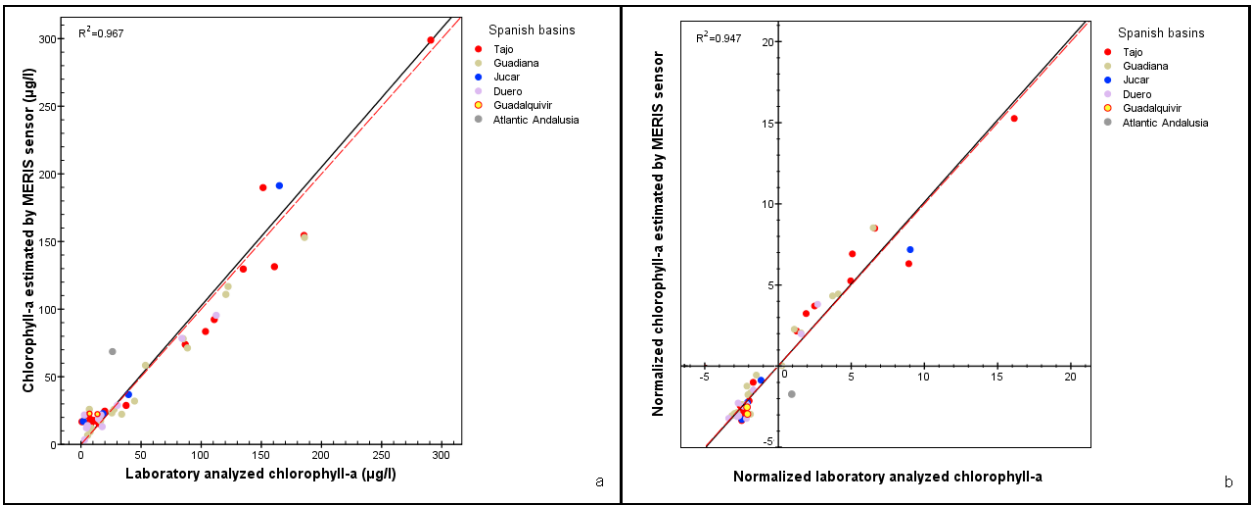


Figure 2. Chlorophyll-a concentration comparisons between both methodologies. Both linear regressions were forced to begin in the origin (0,0) for validation purposes. Broken red line represents y=x (RMSE = mean square error).

a) Chlorophyll-a concentration measuring methodologies comparison. n=52; p<0.001; RMSE=0.024.

b) Normalized chlorophyll-a concentration measuring methodologies comparison. n=52; p<0.001; RMSE=0.031.

Other possible cause of error is the vertical distribution of the cyanobacterial community. Field samples were taken from the maximum Chl-a layer within the euphotic zone, which maximum depth equals to 2.5 times the depth of the Secchi disk, However, MERIS sensor covers the first optical thickness which reaches 0.6

times the depth of the Secchi disk. Therefore, when most of the cyanobacterial community is located deeper than the first optical thickness, it might not be detected by MERIS sensor. However, when heterogeneity in the vertical cyanobacterial distribution is not intense, recent publications have found a good agreement between MERIS sensor pigment estimation and field samples (Ruiz-Verdú, *et al.* 2008; Guanter, *et al.* 2010; Domínguez, *et al.* 2011; Matthews 2011; Agha, *et al.* 2012; Dekker and Hestir 2012; Ali, *et al.* 2013; Mishra, *et al.* 2013).

In this work, field samples were taken at a certain distance from the dams, where distribution of the phytoplanktonic communities is traditionally expected to be more homogenous (Kimmel, *et al.* 1990). Moreover, algorithms used to estimate both Chl-*a* and PC in our work have been proved reliable and accurate when compared to field samples (Domínguez, *et al.* 2011).

	Minimum	**Maximum**	**Mean**	**Median**
Days of difference between MERIS image and field sampling (Absolute)	0	14	3.27	3
Matching data information				
Satellite image retrieving not blocked by cloud cover (not including matching sample dates)	**Total times satellite image retrieving was blocked by cloud cover**	**Total times that satellite image retrieving was blocked by cloud cover more than once**	**Matching sample dates**	
24	43	6	8	

Table 2. Summary of time differences between time both methodologies (n=65)

It was considered that major differences between Chl-a levels in field and MERIS data would be due to heterogeneity, at both temporal (Table 2) and spatial scales, in the cyanobacterial community, because given the brief time lag considered between both measurements, no relevant changes in the phytoplankton composition are expected. However, our data show high correlation between both Chl-a datasets (Figure 2), and it can be considered that MERIS data highly reflect the field sampled community.

1.8.2 Phycocyanin and cyanobacterial biovolume correlation

PC estimated by remote sensing and total cyanobacterial biovolume measured in field samples (Figure 3) were found to be significantly correlated (R^2 = 0.6219; $p<0.001$).

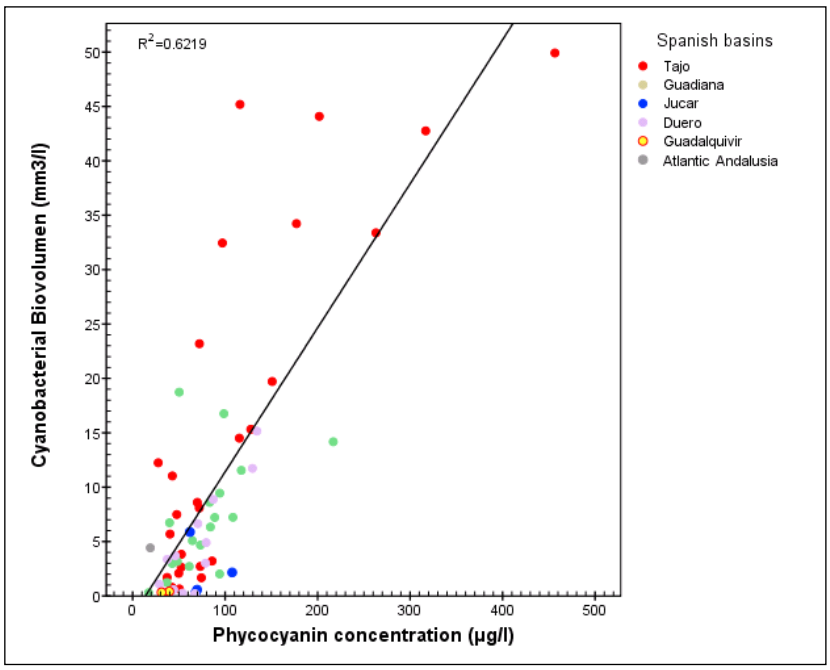


Figure 3. Correlation between phycocyanin concentration estimated by remote sensing and total cyanobacterial biovolume. n=65; p<0.001; RMSE=0.06. (RMSE = mean square error)

The values are related by the following function:

$$\text{Cyanobacterial biovolume} = (132.39 \times PC) - 1825.1 \quad (5)$$

Previous reports also found high correlations between cyanobacterial biovolume and *PC* values estimated by waterborne radiometry analysis (Randolph, *et al.* 2008). The potential regression between the number of cells per volume and *PC* showed the strongest determination coefficient.

However, it still shows a weak correlation (Figure 4), although it was still significant ($R^2 = 0.362$; $p<0.001$). This relationship was slightly weaker than that found in other studies comparing the *PC* estimated by remote sensing with cell counts (Hunter, *et al.* 2010). It is even remarkably weaker than other reported field studies that used probe samples (Brient, *et al.* 2008). However, this may be because this study includes 23 different water bodies with heterogeneous species composition that may

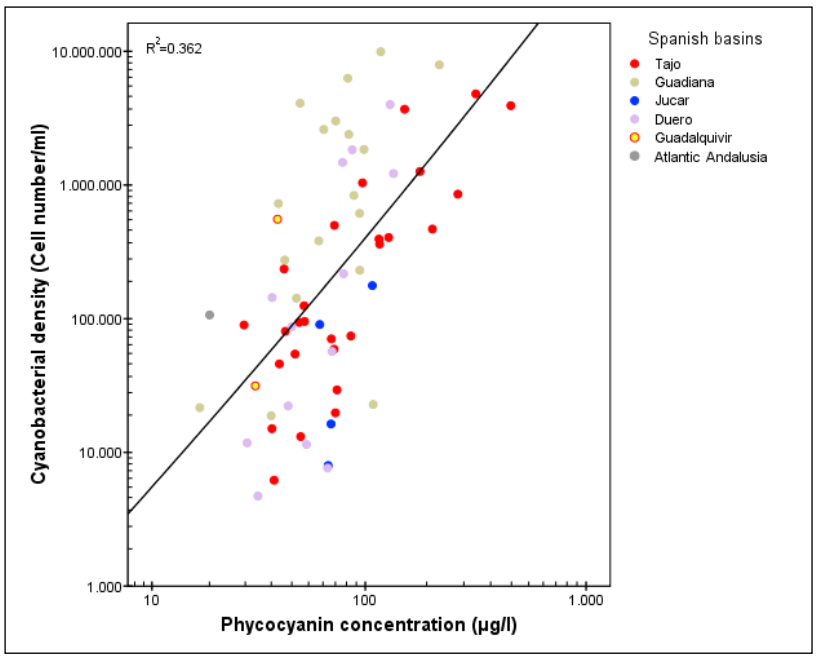


Figure 4. Correlation between phycocyanin concentration estimated by remote sensing and number of cells per millilitre. n=65; p<0.001; RMSE=0.19. (RMSE = mean square error)

scatter the data distribution.

Phycobiliprotein cell quota can vary drastically for different cyanobacteria (Patel, *et al.* 2005). Therefore, our results clearly identify biovolume as the most reliable biomass parameter to be estimated by remote sensing techniques in a diverse group of water bodies.

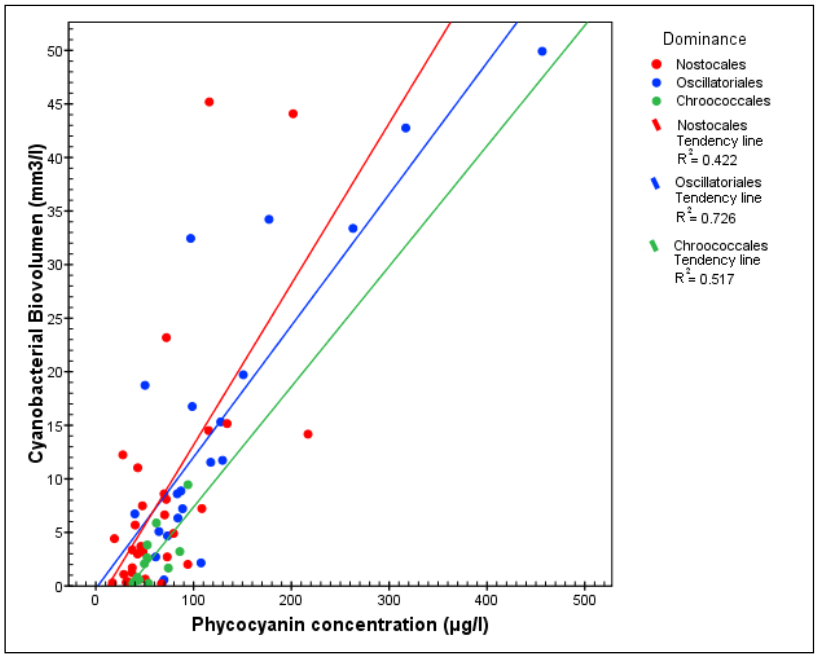


Figure 5. Correlation between phycocyanin concentration and total cyanobacterial biovolume depending on the dominant order (RMSE = mean square error). (Nostocales: n=30, p<0.001, RMSE=0.08; Oscillatoriales: n=30, p<0.001, RMSE=0.07; Chroococcales: n=13, p<0.05, RMSE=0.08)

When we considered the taxonomic distribution of the cyanobacteria found in the water bodies and subdivided the dataset by cyanobacterial order (Figure 5), we found the best correlation between biovolume and PC for the order Oscillatoriales (R^2= 0.726, p<0.001). Nostocales and Chroococcales showed weaker correlations (Nostocales: R^2=0.422, p<0.001; Chroococcales: R^2=0.517; p<0.05). Although the correlation of Nostocales is the weakest of the three orders, the pattern described by

the linear regression and the biovolume/pigment ratio is remarkably similar to that of Oscillatoriales and total cyanobacteria. The weaker correlation of Chroococcales and the different pattern followed by its biovolume/pigment correlation could be caused by the dominance of Microcystis aeruginosa (which was dominant in 6 of the 12 Chroococcales samples).

Microcystis aeruginosa has been described as highly variable in terms of its cellular pigment content, even when grown in laboratory conditions (Banares-Espana, et al. 2007). It is also important to note that Microcystis aeruginosa forms large 3D colonies, which can exhibit unexpected optical behaviour (scattering within the colony) and making cell counting difficult. The relatively small number of samples from water bodies dominated by Chroococcales (Chroococcales = 12; Nostocales = 30; Oscillatoriales = 22) could also partly explain the weaker correlation.

Taking into account the statistical robustness of our results, we can conclude that the equation given by the linear regression between cyanobacterial biovolume, and *PC* estimated by remote sensing is a very good method for calculating cyanobacterial biovolume using remote sensing tools. The dataset used in this study is diverse in terms of cyanobacterial abundance and taxonomic distribution, so our equation should be robust for assessing cyanobacterial biomass (as biovolume) in a wide range of ecosystems. This represents an improvement over

previously published methods, which were based on a very limited number of water bodies and community types (Randolph, *et al.* 2008; Hunter, *et al.* 2010; Matthews, *et al.* 2012).

1.8.3 Validation

The equation obtained with the entire dataset was applied to the MERIS imagery of Rosarito reservoir to obtain an estimated cyanobacterial biovolume time series. These time series was compared to the biovolume data measured in an extensive sampling schedule spanning 4 years.

The results (Figure 6) show a high correlation between the biovolume measured in the field samples and the one estimated by applying the equation to the satellite imagery. However, some discrepancies were found in 2010, most likely due to a significant difference between the sampling dates and the image acquisition dates. In fact, when data are disaggregated by the time lag, the determination coefficients of the respective correlation between the field sampling and the imaging show a clear decrease after 9 days' time difference between the acquisition date (Figure 7).

It is obvious that when sampling and imaging take place on the same day, the correlation is particularly good (R^2 =0.72; p<0.001).

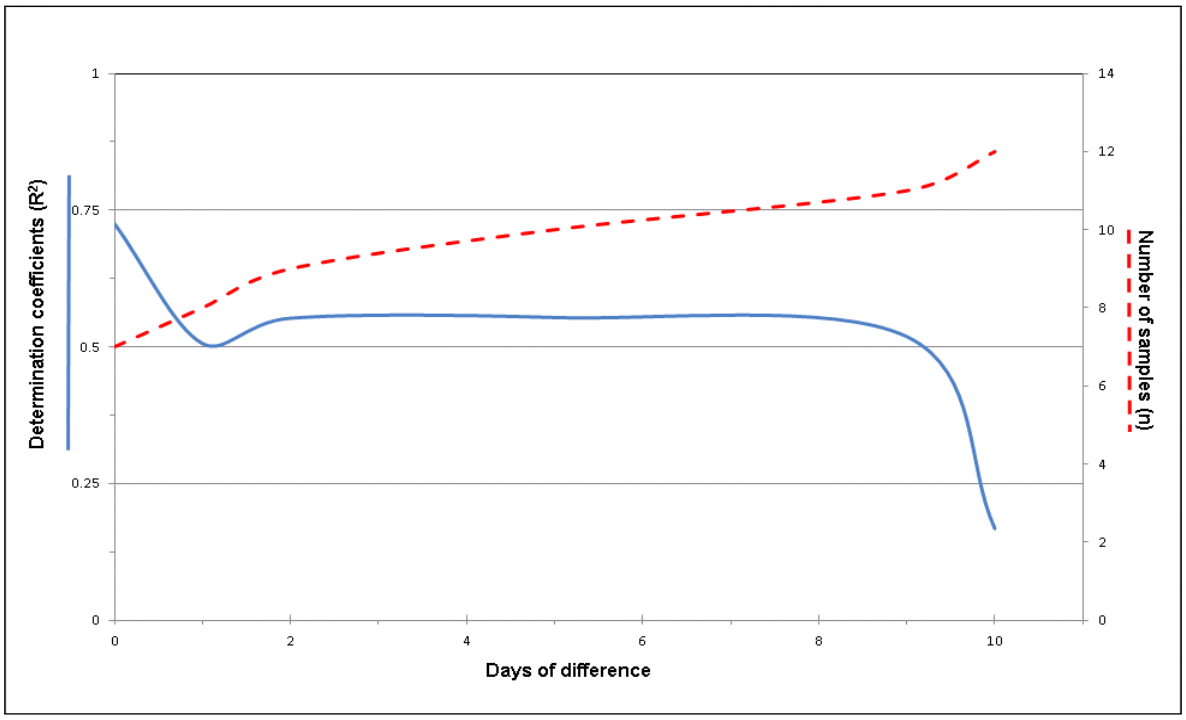


Figure 6. Blue line (left y axis): Determination coefficients (R2) of the correlation between estimated cyanobacterial biovolume with field sampled cyanobacterial biovolume in Rosarito reservoir depending on the days of difference between both data. Red broken line (right y axis): Number of samples for each correlation.

When all time lags are considered, the determination coefficients are weaker, but they are still significant until 9 days of difference (R^2 varies between 0.51 and 0.55; p<0.001). However, when the time lag spans for longer than 9 days, there is no significant correlation (R^2 = 0.1676; p=0.06).

Although the MERIS sensor is now inoperative, future Sentinel-3 is expected to have a similar sensor available which will retrieve also L1b products after 1 hour of scanning (Donlon, *et al.* 2012). Therefore, Sentinel-3 mission can be an especially

useful monitoring tool for larger than 9 ha water bodies, especially for countries with few cloudy days, supporting the management strategy.

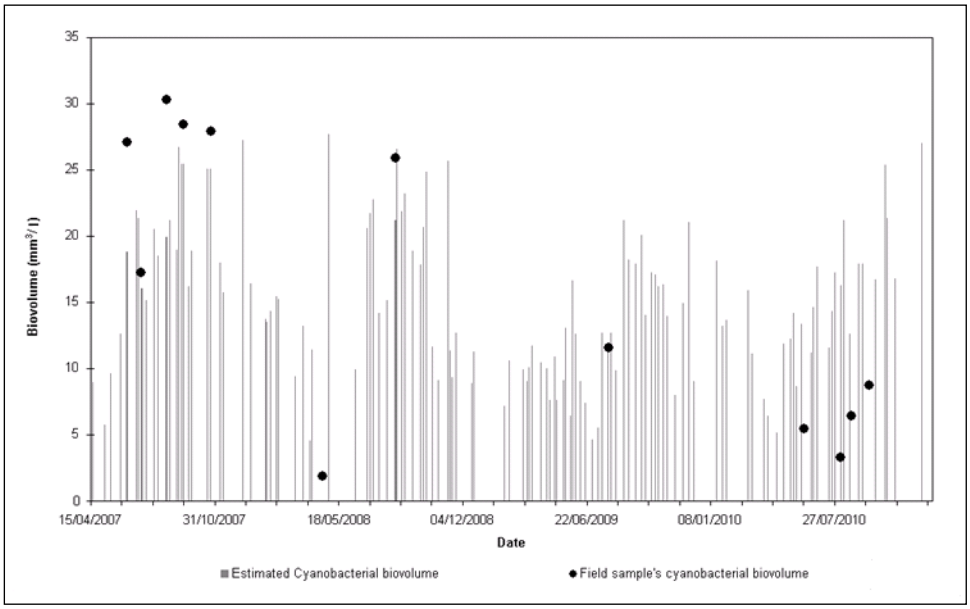


Figure 7. Timeline comparing estimated cyanobacterial biovolume (calculated from phycocyanin estimated by MERIS) and field sample cyanobacterial biovolume.

The already recorded MERIS images can also represent a particularly useful data source for understanding cyanobacterial dynamics in certain waterbodies at long term, because in some cases up to 10 years of high frequency data can become crucial to develop management strategies and increasing the forecasting capabilities.

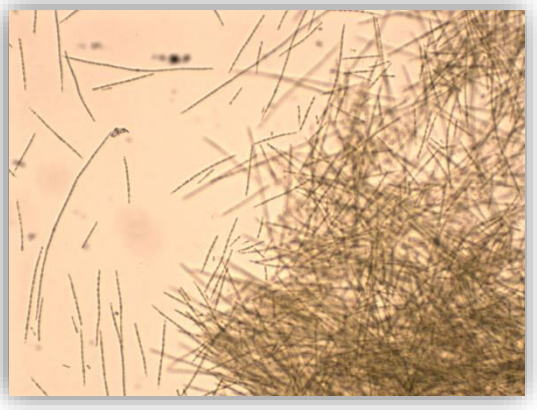
1.9 Conclusions

In this work, we define and validate the use of MERIS imagery to estimate the cyanobacterial biovolume in freshwater ecosystems under non-scum conditions. This approach is based on the detection of the cyanobacterial pigment PC by means of the

MERIS sensor, and we have adapted this tool to calculate cyanobacterial biovolume, an especially important parameter for limnological and water quality studies. In our analysis, the correlation between the measured cyanobacterial biovolumes and the *PC* values estimated from the MERIS imagery was found to be a robust proxy for cyanobacterial biomass estimation. The regression equation was built by using data from 23 aquatic ecosystems in the Iberian Peninsula, which were dominated by diverse cyanobacterial communities.

A validation exercise carried out with time series data from an external water reservoir indicated that the equation was useful for estimating cyanobacterial biomass with excellent results when the time lag between field measurements and MERIS image was lower than 9 days. Considering that our data cover a wide area of different lithological and trophic characteristics, with 89 different cyanobacteria species, from which 23 different species were dominant and the positive results from the validation test we believe that these results could be transferable to other water reservoirs.

Chapter 2. Ecology of invasive cyanobacteria from two contrasting climates: Nordic and Mediterranean habitats



Chapter 2. Ecology of invasive cyanobacteria from two contrasting climates: Nordic and Mediterranean habitats

Abstract

It is crucial to recognize the ecological traits that influence the distribution and growth of cyanobacterial communities in water bodies, especially when these are threatened by potentially toxic invasive species. The aim of this study is to identify the ecological traits that drive the distribution and growth of cyanobacterial species (and thus invasiveness). This purpose was achieved through analysing the databases created from field sampling, comparing their set of physicochemical, lithological, and environmental variables of water bodies located in two different geographic ecosystems in Denmark and Spain. This analysis entailed the inter-comparison of ecological traits' specificities found in temperate Nordic regions and warm Mediterranean environments. The resulting composite database covers more than 1500 water bodies spread on both geographical ecosystems. The role of these variables in influencing the growth and distribution of three potentially threatening toxic invasive cyanobacteria, *Dolichospermum lemmermannii*, *Cylindrospermopsis raciborskii* and *Chrysosporum ovalisporum* was investigated. Those variables were also used to investigate two ubiquitous cyanobacteria: *Cuspidothrix issatschenkoi* and *Planktothrix agardhii*. Our results point to temperature and eutrophication as the two major factors influencing the growth and distribution of potentially toxic invasive

cyanobacteria species. Specifically, *D. lemmermannii* showed a preference for temperate waterbodies with mesotrophic or lower eutrophication levels. On the contrary, *C. raciborskii* frequents mostly shallow water bodies with high-nutrient concentrations. Finally, *Ch. ovalisporum* distribution is apparently not driven by nutrients concentrations but rather by warm water temperatures, reaching its maximum growth at 30ºC. This elevated temperature dependence represents a potential threat considering the increase of overall temperature due to climate change, most particularly affecting warm Mediterranean environments. Contrarily, *C. issatschenkoi* and *P. agardhii* both showed a steady distribution and growth within a wide range of physicochemical, nutritional, and environmental variables, signifying its adaptation capability to various conditions.

2.2 Introduction

There is abundant literature on ecological traits of cyanobacteria under laboratory or mesocosm conditions (e.g., Häder, *et al.* 1998; Kosten, *et al.* 2012). Several studies have analysed the ecology of invasive cyanobacteria species and compared it to local species from the same waterbody where invasive species were found (Mehnert, *et al.* 2010; Thomas and Litchman 2016). However, if we want to define distinct species traits, many samples and waterbodies should be taken into account; therefore, a larger dataset should also be considered. However, although information is available in national monitoring programs, very few datasets have

been analysed specifically for cyanobacterial dispersion across lentic waters at regional or geographic scales (Carvalho, *et al.* 2013; Mantzouki, *et al.* 2018; Donis, *et al.* 2021).

The objective of this work is to address this gap and to identify ecological traits that promote the distribution of certain cyanobacteria taxa at global scale. For this purpose, we compare the environmental conditions of two geographical locations, Mediterranean and northern European locations (Spain and Denmark, respectively). This will add insight into the roles of physicochemical, nutritional, and climatic parameters affecting and distribution of potentially toxic invasive cyanobacteria. It was hypothesized that climate could be critical to determine the potential invasiveness of each cyanobacterium species could achieve. Five different cyanobacteria species were selected in our analyses (Figure 8):

Cylindrospermopsis raciborskii (Padisak 1997) has been reported in tropical and warm climates (De Souza, *et al.* 1998; Bouvy, *et al.* 1999; Bouaicha and Nasri 2004).

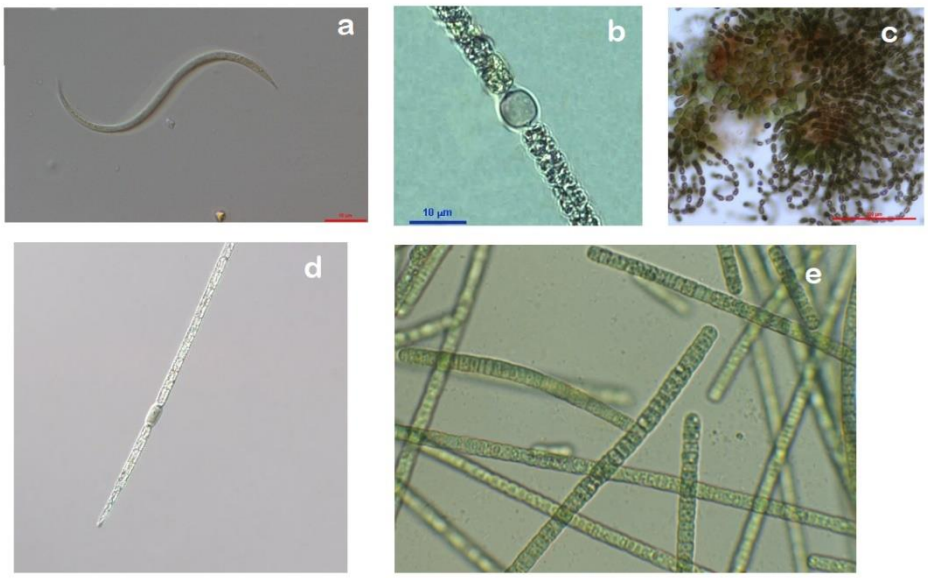


Figure 8. Selected cyanobacteria of interest: a) *Cylindrospermopsis raciborskii*; b) Chrysosporum ovalisporum; c) D. lemmermannii; d) Cuspidothrix issatschenkoi; e) Planktothrix agardhii

This morphospecies is a potential producer of CYN, a cytotoxin (Capelli, *et al.* 2017). However, in some countries, *C. raciborskii* has not been found to be toxic. In European strains this could be caused by the natural deletions of some of the genes that codify the enzymes which partake in cyanotoxin production, such as the cyrJ gene-lacking morphospecies, as this gene is crucial to produce CYN (Cires, *et al.* 2014).

Chrysosporum ovalisporum, formerly named *Aphanizomenon ovalisporum* (Zapomělová, et al. 2012) is a bloom forming toxic cyanobacterium (Fadel, *et al.* 2014; Crawford, *et al.* 2017; Facey, *et al.* 2021) that has been reported in several freshwater bodies, mainly around Mediterranean-climate water bodies (Gkelis, *et al.* 2005;

Quesada, *et al.* 2006; Messineo, *et al.* 2010). Like *C. raciborskii*, *Ch. ovalisporum* also may produce CYN (Berry, *et al.* 2009), with some exceptions (again, like *C. raciborskii*) when some strains only lack the cyrJ gene (Ballot, *et al.* 2011). *Ch. ovalisporum*, has been reported in countries like Australia (Crawford, *et al.* 2017) or Israel (Hadas, *et al.* 2002).

These two previous species of cyanobacteria have been widely described as invasive species in Mediterranean habitats (Ohtani, *et al.* 2002; Messineo, *et al.* 2010; Antunes, *et al.* 2015);

D. lemmermannii (Wacklin, et al. 2009), although reported in tropical climates (Palacio, *et al.* 2015) is suspected to be invasive in Nordic habitats (Onodera, *et al.* 1997; Capelli, *et al.* 2017). It is a potentially hazardous invasive filamentous cyanobacterium in Northern Europe (Wacklin, *et al.* 2009)[43] because it is a potential producer of neurotoxic ATX (Bouaicha and Nasri 2004), which is known to induce paralysis by fixation on acetylcholine receptors without being degraded by acetylcholinesterase. Consequently, death can occur by respiratory arrest when muscles involved in breathing activity are affected (Carota, *et al.* 2016). *D. lemmermannii* is characterized by achieving a high growth rate in a wide range of temperatures and is therefore frequently found forming blooms in both warm and cold environments all over Europe (Salmaso 2003; Li, *et al.* 2016; Capelli, *et al.* 2017).

Cuspidothrix issatschenkoi (Rajaniemi, et al. 2005), spreads all over Europe (Zapomělová, *et al.* 2012; Ballot, *et al.* 2018) and Asia (Ballot, *et al.* 2018). ATX producing strains of *C. issatschenkoi* have been recognized and isolated from two lakes in New Zealand and Germany (Wood, *et al.* 2007; Ballot, *et al.* 2010). In Japan, this species has spread over many lakes and reservoirs, where it becomes dominant from October to December (Hodoki, *et al.* 2012).

Finally, *Planktothrix agardhii*, a commonly toxic filamentous species spread all over Europe (Komárek and Anagnostidis 2005). *P. agardhii* strains are known for producing a variety of MCs (Stefaniak and Kokocinski 2005; Carmichael 2012). *P. agardhii* is common among nutrient enriched, exposed and generally shallow lakes at most latitudes, where it can aspire to monospecific populations persisting throughout the year, constituting 'the third stable state' to which shallow lakes may gravitate (Reynolds, *et al.* 2002). The last two species are used as control groups for invasiveness, as they are spread broadly through the studied countries during the whole timeframe of

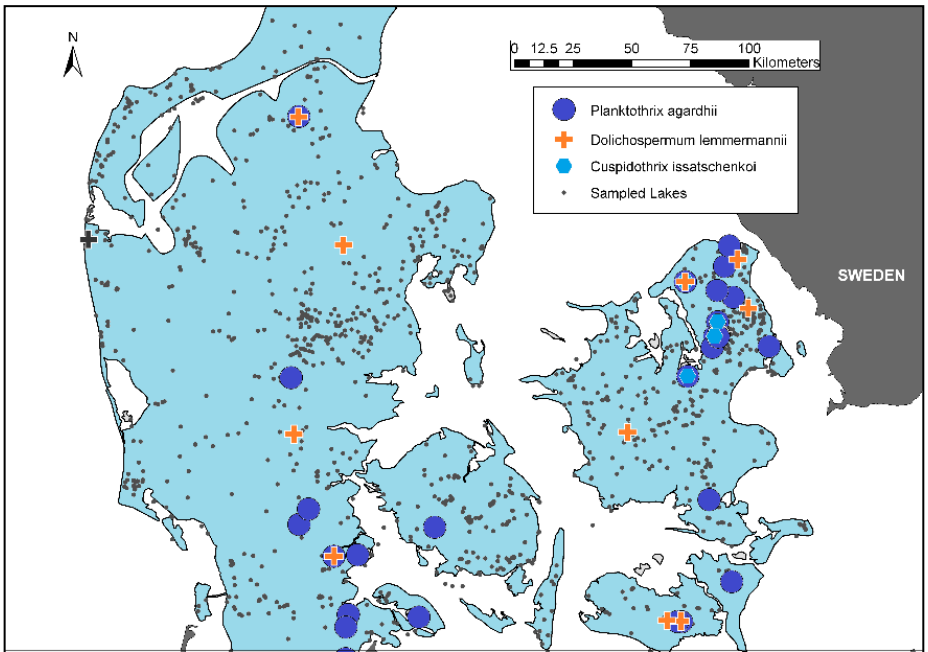


Figure 9. Sampled lakes from Denmark and localization of P. agardhii, D. lemmermannii and C. issatschenkoi. Only data were biovolume was quantified for these species was considered.

the dataset.

2.3 Database comparison

2.3.1 Area of study

For this analysis it was considered the data from samples where at least one of the five cyanobacteria of interest were identified and their biovolume was quantified. The dataset includes many sampling campaigns. Most sampling campaigns were conducted every two weeks or every month. Danish data were extracted from The Danish Environmental Portal, which is the Danish database for all environmental data (D.E.P.). This database covers data from 1258 lakes all over the country from 1987 to 2013 (Figure 9). Approximately 77% of the samples used in further analysis were obtained during the growing season for cyanobacteria (summer and autumn). In Denmark, almost 50% of the samples are from shallow lakes, which implies a maximum depth of 3 m following the Water Framework Directive (Moss, *et al.* 2003).

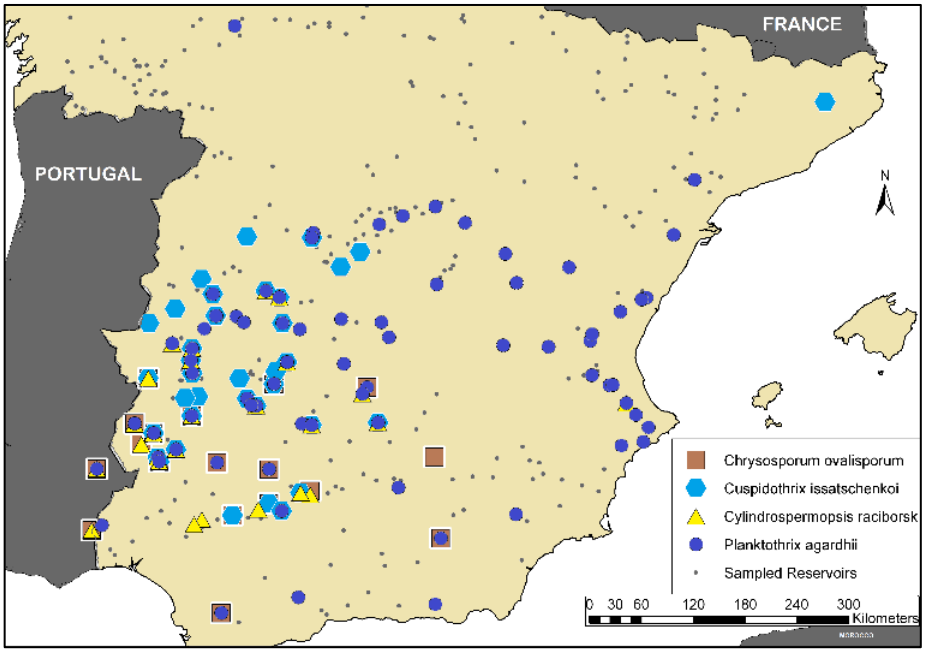


Figure 10. Sampled water reservoirs from Spain and localization of Ch. Ovalisporum, C. issatschenkoi, *C. raciborskii* and P. agardhii. Only data where biovolume was quantified was considered.

Samples from Spain were obtained from the Hydrographic Studies Centre of CEDEX and the Ministry of Environment. The Spanish database spans 12 years, from 1999 until 2011, with samples of 320 different water reservoirs all over Spain (Figure 10).

2.3.2 Sampling methodology

The analytical methodologies followed in each country are summarized in Table 3. In Denmark, the national monitoring program for lakes was established in 1988 (Svendsen and Norup 2005) and includes Chl-a, water temperature profiles and chemistry parameters through the water column monitored from April to October.

Parameter	Methodology Spain	Methodology Denmark
Biovolume	Utermöhl (1958)	Utermöhl (1958)
Chlorophyll-a	Parsons and Strickland (1968)	Jespersen and Christoffersen (1987)
Nitrogen compounds (nitrate and nitrite)	LASA 100 photometer (Hach Lange)	Danish Standard 223
Total phosphorus and orthophosphates	Standard Methods 4500-P F. Automated Ascorbic Acid Reduction Method (Ed 22nd)	Danish Standard 6878
pH, temperature, conductivity, oxygen saturation	Multi sensor Probes	Multi sensor Probes
Alkalinity	UNE-EN ISO 9963-1:1996	Danish Standard 223

Table 3. Methodology for biological and physical and chemical analyses

Discrete water samples for water chemistry and phytoplankton were taken with a standard water sampler from the euphotic zone (defined as 2.5 times the Secchi depth) at three stations (one being the mid-lake), and within each station, the water was pooled together to obtain a composite sample for further subsampling.

The units used for every parameter used in the study can be found in Table A1, in the Supplementary Material section.

In Spain, sampling was performed at least 200 m from the reservoir dam (MAGRAMA 2013) mostly during summer and early autumn (from May until October). Most of the samples were taken as integrated samples from the euphotic zone; however, 34% of the used data come from a single depth point located at the maximum Chl-a concentration depth, as defined by the profile obtained with a multisensor probe. This sample point depth was always within the euphotic zone. Phytoplankton samples were sedimented and counted following the method of Utermöhl (1931). Identification followed (Komarek and Anagnostidis 1999) and was performed to the highest possible taxonomic resolution.

2.3.3 Statistical analysis

For the statistical analysis, data from samples where the selected cyanobacteria were identified and quantified were chosen. Only data from the growing season (defined from June until November) were considered, as 96.5% of data points fall within this period, and the other 3.5% can reduce the focus of the analyses. Therefore, the dataset from Denmark consisted of 271 field samples from 34 different lakes, and the data from Spain represented 291 field samples from 99 different reservoirs. The selection of the cyanobacteria species of interest was based on their potential as invaders. Thus, we selected *Cylindrospermopsis raciborskii*,

Chrysosporum ovalisporum and *Dolichospermum lemmermannii*. *Cuspidothrix issatschenkoi* and *Planktothrix agardhii* were chosen as the control groups because they are described as ubiquitous cyanobacteria in the literature (Chorus 2012) and appear in several water bodies in the dataset of both countries. Due to the heterogeneity of variances, the data were normalized using the average annual value of each parameter for each water body.

All parameters measured in each sample were statistically compared using cyanobacteria species as a grouping factor. For this analysis, Danish and Spanish morphospecies of *P. agardhii* and *C. issatschenkoi* were analysed separately to detect whether they have special adaptations to the climate, or if they have the same specific conditions in its habitat in both regions.

Homoscedasticity, one-way analysis of variance, post hoc analysis and Monte Carlo permutation were performed with SPSS v.20 software. Principal component analysis was performed by XLSTAT software. Post hoc analysis results for nutrient, physical and chemical and weather-related parameters are shown in Figures 12, 13 and 14, respectively. Each significant similarity group is represented accordingly at the top of each boxplot with lowercase letters (a, b, or c) or a combination of them if the analysed set of data is significantly represented in two similarity groups (a/b, a/c, or b/c).

Parameter	Country	Minimum	Maximum	Median
# samples per year in each water body	Denmark	1	13	2
	Spain	1	6	2
Max depth (m)	Denmark	0.8	22.3	3.6
	Spain	5.5	124.4	44
Total Phytoplankton biovolume (mm^3/l)	Denmark	0.02	143.9	2.5
	Spain	0.04	133.6	6.5
Total Cyanobacteria biovolume (mm^3/l)	Denmark	10^{-3}	143.9	1.6
	Spain	10^{-3}	100.1	0.9
Cyanobacteria percentage of biovolume (%)	Denmark	0.1	100	83.4
	Spain	0.1	97.9	27.6
Chlorophyll-a (μg/l)	Denmark	1	381.1	76.1
	Spain	0.6	278	11.2

Table 4. Water bodies abiotic and biological parameters from samples with identification(s) of any cyanobacteria of interest

2.4 Results and discussion

The biological and morphological parameters of the water bodies (biovolume of cyanobacteria, Chl-a concentration, and maximum depth) with the occurrence of any of the five selected cyanobacteria species are summarized in Table 4. This showed that the water bodies selected in Denmark were generally shallower and more eutrophic than the selected Spanish reservoirs. Cyanobacteria tend to be the dominant phylum in the selected Danish lakes (>50% of the total phytoplankton biomass) (Whitton 2012).

Regarding cyanobacteria composition in the dataset summarized in Table 5, it is shown that *P. agardhii* is the species found more frequently in more different water bodies and in larger biovolume quantities, in both countries. As *P. agardhii* seems to thrive in both climates with vastly different environmental limitations, it could be considered an indicator of the species' elevated level of adaptation.

Cyanobacteria species	Country	# of samples	# water bodies	Biovolume (mm^3/l)		
				Minimum	Maximum	Median
Dolichospermum lemmermannii	Denmark	22	17	10^{-3}	5.5	10^{-3}
Cylindrospermopsis raciborskii	Spain	57	27	0.01	18.6	0.2
Chrysosporum ovalisporum	Spain	25	20	0.02	5.3	0.1
Cuspidothrix issatschenkoi	Spain	68	42	10^{-3}	1.5	3×10^{-3}
	Denmark	25	4	0.04	1.5	0.3
Planktothrix agardhii	Spain	145	88	10^{-3}	20.7	0.02
	Denmark	71	33	0.02	35.6	0.8

Table 5. Biovolume Data summary

Considering the whole data on cyanobacterial biovolumes it is evident that the growing season was limited to late summer for all morphospecies, except for P. agardhii, which grew during all seasons. This species peaked in early summer in Spain and late summer in Denmark (Figure 11). Another observation is that some cyanobacteria, such as P. agardhii (in both countries) and C. raciborskii, have the highest values of total biovolume of the five selected species. This elevated amount

of biovolume can be linked to bloom formation (Burford, et al. 2016; Churro, et al. 2017).

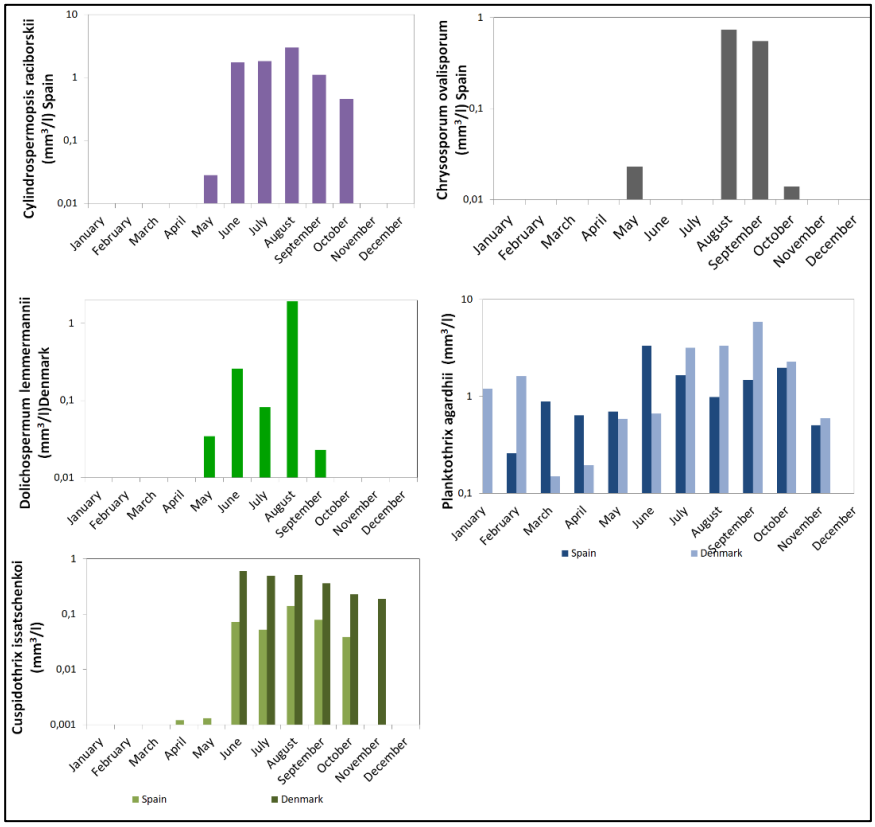


Figure 11. Distribution of the selected cyanobacteria along the year grouped by country. Average biovolume per month from every sample with a quantified measured biovolume of each cyanobacterium

Regarding the concentrations of Chl-a and nutrients, some of the parameters were significantly different in each country (Figure 12). However, high nutrient values, according to the values given by de OCDE in Hart (1984), seem to have very significant impacts for some species despite their origins. *D. lemmermannii* and *Ch. ovalisporum* clearly grow in water bodies with low levels of nitrate, phosphorus and ammonia and high transparency, the latter measured as Secchi disk depth (Figures 12B, 12C, 12D and 12E, respectively).

Most of these low values, which include levels of chlorophyll under 2.6 µg/l, nitrate and nitrite under 0.3 mg/l and phosphorus lower than 12 mg/l, correspond to oligotrophic water bodies (Carlson 1977; Chorus and Bartram 1999). When the nutrient parameters reach the threshold for hypereutrophic water levels, they seem to be ideal for the growth of *C. raciborskii*.

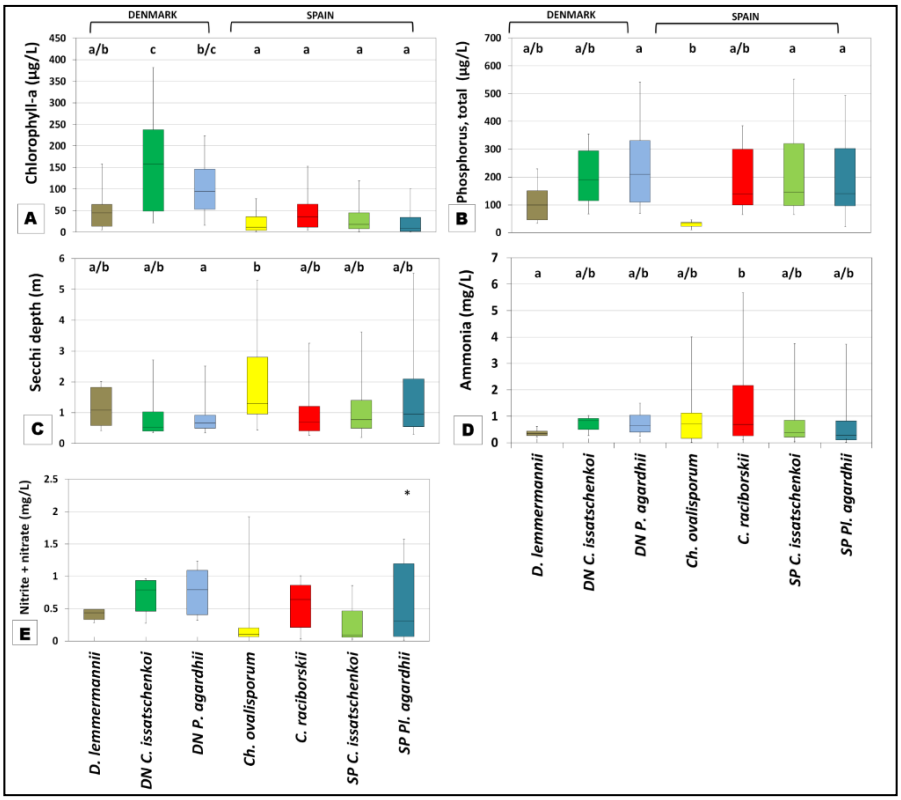


Figure 12. Box plot of: A) Chlorophyll-a; B) Total phosphorus; C) Secchi Depth; D) Ammonia; E) Nitrate+Nitrite. Only for locations with the cyanobacteria in question. Letters on top of the box plots (a, b, c, and combinations significantly represent groupings (p<0.05). (*): Analysis of variance did not find significant differences between any dataset.

The analyses of the physico-chemical and morphological variables (Figure 13) indicated that the maximum depth of the waterbodies where the selected cyanobacteria were sampled (Figure 13E) is vastly different between the two countries due to the different nature of the sampled waterbodies (lakes in Denmark vs. reservoirs in Spain). Another important trend is related to pH levels (Figure 13D).

Post hoc analysis shows that there are significant differences with Danish *C. issatschenkoi*, as it was sampled in waters with basic pH (>8). However, it is worth noting that each species is found in water bodies that cover a very wide pH range, although the vast majority appears in basic conditions where pH is above 7.5.

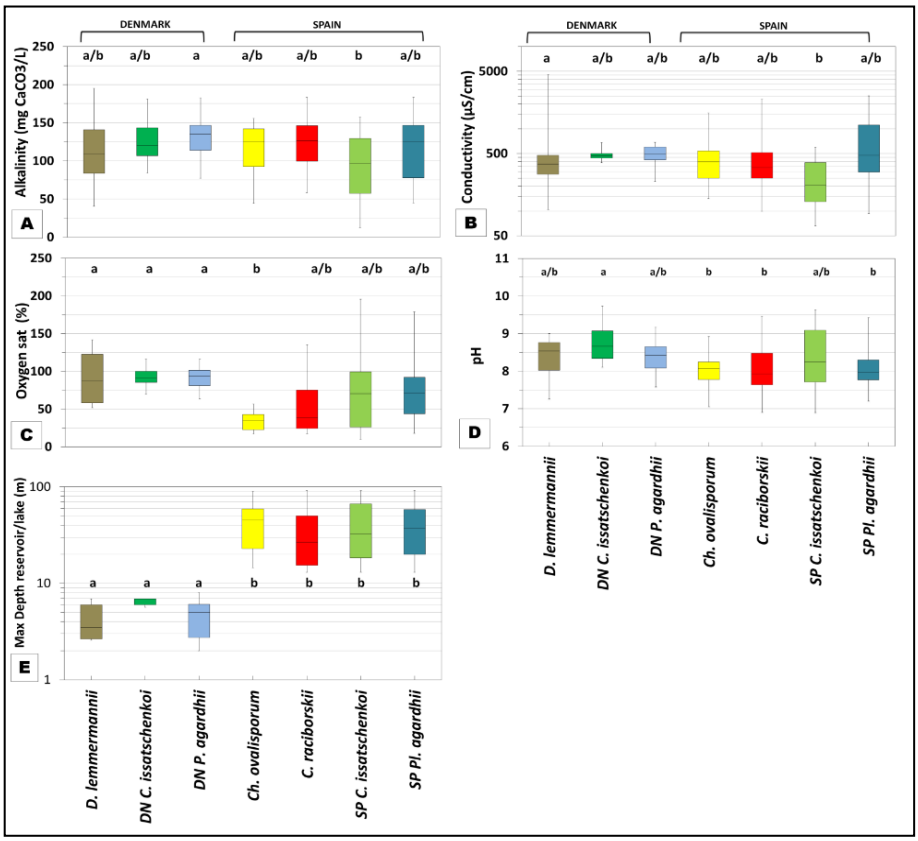


Figure 13. Box plot of: A) Alkalinity; B) Conductivity; C) Oxygen saturation; D) pH; E) Max depth of water bodies. Only for locations with the cyanobacteria in question. Letters on top of the box plots (a, b, c, and combinations significantly represent groupings (p<0.05). (*): Analysis of variance did not find significant differences between any dataset.

The rest of the physicochemical parameters do not follow a general trend, although some details will be discussed in each species section. As expected, the water temperature ranges are quite different in Denmark and Spain's sampled water bodies, with averages of 12 °C in Denmark and 23 °C in Spain.

These differences between countries are shown quite clearly in the post hoc analysis (Figure 14) of parameters such as water surface temperature (Figure 14A),

minimum atmospheric temperature (Figure 14B), maximum atmospheric temperature (Figure 14F) and accumulated precipitation (Figure 14C). However, post hoc analysis also estimates two additional similarity groups within Denmark regarding accumulated precipitation.

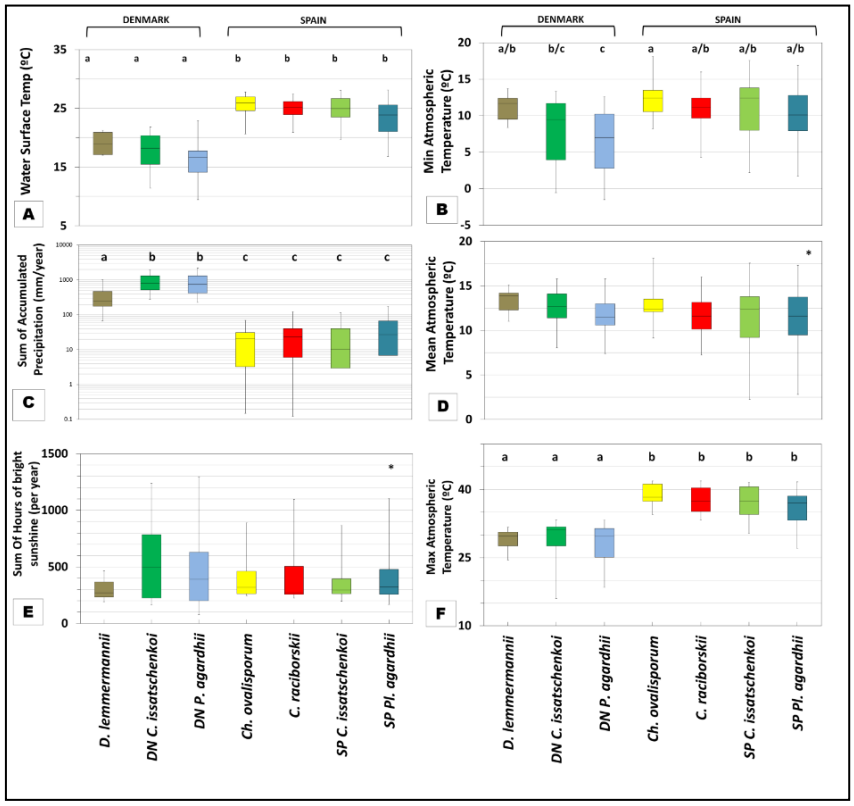


Figure 14. Box plot of: A) surface Water Temperature; B) Minimum Air Temperature (what height); C) Sum of accumulated precipitation during growing season (June – November) in log scale; D) Mean air Temperature; E) Sum of Hours of bright sunshine precipitation during growing season (June – November); F) Maximum air Temperature. Only for locations with the cyanobacteria in question. Letters on top of the box plots (a, b, c, and combinations significantly represent groupings ($p<0.05$). (*): Analysis of variance did not find significant differences between any dataset.

In the case of temperature, post hoc analysis shows that *Ch. ovalisporum* shows a preference for warmer regions, and Danish *P. agardhii* shows a preference for the coldest temperature range.

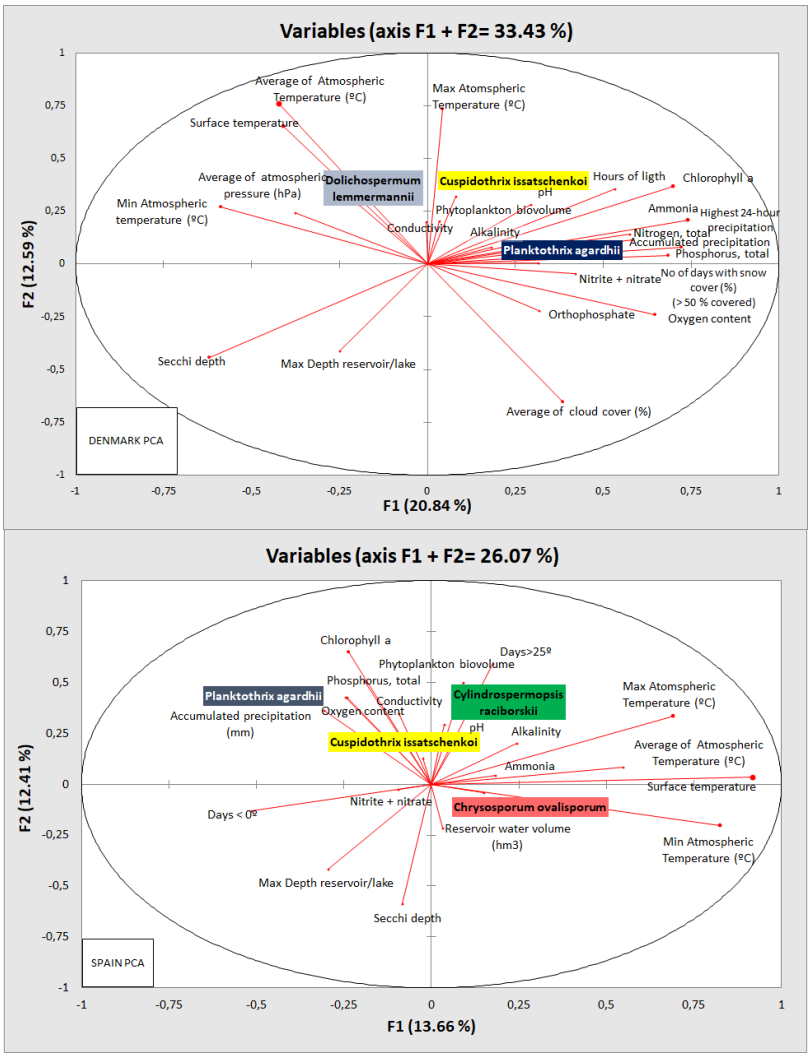


Figure 15. Principal Component Analysis (PCA) for each country dataset

The other cyanobacteria species seem to be found at intermediate temperature levels (Figure 14). It is interesting to point out that some climatological parameters had no significant difference that could be detected by the post hoc analysis, such as

the case of mean atmospheric temperature (Figure 14D) and the sum of hours of bright sunshine (Figure 14E). Conversely, regarding the sum of hours of bright sunshine, the two Danish cyanobacteria (*C. issatschenkoi* and *P. agardhii*) were found under the highest amounts of precipitation and hours of sunshine conditions during the sampling period (Figure 14C and E).

The presence of the selected cyanobacteria species in relation to environmental conditions was evaluated using a Principal Component Analysis PCA (Figure 15). However, the represented variance in the analysis can be considered low (33.4% for the Danish dataset and 26.1% for the Spanish dataset). The first axis (F1) for the Danish lakes represents eutrophication. For Spanish reservoirs, the F1 axis clearly represents atmospheric temperature because all temperature-related variables are aligned along this axis, and F2 represents eutrophication where most of the nutrient-related parameters are aligned. The low significance found in both countries could mean that the proliferation of cyanobacteria is driven by many more variables, as expected given the wide span of ecosystems and environmental conditions in the analyses.

P. agardhii and *C. issatschenkoi* show similar ecological patterns in both countries. In the PCA, both morphospecies appeared in the quadrant representing high eutrophication, as was also found for their biomass graphs (Figure 11). This

could indicate that these morphospecies could be prone to form blooms, as is a usual occurrence for this species when they have sufficient nutrients [65,69].

Finally, the PCA illustrates an interesting pattern between different cyanobacterial species. *Ch. ovalisporum* and *D. lemmermannii* appear in the PCA at high-temperature climates and low eutrophication levels, while *C. raciborskii* also appears at high-temperature climates but with high eutrophication levels.

The results give information about all cyanobacteria species as a whole, but it is interesting to point out interesting ecological traits found for each cyanobacterium.

2.5 Species autecology

2.5.1 Dolichospermum lemmermannii

This cyanobacterium was mostly found during the warmest months (Figure 11 and 6A). As *D. lemmermannii* is a nitrogen-fixing species, it was found in environments with low nitro-gen levels (Figure 12D, Figure 12E), a trend that has been reported previously (Olli, *et al.* 2005).

D. lemmermannii tolerates a great range of alkalinities and conductivities (Figure 13A and B, respectively), allowing this species to colonize a broad range of environments (Callieri, *et al.* 2014). However, according to the findings in this study,

D. lemmermannii seems to prefer temperate waterbodies with mesotrophic or lower eutrophication levels, which include levels of chlorophyll under 20 μg/l, nitrate and nitrite under 0.5 mg/l, phosphorus lower than 24 mg/l. The lack of other obvious ecological restrictions would make *D. lemmermannii* a very potent invasive species of northern and mountainous habitats under the predictions of most climate change scenarios (Sarmento, *et al.* 2013; Rigosi, *et al.* 2014).

2.5.2 Cylindrospermopsis raciborskii

The results confirm that most water bodies where *C. raciborskii* is found are shallow and have high nutrient concentrations (Saker, *et al.* 2003; Komárek 2006). *C. raciborskii* was the most prone of the five studied cyanobacteria to appear in shallow water bodies producing high Chl-a concentrations, as shown in Figure 13E and Figure 12A and by PCA (Figure 15). Temperature has been considered the major limiting factor for the growth of *C. raciborskii* (Wiedner, et al. 2007). However, it has been reported frequently in countries from Central and Northern Europe (Haande, *et al.* 2008; Kokocinski, *et al.* 2009). Its akinete germination is temperature-dependent and occurs primarily from 22–23.5 °C (Briand, *et al.* 2004). This temperature range corresponds well with the median spring and summer values found in the Spanish water bodies evaluated in this study (Figure 14A).

2.5.3 *Chrysosporum ovalisporum*

Ch. ovalisporum ecophysiological characteristics show their maximum growth rate at very warm temperatures (approximately 30 °C) (Barón-Sola, *et al.* 2016), which is in line with the trend encountered in this study (Figure 14A). This explains why it is found in the southernmost and warmest regions of Spain (Figure 10). This cyanobacterium was found in the reservoirs with the lowest nitrogen and total phosphorus concentrations and the highest Secchi depth values (Figure 12E, B and D, respectively), which is also reflected in the PCA (Figure 15), and in areas with low values of eutrophication. This opens an interest path for invasiveness, not driven by nutrients but by warmer temperatures produced by climate change or other anthropogenic pollution (such as warm water waste produced by hydroelectrical factories or nuclear power plants and discarded into rivers).

2.5.4 *Cuspidothrix issatschenkoi*

The results show that in Spain and Denmark, the growing season seems to cover late summer until October-November (Figure 11).

C. issatschenkoi was first recorded in shallow and brackish areas (e.g., the Baltic Sea), and high salinities have been suggested as the key factor driving this species' expansion (Wilk-Woźniak, *et al.* 2015). However, there are also records from freshwater locations such as Lake Volvi in Greece (Ferrari, *et al.* 2011). In this study, *C. issatschenkoi* confirms the second statement, as it is mostly found in water bodies

with low alkalinity, low conductivity (especially the Spanish morphospecies) and at an average high pH level, as in each country, the morphospecies is found within the highest pH mean values (Figure 13A, B and D, respectively). The wide ranges shown for almost every other parameter in this morphospecies could explain the lack of limitations in its expansion and why it is so widespread all over the world (Ballot, *et al.* 2010; Hodoki, *et al.* 2012).

2.5.5 Planktothrix agardhii

P. agardhii in Spain can be found throughout a wide range of water depths (Figure 13E) (Bonilla, *et al.* 2012; Kurmayer, *et al.* 2015). Considering the data compiled in this study, *P. agardhii* can tolerate a wide range of temperatures (8–29 °C; Figure 14A). However, it is well known that *P. agardhii* populations develop optimally in late summer or autumn because growth is optimal above 20 °C [83]. In Spanish reservoirs, the growing season appears to present two peaks, one in June and another in October (Figure 11), and different ecotypes could be responsible for these peaks (Callieri, *et al.* 2014).

P. agardhii had the longest duration throughout the year (Figure 11). Populations of *P. agardhii* have been observed in highly eutrophic water bodies (levels of chlorophyll under 2.6 μg/l, nitrate and nitrite under 0.3 mg/l, phosphorus lower than 12 mg/l all year long in both the Danish and Spanish water bodies, coinciding with behaviour found in previous literature (Poulíčková, *et al.* 2004).

It is interesting that *P. agardhii* appears over a wide range of parameters, such as total phosphorus, Secchi disk depth, nitrates and nitrites, and conductivity. This wide range of adaptation to so many ecological parameters could be one of the reasons this species has the actual worldwide distribution that it shows.

2.6 Conclusions

Although invasiveness is complicated to predict, it is possible to analyse the growing conditions and likelihood of survival of new species in each habitat. In this study, the two most crucial factors or variables that may be related to the dominance of potential invasive cyanobacteria species are temperature and eutrophication.

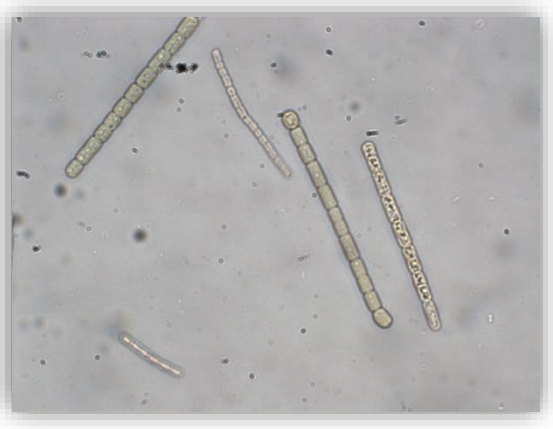
Because temperature plays a significant role, temperate or cold ecosystems facing warmer climates and droughts induced by climate change could be prone to invasion by new cyanobacteria species. *Ch. ovalisporum* is now well established in Spain due to its preference for very warm summers (>30 °C), which can surely help to colonize new oligotrophic warm ecosystems. *C. raciborskii*, a tropical cyanobacterium, is spread all over Europe because of the new niches that climate change and anthropogenic pollution have opened. Because of the warmer climates and higher levels of nutrients in water bodies, ecosystems are more easily invaded by

this cyanobacterium. *D. lemmermannii* is also spreading all over Europe, taking advantage of similar ecological preferences, with the northern morphospecies being the most troubling because they include the cyanotoxin producers [26].

It is also interesting to point out that *P. agardhii* and *C. issatschenkoi* follow similar ecological preferences in different countries, with slight differences in range. This phenomenon could indicate great adaptability because the range of conditions in which they can live is so wide that perhaps the limits shown in the data are not the limitations of the species but instead the whole range of conditions that appear in each country.

Chapter 3. The untapped potential of cyanotoxin production.

Laboratory vs Natural conditions



Chapter 3. The untapped potential of cyanotoxin production. Laboratory vs Natural conditions

3.1 Abstract

The effects of cyanotoxins are widely reported worldwide. Despite the apparent risk they have been shown to cause, very few cyanotoxins are regulated for monitoring in water, including those intended for human consumption. Processes such as blooms, large phytoplankton formations, usually due to natural or artificial excess of nutrients (especially phosphorus and nitrogen), can increase the production of toxins, but it is unclear in the literature the limits of cyanotoxin production. In this chapter it will be analysed the production of toxins in Rosarito's reservoir by cyanobacteria. This objective will be achieved by measuring biovolume and cyanotoxin composition from several field samples and then compare the same biovolume and cyanotoxin composition from cyanobacterial isolates taken from the same field samples and cultivated under optimal laboratory conditions. After analysing the biovolume and cyanotoxin production from field samples it was calculated the Toxin Production Ratio (TPR), which represents how much μg of toxin is produce per each mm^3 of toxin-producer cyanobacteria biovolume. In both field samples and isolates only microcystin (MC) and saxitoxin (STX) were detected. In the isolates MC was produced mostly by *Planktothrix agardhii* and the rest by an isolate of *Limnothrix redekei*. STX was produced only by *Aphanizomenon gracile* in the isolated cultures. STX and MC were produced more in higher concentrations under

laboratory conditions. Therefore the average MC TPR from isolates was 110 times higher than the average MC TPR value found in the field samples. The average STX TPR from isolates was 750 times higher than the average STX TPR value found in the field samples. This creates a problematic scenario were under more optimal growth conditions in the field the cyanotoxins production could reach extremely high values that may compromise the utilization of water resources. NGS also proved to be a great tool, as identified correctly all cyanotoxins productors as the ADN analysed corresponds perfectly with all the toxin producers' isolates.

3.2 Introduction

The term "cyanotoxin" refers to hundreds of compounds that may strongly differ in their chemical structure and toxicological property. They are usually arranged into 4 classes according to their target organ: hepatotoxins that induce liver injuries, neurotoxins that alter the neuromuscular transmission, dermatoxins that induce skin irritation, and cytotoxins (Merel, *et al.* 2013).

Toxins produced by cyanobacteria are increasingly documented around the world and problems related to toxins in water bodies have received more attention in recent years. As a result, awareness of risk posed by cyanotoxins increased substantially and regulatory approaches for cyanotoxin risk management have been put in place (Akcaalan, *et al.* 2014).

Climate change further aggravates this problem. The combination of water scarcity, high temperatures and eutrophication caused by human activity, mainly due to the effects of nutrients (from origins like the industry, cattle or untreated sewer water, among many others) provoke a scenario conducive to the great increase in cyanotoxicity. This increase would be more pronounced in lentic water bodies such as lakes and reservoirs. Less water and more eutrophication can lead to an increase in phytoplankton and rising temperatures can lead to the proliferation of cyanobacteria, by better adapting to more extreme ecosystems, especially invasive species of tropical origin that can be potentially toxic.

In addition to direct analysis for cyanotoxins, surrogate procedures are recommended to indicate whether cyanotoxins concentrations are likely to be approaching concentrations that present health risks via drinking and recreational exposure (Chorus, *et al.* 2021). These surrogates include the determination of cyanobacterial cell concentrations in water and of chlorophyll a concentrations, when cyanobacteria are dominant (Falconer, *et al.* 1999). The estimations of (putative) cyanotoxin concentration from cyanobacterial cell numbers and Chl-a concentrations are based on compare cell counts, pigment analyses and cyanotoxins analyses of environmental samples with the determination of cell number and cyanotoxins concentration in laboratory cultures (Pereira, *et al.* 2004; Akcaalan, *et al.* 2006). Toxicity per volume could be a useful tool to know how dangerous a potentially toxic cyanobacteria could be if it can be compared between different cyanotoxin

producers (or different strains of the same species). However the few cases that exist they compare strains from different origins and it the ratio of cyanotoxin production is usually compared to number of cells per millilitre (Akcaalan, *et al.* 2006). Number of cells per millilitre is widely used in phytoplankton studies. However, to achieve a homogeneous comparative between samples from different species or origins is almost impossible because the cell size of phytoplankton varies greatly, even between the same phytoplanktonic strains (Xiao, *et al.* 2017).

With the purpose of study the potential cyanotoxin production under laboratory and natural conditions, Rosarito reservoir was sampled. This water reservoir in Spain was screened for the presence of diverse toxic cyanobacteria and several cyanotoxins. From the same field samples, many cyanobacterial strains were isolated and identified with the purpose of study the potential toxin production of these strains under laboratory and natural conditions. The results will also be compared with the results of next generation sequencing (NGS) used in the same field samples, analysed and published in other study (Casero, *et al.* 2019). Thanks to NGS, the identity of the isolates was identified in the field samples, being able to attribute toxicity events in natural environments to specific strains in the field.

3.3 Methodology

3.3.1 Field sampling

Field sampling took place in Rosarito reservoir (Figure 16, Table 6) which belongs to the Tietar River located in the Tajo watershed, the dam height is 38 m, has a water surface of approximately 12 km^2 and has a capacity of 92 hm^3. This reservoir was chosen because in previous sampling campaigns it showed that cyanobacteria were by far the most dominant phylum among the phytoplankton community (an average of the 95% of the total biomass), that also showed a high diversity of different species within this phylum (Cirés, *et al.* 2011; Agha, *et al.* 2012; Casero, *et al.* 2019).

Figure 16. Aerial photo of Rosarito reservoir

Field sampling took place once per month from June to October of 2013 (except for August). Sampling point was located 100 meters from the dam, and samples were taken from a single point located at the maximum Chl-*a* concentration depth (as defined by the profile obtained with the probe YSI6600 V2). This was always within the euphotic zone (defined as 2.5 times the Secchi disc depth). No scum episodes were detected during the samplings.

Water reservoir Uses	Water supply, irrigation, fishing, navigation, bath
Year of construction	1958
Water basin	Tajo
River	Tietar
Dam height (m)	38
Water surface (km^2)	1475
Capacity (ha)	92

Table 6. Rosarito's reservoir characteristics

3.3.2 Cyanotoxin quantification

The molecular classification of the cyanotoxins that are going to be analysed is microcystins (MCs), anatoxins (ATX), saxitoxins (STXs), and cylindrospermopsin (CYN) (Merel, et al. 2013). Sestonic concentrations of the cyanotoxins of the field samples ATX, MCs, CYN and STXs were determined from biomass retained in GF/F filters (Whatman, UK) extracted as described in Wormer, *et al.* (2010) for MCs and CYN, Carrasco, *et al.* (2007) for ATX, and Casero, *et al.* (2014) for STXs. The final extracts were vacuum-evaporated and re-suspended into Milli-Q water compatible with commercial enzyme-linked immunosorbent assay (ELISA) kits for the detection and quantification of the hepatotoxins MCs (microcystins-ADDA ELISA kit, Abraxis,

USA), the cytotoxin CYN (cylindrospermopsin ELISA kit, Abraxis, USA) and the neurotoxins STXs (saxitoxins (PSP) ELISA kit, Abraxis, USA). The neurotoxin ATX was analysed by the receptor-binding assay (RBA) kit (Abraxis, USA). All toxin standards and samples were run in duplicate following the manufacturer instructions. Absorbance readings were performed at 450 nmin a Biotek SynergyHYmulti-modemicroplate reader (Biotek Instruments, USA) and converted into toxin concentrations (μg L−1) according to the standard curves following the manufacturer indications. ELISA test results were published in Casero, *et al.* (2019). The same cyanotoxins from both the field campaigns and isolates will be analysed by HPLC/MS/MS using a liquid chromatograph with triple quadrupole Thermo Electron detector.

3.3.3 Genomic DNA extraction and PCR

DNA was extracted from GF/F filters using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with severalmodifications in the cell disruption step. Briefly, GF/F filters were placed in 2mLmicrocentrifuge tubeswith addition of the AP1 lysis buffer fromthe kit. Tubeswere then introduced for 30 s in liquid N2 and thawed at room temperature, repeating the process twice. After thawing, cells were disrupted in a homogenizer using sterile glass beads (212–300 μm, acid-washed, Sigma-Aldrich, St. Louis, MO, USA). RNAse was added to tubes, and they were incubated at 65 °C for 10 min and subsequently centrifuged at 20,000 ×g for 2 min. Supernatantswere then transferred

to newmicrocentrifuge tubes and manufacturer's instructions were followed from this point onwards. Genomic DNAwas dissolved inMilli-Qwater and stored at−20 °C until analysed. The 16S rRNA gene was amplified by PCR using primers specific for cyanobacteria with ample use in the literature (CYA359F/CYA781Ra and CYA781Rb) following reaction mix and PCR conditions from (Nübel et al., 1997) (Table S1 in supplementary data). The presence of genes involved in the biosynthesis of the main cyanotoxins (ATX, CYN, MCs and STXs) was analysed by PCR. The genes targeted were anaF, encoding for a polyketide synthase within ATX-synthesis cluster; cyrJ encoding for a sulfotransferase within CYNbiosynthesis cluster; mcyE, encoding for hybrid polyketide synthasenonribosomal peptide synthetase involved in MCs-biosynthesis; and sxtI encoding for a carbamoyltransferase of STX biosynthesis. These target genes were chosen because of their essential functions in the iosynthesis of the respective cyanotoxins. The PCR amplification was performed using specific primers sets that were carefully selected fromthe literature because theywere annealing in highly conserved regions located at the flanks of variable gene regions, so that each primer pair proved successful in several cyanobacterial genera while providing taxonomic resolution (Table S1 in supplementary data, and references therein). The reaction mixture was performed in a final volume of 20μL and contained 0.1 μL of Taq DNA Polymerase (5 U μL−1), 0.5 μL of deoxynucleoside.triphosphate mix (10 mM), 2 μL of 10 X reaction buffer, 1–1.2 μL of MgCl2 (25 mM), 1 μL of each forward and reverse primer (10 μM) and 0.4 μL of genomic DNA. NGS methodology can be found in Casero, *et al.* (2019)

3.3.4 Isolates and cultures

Isolates from one single filament were incubated in BG11 medium,28ºC (Rippka, *et al.* 1979) until cultures reached enough biomass to be identified. Nostocales cultures were changed to a $BG11_0$ medium while the rest kept growing in BG11 medium. All cultures were irradiated constant light at 6μE/m^2s, except the Planktothrix strains that were irradiated at 3μE/m^2s.

3.3.5 Biovolume quantification and toxin ratio calculation

Phytoplankton was counted in the laboratory according to the Utermöhl sedimentation method (Sournia 1978). Phytoplanktonic organisms were identified at the finest taxonomic level possible. Biovolume was calculated by giving each species an approximate simple or compound geometrical shape (Hillebrand, et al. 1999). Chl-*a* was extracted with 90% acetone and quantified with a spectrophotometer using the trichromatic equations (Parsons and Strickland 1968).

As stated before, most studies, calculate a ratio of cyanotoxin produced per volume of cyanobacteria it is used cell count per millilitre as volume unit. However, cell count is not a homogeneous measure to compare cyanotoxin production, mostly because cell size varies greatly within the cyanobacteria phylum, even between strains of the same species. Biovolume is a better volume indicator because, as is based in the metric system, is the same units for every phytoplanktonic species.

Therefore, in this thesis the cyanotoxin production ratio will be calculated as μg of toxin per mm^3 of biovolume of cyanobacteria.

3.4 Results and discussion

Results from the dominant taxa and their respective biovolume can be found in Figure 17. We can distinguish 2 main cyanobacteria that share dominance: *Planktothrix agardhii* and *Aphanizomenon gracile*. In June there is a co-dominance as *P. agardhii* covers 41% of the total biovolume of the samples from the reservoir while *A. gracile* covers 36%. However, we can see a steadily increasing dominance of *P. agardhii*, because each month has more dominance than before, achieving a total of 87.66% of the total biovolume of the reservoir in October. From all the samples there

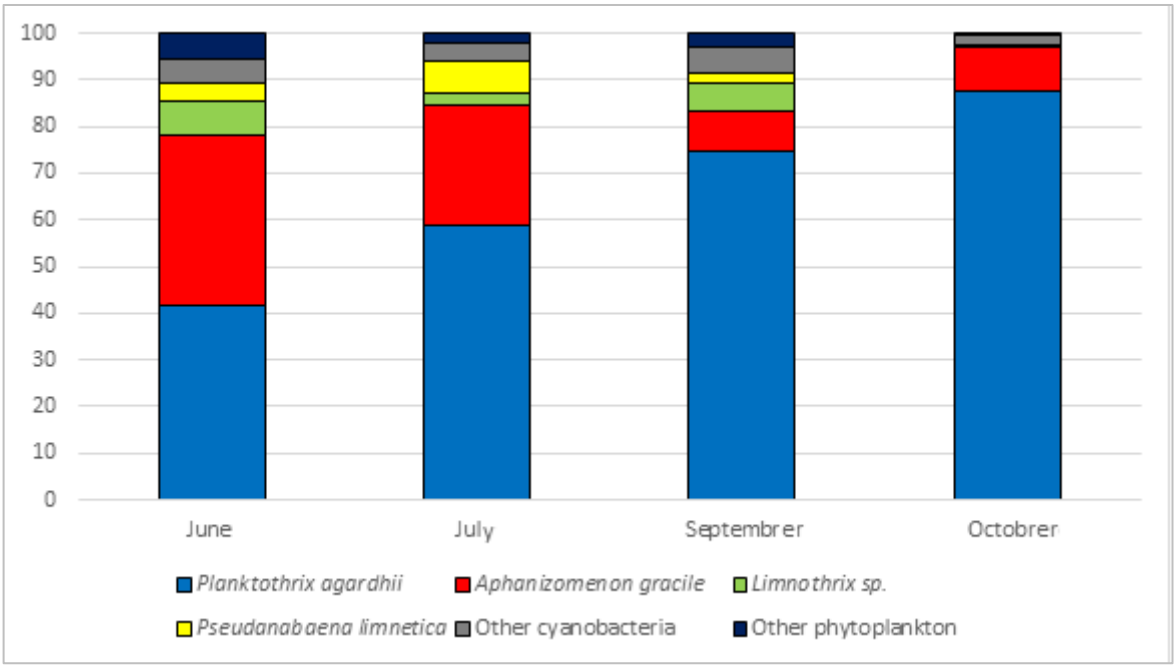


Figure 17. Proportion of phytoplankton in the samples of Rosarito (only showed the species representing above 5% of the total phytoplankton biomass)

were identified 10 phytoplanktonic species, 8 corresponding to the phylum cyanobacteria (Table 7).

Sampling date	June	July	September	October
ATX (μg/L)	0.06	0.62	2.11	0.06
MCs (μg/L)	3.50	7.60	18.60	14.20
STXs (μg/L)	0.07	0.12	0.04	0.02

Table 7 Results of phytoplankton identification and quantification. Biovolume measured in mm3/m3.

From the field samples, only MCs were detected and quantified by HPLC (Table 8). However, MCs, STX and ATX were detected using ELISA (Table 9). STX and ATX values were not detected with the HPLC-MS-MS methodology possibly because the values detected are remarkably close to the detection limit (0.05μg/L).

Regarding the isolates, there were a total of 22 successful isolates, composed by 16 pure cultures (isolates with only one single strain of cyanobacteria) and 6 mixed cultures (those which contained 2 phytoplanktonic strains, being at least one of them from the cyanobacteria phylum). Only 9 cultures produced cyanotoxins (Table 10), 5 of them MCs and the other 4 STXs. Four MCs positives were found in *Planktothrix agardhii* cultures, 3 of them were pure cultures of *P. agardhii* and the

fourth culture was a mixed culture were *P. agardhii* was isolated with *Pseudanabaena limnetica*.

Species	June		July		September		October	
	Biovol. (mm^3/m^3)	cell/ml	Biovol. (mm^3/m)	cell/ml	Biovol. (mm3/m3)	cell/ml	Biovol. (mm3/m3)	cell/ml
Planktothrix agardhii	2137.5	182949.7	3305.4	112912.9	38248.1	1306495	14170.7	785768
Aphanizomenon gracile	1859.2	169831.6	1450.7	41717.4	4390.7	209833.1	1510.4	50799.8
Limnothrix redekei.	377.8	52736.7	138.0	5424.0	3120.0	107115.4	31.7	31694.9
Pseudanabaena sp.	215.3	27116.7	173.1	21931.7	2117.7	72267.2	358.4	84079.4
Pseudanabaena limnetica	206.7	7219.4	373.1	11838.4	1116.9	38151.2		
Anabaena sp.	35.0	484.2			206.7	2567.9		
Aphanizomenon issatschenkoi			58.2	1061.2	474.9	37234.1		
Merispodemia sp.					35.5	26962.6		
Cyanobacteria total biovolume	**4831.5**	**440338**	**5498.6**	**194885.6**	**49710.4**	**1800626.3**	**16071.2**	**952342**
Chlorococcales	294.7	8055.8	1.3	212.2	1469.3	5870.2	95.0	2817.3
Staurastrum cingulum			111.5	23.6				
Total biovolume	**5126.2**	**448394.1**	**5611.3**	**195121.4**	**51179.7**	**1806496**	**16166.2**	**955159**

Table 8. Content in µg/L in the field samples measured with ELISA (Casero, et al. 2019)

There were 2 pure cultures of nontoxic strains of *P. agardhii.* These cultures didn't produce any detectable toxin and they lacked any toxin-producer related genes (mcyE in case of MCs) analysed with the PCR. The fifth MCs positive was from a pure *Limnothrix redekei* culture.

MICROCYSTINS

Strains	Cyanobacteria culture	MC-dmRR	MC-LR	MC-RR	MC-YR	MC-LA	MC-LY	MC-LW	MC-LF	Total MCs equivalents
M1	**Planktothrix agardhii**	33069	n.d	82.1	72.6	n.d	n.d	n.d	n.d	33224.4
M2	**Planktothrix agardhii**	8021.6	n.d	40.5	n.d	n.d	n.d	n.d	n.d	8062.1
M3	**Planktothrix agardhii**	6396.9	n.d	n.d	n.d	n.d	n.d	n.d	n.d	6396.9
M4	***Planktothrix agardhii* /Pseudanabaena limnetica**	460.8	n.d	n.d	n.d	n.d	n.d	n.d	n.d	460.8
M5	**Limnothrix redekei**	n.d	n.d	25.1	n.d	n.d	n.d	n.d	n.d	25.1

SAXITOXINS

Strains	Cyanobacteria culture	saxitoxin	dcsaxitoxin	neosaxitoxin	gonyautoxin	Sum of saxitoxins
S1	**Aphanizomenon gracile**	5733.0	n.d	n.d	n.d	5733.0
S2	**Aphanizomenon gracile**	2473.0	n.d	n.d	n.d	2473.0
S3	**Aphanizomenon gracile**	739.0	n.d	n.d	n.d	739.0
S4	**Aphanizomenon gracile / Chlorococcales**	2106.8	n.d	1902.4	94.4	4103.6

Table 9. Content in cultures isolates. Toxins are measured in µg/L; n.d: not detected or under detection levels; <L.C: Positive result but under reliable quantification levels

There were isolated another 2 nontoxic pure cultures of *L. redekei.* Every STX positives, corresponded with an *Aphanizomenon gracile* culture. Within the toxin producers' cultures found 3 of them were pure cultures of *Aphanizomenon gracile*, and a fourth one was mixed, but in this case with a green alga from the Chloroccocal family. There were isolated 7 nontoxic pure cultures of *Aph. gracile.* The last isolated left was a nontoxic pure culture of *Phormidium sp*.

Cultures origin		June	July	Aug	Sept	M1	M2	M3	M4	M5	S1	S2	S3	S4
HPLC Measurement Tox/Biovol (μg/mm³)	**MC/*P. agardhii* TPR**	1,08	2,01	0,43	1,06	129,8	46,89	234,3	31,22	10^{-3}*				
	PSP/*A. gracile* TPR										22,40	167,5	23,87	2,48
ELISA Measurement Tox/Biovol (μg/mm³)	**MC/*P. agardhii* TPR**	1,64	2,30	0,49	1,00									
	PSP/*A. gracile* TPR	0,038	0,083	0,009	0,013									

Table 10. Toxin quantity and cyanobacteria biovolume ratio (TPR) in field samples and isolates. (*) This ratio was calculated with *Limnothrix redekei* biovolume.

With all these results we calculated the Toxin Production Ratio (TPR) by calculating how many μg correspond to a mm^3 of cyanobacterial biomass (Table 10). For the field samples TPR, *P. agardhii* was considered the only MC producer and *Aph. gracile* the only STX producer. From the TPR calculated (Table 10), it can be estimated that the average MC TPR from isolates was 110 times higher than the average MC TPR value found in the field samples. The average STX TPR from isolates was 750 times higher than the average STX TPR value found in the field samples.

Variations in MCs TPR (Table 10) cannot be explained by physiological regulations of MC synthesis at the individual level (Orr and Jones 1998), as within the same cyanobacteria strains there is a difference of 3 magnitudes in their TPR, meaning than in a more a more optimal metabolic conditions, *P. agardhii* could produce much more quantity of MC . Moreover, the changes in MC TPR are more likely the result of shifts in the composition of toxic (MC-producing) and nontoxic individuals in the population (Kardinaal, *et al.* 2007).

STXs TPR follows a similar production pattern, where it seems to be less efficient producing toxins in nature than in cultures (Table 10). In other STX producing cyanobacteria, temperature can be major contributors in the production of said toxin, peaking toxin production been at 25ºC (Mesquita, *et al.* 2019). All field sampling measurements were taken in the range from 20-25ºC. However, it is important to point out that the STX/biovolume ratio from Table 4 only considers *Aphanizomenon gracile* biovolume. There are reports from the same field samples which points out *A. gracile* as the only STX toxin producer in Rosarito reservoir (Casero, *et al.* 2019).

It is to be expected that no cyanobacteria will produce the same levels of toxins in nature as in the laboratory, even under ideal conditions. However, considering the significant difference in the TPR, the potentiality of extremely high toxin production,

as demonstrated in the laboratory indicates that eventually the toxin concentration in the field could reach harmful concentrations.

In freshwater cyanobacteria, next generation sequencing (NGS) has been used to assess the general diversity mostly based on Operational Taxonomy Units (OTUs) derived from 16S rRNA amplicons (also known as metabarcoding). The same field samples analysed in this study were analysed with NGS and already published (Casero, *et al.* 2019). The OUT's used for this study are listed in Table A2 from the Supplementary material section. The analysis of Rosarito toxic strains detected by NGS with the 16S rRNA coincidence with the toxic isolates, therefore confirming the identification of those cyanobacteria as the toxin producers from Rosarito reservoir (Table 11).

These proves NGS is a great technique when identifying cyanotoxin producers, which would make a great synergy with other field sample methodologies to properly analyse all the ecosystem of waterbodies with the purpose of monitoring health hazards.

3.5 Conclusions

Several noteworthy results were found on the cyanotoxicity of the Rosarito reservoir. Regarding the methodology, emphasize the precise detection of MC by

both HPLC-MS-MS and ELISA techniques. However, the ELISA technique proved to be accurate in detecting and quantifying STX and ATX when the values of these toxins are in low concentration.

Strains	NCBI BLAST Identification	Similitude cover	Percent Identity	Related OTU #1	Similitude cover	Percent Identity	Related OTU#2	Similitude cover	Percent Identity
S1	*Aphanizomenon gracile* UAM540	96%	96.32%	OTU 2	96%	95.79%	OTU 26	96%	96.32%
S2	Aphanizomenon gracile BACA0041	98%	98.58%	OTU 2	98%	98.34%	OTU 26	98%	97.63%
S3	*Aphanizomenon gracile* UAM539	97%	95.20%	OTU 1	95%	92.80%	OTU 26	95%	91.90%
S4	*Aphanizomenon gracile* UAM539	84%	96.69%	OTU 2	84%	96.42%	OTU 26	84%	95.31%
M1	Planktothrix agardhii CHAB643	100%	96.96%	OTU 1	100%	99.00%			
M2	Planktothrix agardhii CHAB643	96%	89.50%	OTU 1	96%	88.22%			
M3	Planktothrix agardhii CHAB643	96%	88.22%	OTU 1	96%	88.22%			
M4	Planktothrix agardhii CHAB643	99%	98.97%	OTU 1	99%	98.97%			
M5	Limnothrix redekei 2LT25S01	98%	99.19%	OTU 6	99%	86.22%			

Table 11 NCI BLAST identification of the cyanotoxin producers' strains and comparison with related strain analysed in Casero et al (2019). Similitude cover is a percentage that describes how much of the sequence of the isolate's DNA is covered by the target sequence (strains from the NGS). Percent identity is how many in each sequence are identical in percentage (the higher the percent identity is, the more significant the match)

The contrast of the production of toxins in the laboratory and in nature should also be considered by the freshwater management administrations. Despite the numerous rules and limits established by different national and supranational bodies like the WHO (Chorus, *et al.* 2021), a review of how to assess the risk that cyanobacteria, especially those that produce cyanotoxins that are not commonly regulated in countries as is the case with STX, may be necessary.

The results accentuate having to consider cyanobacteria in near future challenges we must face, especially climate change. Climate change will demonstratable boost cyanobacterial populations all over the world (Hofer 2018). If these increases in population reach certain threshold, cyanotoxin production could increase tenfold, skyrocketing the potential of serious problems for human health.

Future studies of composition and distribution of the phytoplanktonic flora will improve the understanding of all aquatic ecosystems. More importantly, it will help management administrations of water bodies to establish policies that protect the population even better from these risks to human health

For this purpose, emphasize the value of the NGS as an identifying element of the phytoplanktonic ecosystem, since the isolates coincide, not only in the main species of which the sample was composed, it also identified the main producers of

cyanotoxins, which could become a particularly useful tool for the management of water bodies in the near future.

General Discussion

The results emphasize the need to consider cyanobacteria in future emerging challenges, especially climate change. It not only favours the proliferation of cyanobacteria due to their greater adaptability to extreme temperatures, but also the invasion of ecosystems by invasive tropical cyanobacteria, especially dangerous when capable of producing bacteria. Future studies in the composition and distribution of the phytoplanktonic flora will help to advance in the ecosystems knowledge to provide water management administrations with scientific information to better establish tailored policies that protect citizens from cyanobacteria damages to human health.

The power of remote sensing

In this thesis, it was defined and validated the use of MERIS imagery to estimate the cyanobacterial biovolume in freshwater ecosystems under non-scum conditions. This approach is based on the detection of the cyanobacterial pigment PC by means of the MERIS sensor, and we have adapted this tool to calculate cyanobacterial biovolume, a particularly important parameter for limnological and water quality studies. In our analysis, the correlation between the measured cyanobacterial biovolumes and the *PC* values estimated from the MERIS imagery was found to be a robust proxy for cyanobacterial biomass estimation. The regression

equation was built by using data from 23 aquatic ecosystems in the Iberian Peninsula, which were dominated by diverse cyanobacterial communities.

A validation exercise carried out with time series data from an external water reservoir indicated that the equation was useful for estimating cyanobacterial biomass with excellent results when the time lag between field measurements and MERIS image was lower than 9 days. Considering that our data cover a wide area of different lithological and trophic characteristics, with 89 different cyanobacteria species, from which 23 distinct species were dominant and the positive results from the validation test we believe that these results could be transferable to other water reservoirs.

Lastly, if something is demonstrated in this thesis is the need for a multidisciplinary study process in the study of cyanobacteria in freshwater bodies. Remote sensing, databases, classic biovolume analysis, quantification of cyanotoxins (especially by ELISA), NGS, sample isolates. Each of these methodologies reveals new aspects of the ecology of cyanobacteria that make a great synergy when comparing results. The study of the environment is essential to face challenges such as climate change and be prepared for the future challenges that cyanobacteria may cause, especially when they are potentially toxic. Thanks to a multidisciplinary methodology, we will be prepared.

The dangers of invasiveness

Although invasiveness is complicated to predict, it is possible to analyse the growing conditions and likelihood of survival of new species in each habitat. In this thesis, the two most key factors or variables that may be related to the dominance of potential invasive cyanobacteria species are temperature and eutrophication.

Because temperature plays a significant role, temperate or cold ecosystems facing warmer climates and droughts induced by climate change could be prone to invasion by new cyanobacteria species. *Ch. ovalisporum* is now well established in Spain due to its preference for very warm summers (>30 °C), which can surely help to colonize new oligotrophic warm ecosystems. *C. raciborskii,* a tropical cyanobacterium, is spread all over Europe because of the new niches that climate change and anthropogenic pollution have opened. Because of the warmer climates and higher levels of nutrients in water bodies, ecosystems are more easily invaded by this cyanobacterium. *D. lemmermannii* is also spreading all over Europe, taking advantage of similar ecological preferences, with the northern morphospecies being the most troubling because they include the cyanotoxin producers [26].

It is also interesting to point out that *P. agardhii* and *C. issatschenkoi* follow similar ecological preferences in different countries, with slight differences in range. This phenomenon could indicate great adaptability because the range of conditions in which they can live is so wide that perhaps the limits shown in the data are not the

limitations of the species but instead the whole range of conditions that appear in each country.

The dangerous peak of cyanotoxin production

Several significant results were found on the cyanotoxicity of the Rosarito reservoir. Regarding the methodology, emphasize the good detection of MC by HPLC-MS-MS and ELISA techniques. However, the later proved to be more accurate in detecting and quantifying STX and ATX when the values of these toxins are low.

On the other hand, the promising value of the NGS as an identifying element of the phytoplanktonic ecosystem composition is noteworthy. The isolates coincided not only in identifying the main species that made up the sample, but also in identifying the main producers of cyanotoxins. This could become a particularly useful tool for water body management in the future.

The contrast of the production of toxins in the laboratory and in nature should also be considered by freshwater management administrations. It is to be expected that no cyanobacteria will produce the same levels of toxins in nature as in the laboratory, even under ideal conditions. However, given that we are talking about three orders of magnitude of difference, the potential of toxic cyanobacteria to produce many more cyanobacteria than expected when sampling is worrying. For many years, population health has been at risk due to high toxicity caused by

cyanotoxins. Despite the numerous regulations and limits established by national and supranational bodies, a review of how to assess the risk of cyanobacteria evolution, especially those that produce cyanotoxins that are not commonly regulated as is the case with STX, may be necessary.

Conclusions and further work

1. The estimation of cyanobacterial biovolume through the calculation of phycocyanin concentration by remote sensing is an important advance in the massive study of cyanobacterial communities. In addition to having the potential to provide fast and accurate data on large water bodies, mainly under human use, through satellite imagery, it also allows to analyze the historical evolution of cyanobacterial communities based on the set of thousands of images captured by the MERIS sensor throughout the period of time in orbit.

La estimación de biovolumen cianobacteriano a través del cálculo de la concentración de ficocianina mediante teledetección es un importante avance en el estudio masivo de comunidades de cianobacterias. Además de tener el potencial de dar datos rápidos y precisos de grandes masas de agua por satélite, aguas que seguramente estén en mayor o menor medida bajo uso humano, también nos permite analizar comunidades del pasado con los miles de imágenes que el sensor MERIS captó durante toda su historia.

2. Now the Sentinel-3A and 3B, launched in 2016 and 2018 respectively, with a sensor similar to MERIS are capable of capturing wavelengths similar to those used in this thesis. This, together with an orbital revisit period of less than 2

days combining both satellites, opens a unique range of possibilities for the study of phytoplankton by remote sensing.

Los Sentinel-3A y 3B, lanzados en 2016 y 2018 respectivamente, con un sensor parecido al MERIS son capaces de captar longitudes de onda similares a las usadas en esta tesis. Eso sumado a un periodo orbital de revisita menor a 2 días combinando ambos satélites, abre un abanico de posibilidades único para el estudio de fitoplancton por teledetección.

3. The future study of remote sensing should involve synchronized sampling with the twin Sentinel-3 satellites on non-cloudy days. The ideal would be to obtain the concentration of phycocyanin and biovolume analysed in the field and compare them with the estimates made through the images captured by the satellites. In this thesis it is shown that to achieve the best possible approximation, results can be very close to reality when comparing samples from the same day, advancing even further in the possible automation of the process to obtain large amounts of information from the communities of daily cyanobacteria at global scale.

El futuro estudio de la teledetección debería pasar por un muestreo sincronizado con los satélites gemelos Sentinel-3 en días no nublados. Lo ideal sería obtener la concentración de ficocianina y biovolumen analizados en campo y

compararlos con las estimaciones hechas a través de las imágenes captadas por los satélites. En esta tesis se demuestra que para conseguir la mejor aproximación posible, los resultados pueden ser muy aproximados a la realidad si se comparan muestras del mismo día, avanzando todavía más en la posible automatización del proceso para poder obtener grandes cantidades de información de las comunidades de cianobacterias diarias en todo el planeta.

4. Thanks to the results of this thesis, the following can be stated as regards cyanobacteria behaviour:

a) ***D. lemmermannii*** showed a preference for temperate waterbodies with mesotrophic or lower eutrophication levels. In contrast,

b) ***C. raciborskii*** mainly frequents shallow water bodies with high nutrient concentrations.

c) ***Ch. ovalisporum*** distribution is apparently not driven by nutrient concentrations but rather by warm water temperatures, reaching its maximum growth at 30°C. This high temperature dependence represents a potential threat considering the increase in overall temperature due to climate change.

d) ***C. issatschenkoi*** is mostly found in water bodies with low alkalinity, low conductivity (especially the Spanish morphospecies) and at an average high pH level, as in each country, the morphospecies is found within the highest pH mean values.

e) ***P. agardhii*** appears over a wide range of parameters, such as total phosphorus, Secchi disk depth, nitrates and nitrites, and conductivity. This wide range of adaptation to so many ecological parameters could be one of the reasons why this species present the current worldwide distribution .

f) ***P. agardhii*** *and* ***C. issatschenkoi*** follow similar ecological preferences in different countries, with slight differences in range. This phenomenon could indicate great adaptability because the range of conditions in which they can live is so wide that perhaps the limits shown in the data are not due to the limitations of the species but rather the entire range of intrinsic conditions in each specific area at the country level.

Gracias a los resultados de esta tesis, se puede afirmar lo siguiente del comportamiento de las cianobacterias:

a) ***D. lemmermannii*** mostró preferencia por cuerpos de agua templados con niveles mesotróficos o de eutrofización más bajos. En contraste,

b) *C. raciborskii* frecuenta en su mayoría cuerpos de agua poco profundos con altas concentraciones de nutrientes.

c) La distribución de ***Ch. ovalisporum*** aparentemente no está impulsada por concentraciones de nutrientes, sino más bien por temperaturas cálidas del agua, alcanzando su crecimiento máximo a 30 ° C. Esta dependencia de las altas temperaturas representa una amenaza potencial teniendo en cuenta el aumento de la temperatura general debido al cambio climático.

d) ***C. issatschenkoi*** se encuentra principalmente en cuerpos de agua con baja alcalinidad, baja conductividad (especialmente las morfoespecies españolas) y a un nivel de pH medio alto, ya que en cada país, la morfoespecie se encuentra dentro de los valores medios de pH más altos.

e) ***P. agardhii*** aparece en una amplia gama de parámetros, como el fósforo total, la profundidad del disco de Secchi, los nitratos y nitritos, y la conductividad. Este amplio abanico de adaptación a tantos parámetros ecológicos podría ser una de las razones por las que esta especie tiene la distribución mundial real que muestra.

f) ***P. agardhii y C. issatschenkoi*** siguen preferencias ecológicas similares en diferentes países, con ligeras diferencias en el rango. Este fenómeno podría indicar una gran adaptabilidad porque el rango de condiciones en las que pueden vivir es tan amplio que quizás los límites mostrados en los datos no sean las limitaciones de la especie sino toda la gama de condiciones intrínsecas en cada zona concreta a nivel país.

5. The main difference between invasive and ubiquitous cyanobacteria is that the invasive ones seem to have more niche ecological needs (temperature ranges, level of eutrophication, etc). Meanwhile, ubiquitous cyanobacteria thrive in a very wide range of environments, even if those environments feature very different regions and climates as is the case in Denmark and Spain.

La principal diferencia entre las cianobacterias invasoras y las ubicuas es que las invasivas parecen tener necesidades ecológicas de nicho (cierta temperatura, nivel de eutrofización, etc..). Mientras tanto, las cianobacterias ubicuas prosperan en una amplia gama de entornos, incluso si esos entornos tienen regiones y climas muy diferentes como es el caso de Dinamarca y España.

6. Recreating controlled scenarios that could occur in the future and comparing them to reality gives us promising results. We have described cyanobacteria with the potential to produce between 100 and 200 times more cyanotoxins per unit biomass that those they are producing in the field, a scenario that is worrying considering that this could occur in water bodies linked to human health.

Recrear escenarios controlados que podrían suceder en el futuro y compararlo con la realidad nos da resultados increíbles. Hemos descrito cianobacterias con el potencial de producir entre 100 y 200 veces más cianotoxinas por unidad de biomasa que están produciendo en el campo, un escenario que es preocupante cuando esto podría suceder en cuerpos de agua vinculados a la salud humana.

7. Database comparison, although descriptive in nature, has the potential to reveal aspects of cyanobacterial distribution dynamics that would be impossible to be achieved otherwise. The work of thousands of samples conducted by

experts from all over the world can bear fruit that could not be obtained in any other way.

La comparación de bases de datos, aunque descriptiva en naturaleza, tiene el potencial de descubrirnos aspectos de la dinámica de distribución de cianobacterias que sería imposible hacer de otra forma. El trabajo de miles de muestreos realizados por expertos de todo el mundo puede dar frutos que no se podrían obtener de ninguna otra forma. Comparing field and laboratory work is essential for the advancement of science.

Bibliography

Agha, R., Cires, S., Wormer, L., Dominguez, J. A. and Quesada, A. (2012). Multi-scale strategies for the monitoring of freshwater cyanobacteria: reducing the sources of uncertainty. *Water Res* 46(9): 3043-53.

Akcaalan, R., Koker, L., Oguz, A., Spoof, L., Meriluoto, J. and Albay, M. (2014). First report of cylindrospermopsin production by two cyanobacteria (Dolichospermum mendotae and Chrysosporum ovalisporum) in Lake Iznik, Turkey. *Toxins (Basel)* 6(11): 3173-86.

Akcaalan, R., Young, F. M., Metcalf, J. S., Morrison, L. F., Albay, M. and Codd, G. A. (2006). Microcystin analysis in single filaments of Planktothrix spp. in laboratory cultures and environmental blooms. *Water Res* 40(8): 1583-90.

Al-Awadhi, F. H., Law, B. K., Paul, V. J. and Luesch, H. (2017). Grassystatins D-F, Potent Aspartic Protease Inhibitors from Marine Cyanobacteria as Potential Antimetastatic Agents Targeting Invasive Breast Cancer. *Journal of Natural Products* 80(11): 2969-2986.

Ali, K., Witter, D. and Ortiz, J. (2013). Application of empirical and semi-analytical algorithms to MERIS data for estimating chlorophyll a in Case 2 waters of Lake Erie. *Environmental Earth Sciences* 71(9): 4209-4220.

Almuhtaram, H., Kibuye, F. A., Ajjampur, S., Glover, C. M., Hofmann, R., Gaget, V., Owen, C., Wert, E. C. and Zamyadi, A. (2021). State of knowledge on early warning tools for cyanobacteria detection. *Ecological Indicators* 133: 108442.

Antunes, J. T., Leao, P. N. and Vasconcelos, V. M. (2015). Cylindrospermopsis raciborskii: review of the distribution, phylogeography, and ecophysiology of a global invasive species. *Front Microbiol* 6: 473.

Ballot, A., Fastner, J., Lentz, M. and Wiedner, C. (2010). First report of anatoxin-a-producing cyanobacterium Aphanizomenon issatschenkoi in northeastern Germany. *Toxicon* 56(6): 964-71.

Ballot, A., Ramm, J., Rundberget, T., Kaplan-Levy, R. N., Hadas, O., Sukenik, A. and Wiedner, C. (2011). Occurrence of non-cylindrospermopsin-producing Aphanizomenon ovalisporum and Anabaena bergii in Lake Kinneret (Israel). *Journal of Plankton Research* 33(11): 1736-1746.

Ballot, A., Scherer, P. I. and Wood, S. A. (2018). Variability in the anatoxin gene clusters of Cuspidothrix issatschenkoi from Germany, New Zealand, China and Japan. *Plos One* 13(7): e0200774.

Banares-Espana, E., Lopez-Rodas, V., Costas, E., Salgado, C. and Flores-Moya, A. (2007). Genetic variability associated with photosynthetic pigment concentration, and photochemical and nonphotochemical quenching, in strains of the cyanobacterium Microcystis aeruginosa. *FEMS Microbiol Ecol* 60(3): 449-55.

Barón-Sola, Á., Campo, F. F. d. and Sanz-Alférez, S. (2016). Dynamics of Cylindrospermopsin Production and Toxin Gene Expression in <i>Aphanizomenon ovalisporum</i&gt. *Advances in Microbiology* 06(05): 381-390.

Berry, J. P., Gibbs, P. D., Schmale, M. C. and Saker, M. L. (2009). Toxicity of cylindrospermopsin, and other apparent metabolites from Cylindrospermopsis raciborskii and Aphanizomenon ovalisporum, to the zebrafish (Danio rerio) embryo. *Toxicon* 53(2): 289-99.

Binding, C. E., Greenberg, T. A. and Bukata, R. P. (2011). Time series analysis of algal blooms in Lake of the Woods using the MERIS maximum chlorophyll index. *Journal of Plankton Research* 33(12): 1847-1852.

Bohunicka, M. (2015). Towards a modern revision of the cyanobacteria, a critically important prokaryotic phylum, Phd. Thesis, University of South Bohemia, Faculty of Science, School of ….

Bonilla, S., Aubriot, L., Soares, M. C., Gonzalez-Piana, M., Fabre, A., Huszar, V. L., Lurling, M., Antoniades, D., Padisak, J. and Kruk, C. (2012). What drives the distribution of the bloom-forming cyanobacteria Planktothrix agardhii and Cylindrospermopsis raciborskii? *FEMS Microbiol Ecol* 79(3): 594-607.

Boopathi, T. and Ki, J. S. (2014). Impact of environmental factors on the regulation of cyanotoxin production. *Toxins (Basel)* 6(7): 1951-78.

Bouaicha, N. and Nasri, A. B. (2004). First report of cyanobacterium Cylindrospermopsis raciborskii from Algerian freshwaters. *Environ Toxicol* 19(5): 541-3.

Bouvy, M., Molica, R., De Oliveira, S., Marinho, M. and Beker, B. (1999). Dynamics of a toxic cyanobacterial bloom (Cylindrospermopsis raciborskii) in a shallow reservoir in the semi-arid region of northeast Brazil. *Aquatic Microbial Ecology* 20(3): 285-297.

Briand, J.-F., Leboulanger, C., Humbert, J.-F., Bernard, C. and Dufour, P. (2004). Cylindrospermopsis Raciborskii (Cyanobacteria) Invasion at Mid-Latitudes: Selection, Wide Physiological Tolerance, Orglobalwarming?1. *Journal of Phycology* 40(2): 231-238.

Brient, L., Lengronne, M., Bertrand, E., Rolland, D., Sipel, A., Steinmann, D., Baudin, I., Legeas, M., Le Rouzic, B. and Bormans, M. (2008). A phycocyanin probe as a tool for monitoring cyanobacteria in freshwater bodies. *J Environ Monit* 10(2): 248-55.

Burford, M. A., Beardall, J., Willis, A., Orr, P. T., Magalhaes, V. F., Rangel, L. M., Azevedo, S. and Neilan, B. A. (2016). Understanding the winning strategies used by the bloom-forming cyanobacterium Cylindrospermopsis raciborskii. *Harmful Algae* 54: 44-53.

Callieri, C., Bertoni, R., Contesini, M. and Bertoni, F. (2014). Lake level fluctuations boost toxic cyanobacterial "oligotrophic blooms". *PLoS One* 9(10): e109526.

Capelli, C., Ballot, A., Cerasino, L., Papini, A. and Salmaso, N. (2017). Biogeography of bloom-forming microcystin producing and non-toxigenic populations of Dolichospermum lemmermannii (Cyanobacteria). *Harmful Algae* 67: 1-12.

Carlson, R. E. (1977). A trophic state index for lakes 1. *Limnology and oceanography* 22(2): 361-369.

Carmichael, W. W. (2012). Health Effects of Toxin-Producing Cyanobacteria: "The CyanoHABs". *Human and Ecological Risk Assessment: An International Journal* 7(5): 1393-1407.

Carota, A., Calabrese, P. and Bogousslavsky, J. (2016). Neurotoxic Weapons and Syndromes. *War Neurology*. L. Tatu and J. Bogousslavsky. Basel, Karger. 38**:** 214-227.

Carrasco, D., Moreno, E., Paniagua, T., Hoyos, C. d., Wormer, L., Sanchis, D., Cirés, S., Martín-del-Pozo, D., Codd, G. A. and Quesada, A. (2007). Anatoxin-a occurrence and potential cyanobacterial anatoxin-a producers in Spanish reservoirs1. *Journal of Phycology* 43(6): 1120-1125.

Carvalho, L., McDonald, C., de Hoyos, C., Mischke, U., Phillips, G., Borics, G., Poikane, S., Skjelbred, B., Solheim, A. L., Van Wichelen, J., Cardoso, A. C. and Cadotte, M. (2013). Sustaining recreational quality of European lakes: minimizing the health risks from algal blooms through phosphorus control. *Journal of Applied Ecology* 50(2): 315-323.

Casero, M. C., Ballot, A., Agha, R., Quesada, A. and Cirés, S. (2014). Characterization of saxitoxin production and release and phylogeny of sxt genes in paralytic shellfish poisoning toxin-producing Aphanizomenon gracile. *Harmful Algae* 37: 28-37.

Casero, M. C., Velazquez, D., Medina-Cobo, M., Quesada, A. and Cires, S. (2019). Unmasking the identity of toxigenic cyanobacteria driving a multi-toxin bloom by high-throughput sequencing of cyanotoxins genes and 16S rRNA metabarcoding. *Sci Total Environ* 665: 367-378.

Cavalier-Smith, T. (2006). Cell evolution and Earth history: stasis and revolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* 361(1470): 969-1006.

Chorus, I. (2005). *Current approaches to cyanotoxin risk assessment, risk management and regulations in different countries*, Fed. Environmental Agency

Chorus, I. (2012). *Current approaches to Cyanotoxin risk assessment, risk management and regulations in different countries*. Berlin, Federal Environment Agency

Chorus, I. and Bartram, J. (1999). *Toxic Cyanobacteria in water*, Spon Press.416

Chorus, I., Fastner, J. and Welker, M. (2021). Cyanobacteria and Cyanotoxins in a Changing Environment: Concepts, Controversies, Challenges. *Water* 13(18).

Churro, C., Azevedo, J., Vasconcelos, V. and Silva, A. (2017). Detection of a Planktothrix agardhii Bloom in Portuguese Marine Coastal Waters. *Toxins (Basel)* 9(12): 13.

Cires, S., Wormer, L., Ballot, A., Agha, R., Wiedner, C., Velazquez, D., Casero, M. C. and Quesada, A. (2014). Phylogeography of cylindrospermopsin and paralytic shellfish toxin-producing nostocales cyanobacteria from mediterranean europe (Spain). *Appl Environ Microbiol* 80(4): 1359-70.

Cirés, S., Wörmer, L., Timón, J., Wiedner, C. and Quesada, A. (2011). Cylindrospermopsin production and release by the potentially invasive cyanobacterium Aphanizomenon ovalisporum under temperature and light gradients. *Harmful Algae* 10(6): 668-675.

Colautti, R. I. and MacIsaac, H. J. (2004). A neutral terminology to define 'invasive' species. *Diversity and Distributions* 10(2): 135-141.

Conley, D. J., Paerl, H. W., Howarth, R. W., Boesch, D. F., Seitzinger, S. P., Havens, K. E., Lancelot, C. and Likens, G. E. (2009). ECOLOGY Controlling Eutrophication: Nitrogen and Phosphorus. *Science* 323(5917): 1014-1015.

Cook, R. (2021). The feasibility of using satellite data (MERIS) to assess water quality using data retrieved from coastal waters in Vuosaari, Eastern Helsinki region.

Cottingham, K. L., Weathers, K. C., Ewing, H. A., Greer, M. L. and Carey, C. C. (2020). Predicting the effects of climate change on freshwater cyanobacterial blooms requires consideration of the complete cyanobacterial life cycle. *Journal of Plankton Research* 43(1): 10-19.

Crawford, A., Holliday, J., Merrick, C., Brayan, J., van Asten, M. and Bowling, L. (2017). Use of three monitoring approaches to manage a major Chrysosporum ovalisporum bloom in the Murray River, Australia, 2016. *Environmental monitoring and assessment* 189(4): 202.

D.E.P. "Danish Environmental Portal." (Accessed: 1st of August, 2014), from https://miljoeportal.dk/english/.

De Hoyos, C., Negro, A. I. and Aldasoro, J. J. (2004). Cyanobacteria distribution and abundance in the Spanish water reservoirs during thermal stratification. *Limnetica 23 (1-2)*: 119-132.

De Souza, R. C. R., Carvalho, M. C. and Truzzi, A. C. (1998). Cylindrospermopsis raciborskii (Wolosz.) Seenaya and Subba Raju (Cyanophyceae) dominance and a contribution to the knowledge of Rio Pequeno Arm, Billings Reservoir, Brazil. *Environmental Toxicology and Water Quality* 13(1): 73-81.

Dekker, A. G. and Hestir, E. L. (2012). *Evaluating the feasibility of systematic inland water quality monitoring with satellite remote sensing*

Domínguez, J. A., Alonso, C. and García, A. A. (2011). Remote sensing as a tool for monitoring water quality parameters for Mediterranean Lakes of European Union water framework directive (WFD) and as a system of surveillance of cyanobacterial harmful algae blooms (SCyanoHABs). *Environmental monitoring and assessment* 181(1): 317-334.

Donis, D., Mantzouki, E., McGinnis, D. F., Vachon, D., Gallego, I., Grossart, H. P., de Senerpont Domis, L. N., Teurlincx, S., Seelen, L. and Lürling, M. (2021). Stratification strength and light climate explain variation in chlorophyll a at the continental scale in a European multilake survey in a heatwave summer. *Limnology and Oceanography*.

Donlon, C., Berruti, B., Buongiorno, A., Ferreira, M. H., Féménias, P., Frerick, J., Goryl, P., Klein, U., Laur, H., Mavrocordatos, C., Nieke, J., Rebhan, H., Seitz, B., Stroede, J. and Sciarra, R. (2012). The Global Monitoring for Environment and Security (GMES) Sentinel-3 mission. *Remote Sensing of Environment* 120: 37-57.

Dörnhöfer, K. and Oppelt, N. (2016). Remote sensing for lake research and monitoring–Recent advances. *Ecological Indicators* 64: 105-122.

Ergon, Å., Seddaiu, G., Korhonen, P., Virkajärvi, P., Bellocchi, G., Jørgensen, M., Østrem, L., Reheul, D. and Volaire, F. (2018). How can forage production in Nordic and Mediterranean Europe adapt to the challenges and opportunities arising from climate change? *European Journal of Agronomy* 92: 97-106.

(2014). Regulation (EU) No 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species.

Facey, J. A., Rogers, T. A., Apte, S. C. and Mitrovic, S. M. (2021). Micronutrients as growth limiting factors in cyanobacterial blooms; a survey of freshwaters in South East Australia. *Aquatic Sciences* 83(2): 1-11.

Fadel, A., Atoui, A., Lemaire, B. J., Vinçon-Leite, B. and Slim, K. (2014). Dynamics of the toxin cylindrospermopsin and the cyanobacterium Chrysosporum (Aphanizomenon) ovalisporum in a Mediterranean eutrophic reservoir. *Toxins* 6(11): 3041-3057.

Falconer, J. B., Chorus, I., Kuiper-Goodman, T., Utkilen, H., Burch, M. and Codd, G. A. (1999). SAFE LEVELS AND SAFE PRACTICES.

Ferrari, G., del Carmen Perez, M., Dabezies, M., Miguez, D. and Saizar, C. (2011). Planktic Cyanobacteria in the Lower Uruguay River, South America. *Fottea* 11(1): 225-234.

Gkelis, S., Moustaka-Gouni, M., Sivonen, K. and Lanaras, T. (2005). First report of the cyanobacterium Aphanizomenon ovalisporum Forti in two Greek lakes and cyanotoxin occurrence. *Journal of plankton research* 27(12): 1295-1300.

Guanter, L., Ruiz-Verdú, A., Odermatt, D., Giardino, C., Simis, S., Estellés, V., Heege, T., Domínguez-Gómez, J. A. and Moreno, J. (2010). Atmospheric correction of ENVISAT/MERIS data over inland waters: Validation for European lakes. *Remote Sensing of Environment* 114(3): 467-480.

Guo, Y. L., Huang, C. C., Li, Y. M., Du, C. G., Shi, L. F., Li, Y., Chen, W. Q., Wei, H. J., Cai, E. X. and Ji, G. X. (2022). Hyperspectral reconstruction method for optically complex inland waters based on bio-optical model and sparse representing. *Remote Sensing of Environment* 276.

Haande, S., Rohrlack, T., Ballot, A., Røberg, K., Skulberg, R., Beck, M. and Wiedner, C. (2008). Genetic characterisation of Cylindrospermopsis raciborskii (Nostocales, Cyanobacteria) isolates from Africa and Europe. *Harmful Algae* 7(5): 692-701.

Hadas, O., Pinkas, R., Malinsky-Rushansky, N., Shalev-Alon, G., Delphine, E., Berner, T., Sukenik, A. and Kaplan, A. (2002). Physiological variables determined under laboratory conditions may explain the bloom of Aphanizomenon ovalisporum in Lake Kinneret. *European Journal of Phycology* 37:2: 9.

Häder, D. P., Kumar, H. D., Smith, R. C. and Worrest, R. C. (1998). Effects on aquatic ecosystems. *Journal of Photochemistry and Photobiology B: Biology* 46(1-3): 53-68.

Harke, M. J., Steffen, M. M., Gobler, C. J., Otten, T. G., Wilhelm, S. W., Wood, S. A. and Paerl, H. W. (2016). A review of the global ecology, genomics, and biogeography of the toxic cyanobacterium, Microcystis spp. *Harmful algae* 54: 4-20.

Härmä, P., Vepsäläinen, J., Hannonen, T., Pyhälahti, T., Kämäri, J., Kallio, K., Eloheimo, K. and Koponen, S. (2001). Detection of water quality using simulated satellite data and semi-empirical algorithms in Finland. *Science of the Total Environment* 268(1-3): 107-121.

Hart, B. T. (1984). Eutrophication of Waters - Monitoring, Assessment and Control - Oecd. *Search* 15(1-2): 50-50.

Hillebrand, H., Dürselen, C.-D., Kirschtel, D., Pollingher, U. and Zohary, T. (1999). Biovolume Calculation for Pelagic and Benthic Microalgae. *Journal of Phycology* 35(2): 403-424.

Hodoki, Y., Ohbayashi, K., Kobayashi, Y., Okuda, N. and Nakano, S.-i. (2012). Detection and identification of potentially toxic cyanobacteria: Ubiquitous distribution of Microcystis aeruginosa and Cuspidothrix issatschenkoi in Japanese lakes. *Harmful Algae* 16: 49-57.

Hofer, U. (2018). Climate change boosts cyanobacteria. *Nature Reviews Microbiology* 16(3): 122-122.

Hoffmann, L. (1996). Geographic distribution of freshwater blue-green algae. *Biogeography of freshwater algae*, Springer**:** 33-40.

Huisman, J., Codd, G. A., Paerl, H. W., Ibelings, B. W., Verspagen, J. M. and Visser, P. M. (2018). Cyanobacterial blooms. *Nature Reviews Microbiology* 16(8): 471-483.

Hunter, P. D., Gilvear, D. J., Tyler, A. N., Willby, N. J. and Kelly, A. (2010). Mapping macrophytic vegetation in shallow lakes using the Compact Airborne Spectrographic Imager (CASI). *Aquatic Conservation: Marine and Freshwater Ecosystems* 20(7): 717-727.

Hunter, P. D., Tyler, A. N., Carvalho, L., Codd, G. A. and Maberly, S. C. (2010). Hyperspectral remote sensing of cyanobacterial pigments as indicators for cell populations and toxins in eutrophic lakes. *Remote Sensing of Environment* 114: 2705-2718.

Jiang, T., Zhang, J. P., Chang, W. R. and Liang, D. C. (2001). Crystal structure of R-phycocyanin and possible energy transfer pathways in the phycobilisome. *Biophys J* 81(2): 1171-9.

Jöhnk, K. D., Cherukuru, N., Anstee, J., ROBSON, B. and BOTHA, E. (2016). *Model-data assimilation framework for harmful algal bloom (CyanoHAB) prediction in inland waters on a continental scale*. Proceedings of the 11th international symposium on ecohydraulics. Melbourne, Australia.

Kaloudis, T., Hiskia, A. and Triantis, T. M. (2022). Cyanotoxins in Bloom: Ever-Increasing Occurrence and Global Distribution of Freshwater Cyanotoxins from Planktic and Benthic Cyanobacteria. *Toxins* 14(4).

Kardinaal, W. E. A., Janse, I., Kamst-van Agterveld, M., Meima, M., Snoek, J., Mur, L. R., Huisman, J., Zwart, G. and Visser, P. (2007). Microcystis genotype succession in relation to microcystin concentrations in freshwater lakes. *Aquatic Microbial Ecology* 48(1): 1-12.

Kim, I. S., Park, H. K. and Kim, Y. J. (2021). Development of Genus-Specific PCR Primers for Molecular Monitoring of Invasive Nostocalean Cyanobacteria. *International Journal of Environmental Research and Public Health* 18(11).

Kimmel, B. L., Lind, O. T. and Paulson, L. J. (1990). Reservoir primary production. *Reservoir limnology: Ecological perspectives*. K. W. Thorton, B. L. Kimmel and F. E. Payne, John Wiley & Sons, Inc.**:** 133-193.

Kokocinski, M., Dziga, D., Spoof, L., Stefaniak, K., Jurczak, T., Mankiewicz-Boczek, J. and Meriluoto, J. (2009). First report of the cyanobacterial toxin cylindrospermopsin in the shallow, eutrophic lakes of western Poland. *Chemosphere* 74(5): 669-75.

Kokociński, M. and Soininen, J. (2012). Environmental factors related to the occurrence ofCylindrospermopsis raciborskii(Nostocales, Cyanophyta) at the north-eastern limit of its geographical range. *European Journal of Phycology* 47(1): 12-21.

Komárek, J. (1995). Current trends and species delimitation in the cyanoprokaryote taxonomy. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*: 11-29.

Komárek, J. (2006). Cyanobacterial Taxonomy: Current Problems and Prospects for the Integration of Traditional and Molecular Approaches. *Algae* 21(4): 349-375.

Komarek, J. and Anagnostidis, K. (1999). Subwasserflora von mitteleuropa: cyanoprokaryota. *Subwasserflora von mitteleuropa: cyanoprokaryota*, Gustav Fischer.

Komárek, J. and Anagnostidis, K. (2005). *Cyanoprokaryota 2. Teil/ 2nd Part: Oscillatoriales*. Heidelberg, Elsevier/Spektrum

Koponen, S., Pulliainen, J., Servomaa, H., Zhang, Y., Hallikainen, M., Kallio, K., Vepsäläinen, J., Pyhälahti, T. and Hannonen, T. (2001). Analysis on the feasibility of multi-source remote sensing observations for chl-a monitoring in Finnish lakes. *Science of the total environment* 268(1-3): 95-106.

Kosten, S., Huszar, V. L. M., Bécares, E., Costa, L. S., Donk, E., Hansson, L.-A., Jeppesen, E., Kruk, C., Lacerot, G., Mazzeo, N., Meester, L., Moss, B., Lürling, M., Nõges, T., Romo, S. and Scheffer, M. (2012). Warmer climates boost cyanobacterial dominance in shallow lakes. *Global Change Biology* 18(1): 118-126.

Kurmayer, R., Blom, J. F., Deng, L. and Pernthaler, J. (2015). Integrating phylogeny, geographic niche partitioning and secondary metabolite synthesis in bloom-forming Planktothrix. *ISME J* 9(4): 909-21.

Kutser, T. (2004). Quantitative detection of chlorophyll in cyanobacterial blooms by satellite remote sensing. *Limnology and Oceanography* 49(6): 2179-2189.

Kutser, T., Metsamaa, L., Strömbeck, N. and Vahtmäe, E. (2006). Monitoring cyanobacterial blooms by satellite remote sensing. *Estuarine, Coastal and Shelf Science* 67(1-2): 303-312.

Lee, J., Lee, S. and Jiang, X. (2017). Cyanobacterial toxins in freshwater and food: important sources of exposure to humans. *Annual review of food science and technology* 8: 281-304.

Lezcano, M. Á., Velázquez, D., Quesada, A. and El-Shehawy, R. (2017). Diversity and temporal shifts of the bacterial community associated with a toxic cyanobacterial bloom: An interplay between microcystin producers and degraders. *Water research* 125: 52-61.

Li, H. Y., Miller, T., Lu, J. R. and Goel, R. (2022). Nitrogen fixation contribution to nitrogen cycling during cyanobacterial blooms in Utah Lake. *Chemosphere* 302.

Li, X., Dreher, T. W. and Li, R. (2016). An overview of diversity, occurrence, genetics and toxin production of bloom-forming Dolichospermum (Anabaena) species. *Harmful Algae* 54: 54-68.

MAGRAMA (2013). *Ministerio de Agricultura, Alimentación y Medio Ambiente. Muestreo Fitoplancton Lagos y Embalses*. Spain

Mantzouki, E.!Lürling, M.!Fastner, J.!De Senerpont Domis, L.!Wilk-Woźniak, E.!Koreivienė, J.!Seelen, L.!Teurlincx, S.!Verstijnen, Y.!Krztoń, W.!Walusiak, E.!Karosienė, J.!Kasperovičienė, J.!Savadova, K.!Vitonytė, I.!Cillero-Castro, C.!Budzyńska, A.!Goldyn, R.!Kozak, A.!Rosińska, J.!Szeląg-Wasielewska, E.!Domek, P.!Jakubowska-Krepska, N.!Kwasizur, K.!Messyasz, B.!Pełechata, A.!Pełechaty, M.!Kokocinski, M.!García-Murcia, A.!Real, M.!Romans, E.!Noguero-Ribes, J.!Duque, D. P.!Fernández-Morán, E.!Karakaya, N.!Häggqvist, K.!Demir, N.!Beklioğlu, M.!Filiz, N.!Levi, E. E.!Iskin, U.!Bezirci, G.!Tavşanoğlu, Ü. N.!Özhan, K.!Gkelis, S.!Panou, M.!Fakioglu, Ö.!Avagianos, C.!Kaloudis, T.!Çelik, K.!Yilmaz, M.!Marcé, R.!Catalán, N.!Bravo, A. G.!Buck, M.!Colom-Montero, W.!Mustonen, K.!Pierson, D.!Yang, Y.!Raposeiro, P. M.!Gonçalves, V.!Antoniou, M. G.!Tsiarta, N.!McCarthy, V.!Perello, V. C.!Feldmann, T.!Laas, A.!Panksep, K.!Tuvikene, L.!Gagala, I.!Mankiewicz-Boczek, J.!Yağcı, M. A.!Çınar, Ş.!Çapkın, K.!Yağcı, A.!Cesur, M.!Bilgin, F.!Bulut, C.!Uysal, R.!Obertegger, U.!Boscaini, A.!Flaim, G.!Salmaso, N.!Cerasino, L.!Richardson, J.!Visser, P. M.!Verspagen, J. M. H.!Karan, T.!Soylu, E. N.!Maraşlıoğlu, F.!Napiórkowska-Krzebietke, A.!Ochocka, A.!Pasztaleniec, A.!Antão-Geraldes, A. M.!Vasconcelos, V.!Morais, J.!Vale, M.!Köker, L.!Akçaalan, R.!Albay, M.!Špoljarić Maronić, D.!Stević, F.!Žuna Pfeiffer, T.!Fonvielle, J.!Straile, D.!Rothhaupt, K.-O.!Hansson, L.-A.!Urrutia-Cordero, P.!Bláha, L.!Geriš, R.!Fránková, M.!Koçer, M. A. T.!Alp, M. T.!Remec-Rekar, S.!Elersek, T.!Triantis, T.!Zervou, S.-K.!Hiskia, A.!Haande, S.!Skjelbred, B.!Madrecka, B.!Nemova, H.!Drastichova, I.!Chomova, L.!Edwards, C.!Sevindik, T. O.!Tunca, H.!Önem, B.!Aleksovski, B.!Krstić, S.!Vucelić, I. B.!Nawrocka, L.!Salmi, P.!Machado-Vieira, D.!De Oliveira, A. G.!Delgado-Martín, J.!García, D.!Cereijo, J. L.!Gomà, J.!Trapote, M. C.!Vegas-Vilarrúbia, T.!Obrador, B.!Grabowska, M.!Karpowicz, M.!Chmura, D.!Úbeda, B.!Gálvez, J. Á.!Özen, A.!Christoffersen, K. S.!Warming, T. P.!Kobos, J.!Mazur-Marzec, H.!Pérez-Martínez, C.!Ramos-Rodríguez, E.!Arvola, L.!Alcaraz-Párraga, P.!Toporowska, M.!Pawlik-Skowronska, B.!Niedźwiecki, M.!Pęczuła, W.!Leira, M.!Hernández, A.!Moreno-Ostos, E.!Blanco, J. M.!Rodríguez, V.!Montes-Pérez, J. J.!Palomino, R. L.!Rodríguez-Pérez, E.!Carballeira, R.!Camacho, A.!Picazo, A.!Rochera, C.!Santamans, A. C.!Ferriol, C.!Romo, S.!Soria, J. M.!Dunalska, J.!Sieńska, J.!Szymański, D.!Kruk, M.!Kostrzewska-Szlakowska, I.!Jasser, I.!Žutinić, P.!Gligora Udovič, M.!Plenković-Moraj, A.!Frąk, M.!Bańkowska-Sobczak, A.!Wasilewicz, M.!Özkan, K.!Maliaka, V.!Kangro, K.!Grossart, H.-P.!Paerl, H. W.!Carey, C. C. and Ibelings, B. W. (2018). Temperature Effects Explain Continental Scale Distribution of Cyanobacterial Toxins. *Toxins* 10(4): 156.

Matthews, M. W. (2011). A current review of empirical procedures of remote sensing in inland and near-coastal transitional waters. *International Journal of Remote Sensing* 32(21): 6855-6899.

Matthews, M. W., Bernard, S. and Robertson, L. (2012). An algorithm for detecting trophic status (chlorophyll-a), cyanobacterial-dominance, surface scums and floating vegetation in inland and coastal waters. *Remote Sensing of Environment* 124: 637-652.

Mehnert, G., Leunert, F., Cires, S., Johnk, K. D., Rucker, J., Nixdorf, B. and Wiedner, C. (2010). Competitiveness of invasive and native cyanobacteria from temperate freshwaters under various light and temperature conditions. *Journal of Plankton Research* 32(7): 1009-1021.

Mehnert, G., Rücker, J. and Wiedner, C. (2014). Population dynamics and akinete formation of an invasive and a native cyanobacterium in temperate lakes. *Journal of Plankton Research* 36(2): 378-387.

Merel, S., Villarin, M. C., Chung, K. and Snyder, S. (2013). Spatial and thematic distribution of research on cyanotoxins. *Toxicon* 76: 118-31.

Merel, S., Walker, D., Chicana, R., Snyder, S., Baures, E. and Thomas, O. (2013). State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environ Int* 59: 303-27.

Meriluoto, J., Blaha, L., Bojadzija, G., Bormans, M., Brient, L., Codd, G. A., Drobac, D., Faassen, E. J., Fastner, J. and Hiskia, A. (2017). Toxic cyanobacteria and cyanotoxins in European waters-recent progress achieved through the CYANOCOST Action and challenges for further research. *Advances in Oceanography & Limnology* 8(1).

Meriluoto, J., Blaha, L., Bojadzija, G., Bormans, M., Brient, L., Codd, G. A., Drobac, D., Faassen, E. J., Fastner, J. and Hiskia, A. (2017). Toxic cyanobacteria and cyanotoxins in European waters–recent progress achieved through the CYANOCOST Action and challenges for further research. *Advances in Oceanography and Limnology* 8(1): 161-178.

Meroni, M., Rossini, M., Guanter, L., Alonso, L., Rascher, U., Colombo, R. and Moreno, J. (2009). Remote sensing of solar-induced chlorophyll fluorescence: Review of methods and applications. *Remote Sensing of Environment* 113(10): 2037-2051.

Mesquita, M. C. B., Lurling, M., Dorr, F., Pinto, E. and Marinho, M. M. (2019). Combined Effect of Light and Temperature on the Production of Saxitoxins in Cylindrospermopsis raciborskii Strains. *Toxins* 11(1).

Messineo, V., Melchiorre, S., Di Corcia, A., Gallo, P. and Bruno, M. (2010). Seasonal succession of Cylindrospermopsis raciborskii and Aphanizomenon ovalisporum blooms with cylindrospermopsin occurrence in the volcanic Lake Albano, Central Italy. *Environ Toxicol* 25(1): 18-27.

Mishra, S., Mishra, D. R., Lee, Z. and Tucker, C. S. (2013). Quantifying cyanobacterial phycocyanin concentration in turbid productive waters: A quasi-analytical approach. *Remote Sensing of Environment* 133: 141-151.

Mishra, S., Stumpf, R. P., Schaeffer, B. A., Werdell, P. J., Loftin, K. A. and Meredith, A. (2019). Measurement of Cyanobacterial Bloom Magnitude using Satellite Remote Sensing. *Scientific Reports* 9(1): 18310.

Mishra, S., Stumpf, R. P., Schaeffer, B. A., Werdell, P. J., Loftin, K. A. and Meredith, A. (2019). Measurement of cyanobacterial bloom magnitude using satellite remote sensing. *Scientific reports* 9(1): 1-17.

Moss, B., Stephen, D., Alvarez, C., Becares, E., Bund, W. V. D., Collings, S. E., Donk, E. V., Eyto, E. D., Feldmann, T., Fernández-Aláez, C., Fernández-Aláez, M., Franken, R. J. M., García-Criado, F., Gross, E. M., Gyllström, M., Hansson, L.-A., Irvine, K., Järvalt, A., Jensen, J.-P., Jeppesen, E., Kairesalo, T., Kornijów, R., Krause, T., Künnap, H., Laas, A., Lill, E., Lorens, B., Luup, H., Rosa Miracle, M., Nõges, P., Nõges, T., Nykänen, M., Ott, I., Peczula, W., Peeters, E. T. H. M., Phillips, G., Romo, S., Russell, V., Salujõe, J., Scheffer, M., Siewertsen, K., Smal, H., Tesch, C., Timm, H., Tuvikene, L., Tonno, I., Virro, T., Vicente, E. and Wilson, D. (2003). The determination of ecological status in shallow lakes - a tested system (ECOFRAME) for implementation of the European Water Framework Directive. *Aquatic Conservation: Marine and Freshwater Ecosystems* 13(6): 507-549.

Njagi, D. M., Routh, J., Odhiambo, M., Luo, C., Basapuram, L. G., Olago, D., Klump, V. and Stager, C. (2022). A century of human-induced environmental changes and the combined roles of nutrients and land use in Lake Victoria catchment on eutrophication. *Science of the Total Environment* 835.

Ohtani, I., Moore, R. E. and Runnegar, M. T. C. (2002). Cylindrospermopsin: a potent hepatotoxin from the blue-green alga Cylindrospermopsis raciborskii. *Journal of the American Chemical Society* 114(20): 7941-7942.

Olli, K., Kangro, K. and Kabel, M. (2005). Akinete Production of Anabaena Lemmermannii and A. Cylindrica (Cyanophyceae) in Natural Populations of N- and P-Limited Coastal Mesocosms1. *Journal of Phycology* 41(6): 1094-1098.

Olofsson, M., Suikkanen, S., Kobos, J., Wasmund, N. and Karlson, B. (2020). Basin-specific changes in filamentous cyanobacteria community composition across four decades in the Baltic Sea. *Harmful Algae* 91: 101685.

Onodera, H., Oshima, Y., Henriksen, P. and Yasumoto, T. (1997). Confirmation of anatoxin-a(s), in the cyanobacterium Anabaena lemmermannii, as the cause of bird kills in Danish Lakes. *Toxicon* 35(11): 1645-1648.

Orr, P. T. and Jones, G. J. (1998). Relationship between microcystin production and cell division rates in nitrogen-limited Microcystis aeruginosa cultures. *Limnology and oceanography* 43(7): 1604-1614.

Padisak, J. (1997). Cylindrospermopsis raciborskii (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology. *Archiv Für Hydrobiologie Supplementband Monographische Beitrage* 107(4): 563-593.

Paerl, H. W. and Barnard, M. A. (2020). Mitigating the global expansion of harmful cyanobacterial blooms: Moving targets in a human-and climatically-altered world. *Harmful algae* 96: 101845.

Paerl, H. W. and Huisman, J. (2008). Blooms like it hot. *Science* 320(5872): 57-58.

Paerl, H. W. and Huisman, J. (2009). Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. *Environ Microbiol Rep* 1(1): 27-37.

Palacio, H. M., Palacio, J. A., Echenique, R. O., SanT'Anna, C. L. and Ramirez, J. J. (2015). DOLICHOSPERMUM LEMMERMANNII (CYANOBACTERIA): A TEMPERATE SPECIES IN A NEOTROPICAL, EUTROPHIC RESERVOIR. *Boletin De La Sociedad Argentina De Botanica* 50(3): 309-321.

Parsons, T. R. and Strickland, J. D. H. (1968). *A practical handbook of seawater analysis. Pigment analysis*. Otawa, Supply and Services Canada

Patel, A., Mishra, S., Pawar, R. and Ghosh, P. K. (2005). Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. *Protein Expr Purif* 40(2): 248-55.

Pereira, P., Li, R., Carmichael, W., Dias, E. and Franca, S. (2004). Taxonomy and production of paralytic shellfish toxins by the freshwater cyanobacterium Aphanizomenon gracile LMECYA40. *European Journal of Phycology* 39(4): 361-368.

Pitois, F., Thoraval, I., Baurès, E. and Thomas, O. (2014). Geographical Patterns in Cyanobacteria Distribution: Climate Influence at Regional Scale. *Toxins* 6: 509-22.

Poulíčková, A., Hašler, P. and Kitner, M. (2004). Annual Cycle ofPlanktothrix agardhii(Gom.)Anagn. &Kom. Nature Population. *International Review of Hydrobiology* 89(3): 278-288.

Quesada, A., Moreno, E., Carrasco, D., Paniagua, T., Wormer, L., Hoyos, C. d. and Sukenik, A. (2006). Toxicity ofAphanizomenon ovalisporum(Cyanobacteria) in a Spanish water reservoir. *European Journal of Phycology* 41(1): 39-45.

Quesada, A., Sanchis, D. and Carrasco., D. (2004). Cyanobacteria in Spanish reservoirs. How frequently are toxic? *Limnetica 23 (1-2)*: 109-118.

Rajaniemi, P., Komarek, J., Willame, R., Hrouzek, P., Kastovska, K., Hoffmann, L. and Sivonen, K. (2005). *Taxonomic consequences from the combined molecular and phenotype evaluation of selected Anabaena and Aphanizomenon strains*. International Association for Cyanophyte Research (Symposium), Schweizerbart.

Randolph, K., Wilson, J., Tedesco, L., Li, L., Pascual, D. L. and Soyeux, E. (2008). Hyperspectral remote sensing of cyanobacteria in turbid productive water using optically active pigments, chlorophyll a and phycocyanin. *Remote Sensing of Environment* 112: 4009-4019.

Rast, M., Bezy, J. and Bruzzi, S. (1999). The ESA Medium Resolution Imaging Spectrometer MERIS a review of the instrument and its mission. *International Journal of Remote Sensing* 20(9): 1681-1702.

Rast, M., Bezy, J. L. and Bruzzi, S. (2010). The ESA Medium Resolution Imaging Spectrometer MERIS a review of the instrument and its mission. *International Journal of Remote Sensing* 20(9): 1681-1702.

Reynolds, C. S., Huszar, V., Kruk, C., Naselli-Flores, L. and Melo, S. (2002). Towards a functional classification of the freshwater phytoplankton. *Journal of Plankton Research* 24(5): 417-428.

Rigosi, A., Carey, C. C., Ibelings, B. W. and Brookes, J. D. (2014). The interaction between climate warming and eutrophication to promote cyanobacteria is dependent on trophic state and varies among taxa. *Limnology and Oceanography* 59(1): 99-114.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R. Y. (1979). GENERIC ASSIGNMENTS, STRAIN HISTORIES AND PROPERTIES OF PURE CULTURES OF CYANOBACTERIA. *Journal of General Microbiology* 111(MAR): 1-61.

Rowan, K. S. (1989). *Photosynthetics pigments of algae*. Cambridge, Cambridge University Press

Ruiz-Verdú, A., Simis, S. G. H., de Hoyos, C., Gons, H. J. and Peña-Martínez, R. (2008). An evaluation of algorithms for the remote sensing of cyanobacterial biomass. *Remote Sensing of Environment* 112(11): 3996-4008.

Sagoff, M. (2018). What Is Invasion Biology? *Ecological Economics* 154: 22-30.

Saker, M. L., Nogueira, I. C., Vasconcelos, V. M., Neilan, B. A., Eaglesham, G. K. and Pereira, P. (2003). First report and toxicological assessment of the cyanobacterium Cylindrospermopsis raciborskii from Portuguese freshwaters. *Ecotoxicol Environ Saf* 55(2): 243-50.

Salmaso, N. (2003). Life strategies, dominance patterns and mechanisms promoting species coexistence in phytoplankton communities along complex environmental gradients. *Hydrobiologia* 502(1-3): 13-36.

Sánchez-Baracaldo, P. and Cardona, T. (2020). On the origin of oxygenic photosynthesis and Cyanobacteria. *New Phytologist* 225(4): 1440-1446.

Sarmento, H., Amado, A. M. and Descy, J.-P. (2013). Climate change in tropical fresh waters (comment on the paper 'Plankton dynamics under different climatic conditions in space and time' by de Senerpont Domiset al.,). *Freshwater Biology* 58(10): 2208-2210.

Simis, S. G. H., Ruiz-Verdú, A., Domínguez-Gómez, J. A., Peña-Martinez, R., Peters, S. W. M. and Gons, H. J. (2007). Influence of phytoplankton pigment composition on remote sensing of cyanobacterial biomass. *Remote Sensing of Environment* 106(4): 414-427.

Sournia, A. (1978). *Phytoplankton manual*. Paris.337

Spoerke, D. G. and Rumack, B. H. (1985). Blue-green algae poisoning. *J Emerg Med* 2(5): 353-5.

Stefaniak, K. and Kokocinski, M. (2005). Occurrence of invasive cyanobacteria speceies in polimictic lakes of the Wielkopolska Region (Western Poland). *Oceanological and Hydrobiological Studies* 34(3): 137-148.

Sukenik, A., Quesada, A. and Salmaso, N. (2015). Global expansion of toxic and non-toxic cyanobacteria: effect on ecosystem functioning. *Biodiversity and Conservation* 24(4): 889-908.

Svendsen, L. M. and Norup, B. (2005). *NOVANA. Nationwide Monitoring and Assessment Programme for the Aquatic and Terrestrial Environments. Programme Description – Part 1*. National Environmental Research Institute, Denmark.53

Thomas, M. K. and Litchman, E. (2016). Effects of temperature and nitrogen availability on the growth of invasive and native cyanobacteria. *Hydrobiologia* 763(1): 357-369.

Utermöhl, H. (1931). Neue Wege in der quantitativen Erfassung des Planktons (Mit besoderer Ber.cksichtigung des Ultraplanktons). *Verhandlungen der Internationalen Vereiningung f.r theoretische und angewandte Limnologie* 5: 567-596.

Vasconcelos, V. (2006). Eutrophication, toxic cyanobacteria and cyanotoxins: when ecosystems cry for help. *Limnetica* 25(1-2): 425-432.

Vilar, M. C. P. and Molica, R. J. R. (2020). Changes in pH and dissolved inorganic carbon in water affect the growth, saxitoxins production and toxicity of the cyanobacterium Raphidiopsis raciborskii ITEP-A1. *Harmful Algae* 97: 101870.

Wacklin, P., Hoffmann, L. and Komarek, J. (2009). Nomenclatural validation of the genetically revised cyanobacterial genus Dolichospermum (RALFS ex BORNET et FLAHAULT) comb. nova. *Fottea* 9(1): 59-64.

Wacklin, P., Hoffmann, L. and Komárek, J. (2009). Nomenclatural validation of the genetically revised cyanobacterial genus Dolichospermum (Ralfs ex Bornet et Flahault) comb. nova. *Fottea* 9(1): 59-64.

Walter, J. M., Coutinho, F. H., Dutilh, B. E., Swings, J., Thompson, F. L. and Thompson, C. C. (2017). Ecogenomics and Taxonomy of Cyanobacteria Phylum. *Frontiers in Microbiology* 8.

Weber, S. J., Mishra, D. R., Wilde, S. B. and Kramer, E. (2020). Risks for cyanobacterial harmful algal blooms due to land management and climate interactions. *Science of the Total Environment* 703: 134608.

Whitton, B. A. (2012). *Ecology of Cyanobacteria II*, Springer Science

Whitton, B. A. and Potts, M. (2002). *The Ecology of Cyanobacteria*, Springer Netherlands

Wiedner, C., Rucker, J., Bruggemann, R. and Nixdorf, B. (2007). Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152(3): 473-84.

Wiedner, C., Rücker, J., Brüggemann, R. and Nixdorf, B. (2007). Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152(3): 473-484.

Wilk-Woźniak, E. and Najberek, K. (2013). Towards clarifying the presence of alien algae in inland waters — can we predict places of their occurrence? *Biologia* 68(5): 838-844.

Wilk-Woźniak, E., Solarz, W., Najberek, K. and Pociecha, A. (2015). Alien cyanobacteria: an unsolved part of the "expansion and evolution" jigsaw puzzle? *Hydrobiologia* 764(1): 65-79.

Willen, E. (1997). Phytoplankton in water quality assessment, an indicator concept. *Phycologia* 36(4): 123-124.

Wood, S. A., Selwood, A. I., Rueckert, A., Holland, P. T., Milne, J. R., Smith, K. F., Smits, B., Watts, L. F. and Cary, C. S. (2007). First report of homoanatoxin-a and associated dog neurotoxicosis in New Zealand. *Toxicon* 50(2): 292-301.

Wormer, L., Huerta-Fontela, M., Cires, S., Carrasco, D. and Quesada, A. (2010). Natural photodegradation of the cyanobacterial toxins microcystin and cylindrospermopsin. *Environ Sci Technol* 44(8): 3002-7.

Xiao, M., Willis, A. and Burford, M. A. (2017). Differences in cyanobacterial strain responses to light and temperature reflect species plasticity. *Harmful Algae* 62: 84-93.

Yan, Y., Bao, Z. and Shao, J. (2018). Phycocyanin concentration retrieval in inland waters: A comparative review of the remote sensing techniques and algorithms. *Journal of Great Lakes Research* 44(4): 748-755.

Zapomělová, E., Skácelová, O., Pumann, P., Kopp, R. and Janeček, E. (2012). Biogeographically interesting planktonic Nostocales (Cyanobacteria) in the Czech Republic and their polyphasic evaluation resulting in taxonomic revisions of Anabaena bergii Ostenfeld 1908 (Chrysosporum gen. nov.) and A. tenericaulis Nygaard 1949 (Dolichospermum tenericaule comb. nova). *Phytoplankton responses to human impacts at different scales*, Springer**:** 353-365.

Zapomělová, E., Skácelová, O., Pumann, P., Kopp, R. and Janeček, E. (2012). Biogeographically interesting planktonic Nostocales (Cyanobacteria) in the Czech Republic and their polyphasic evaluation resulting in taxonomic revisions of Anabaena bergii Ostenfeld 1908 (Chrysosporum gen. nov.) and A. tenericaulis Nygaard 1949 (Dolichospermum tenericaule comb. nova). *Hydrobiologia* 698(1): 353-365.

Zong, J.-M., Wang, X.-X., Zhong, Q.-Y., Xiao, X.-M., Ma, J. and Zhao, B. (2019). Increasing outbreak of cyanobacterial blooms in large lakes and reservoirs under pressures from climate change and anthropogenic interferences in the Middle–Lower Yangtze River Basin. *Remote Sensing* 11(15): 1754.

URL's

[1]http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32000L0060:en:HTML

[2]https://earth.esa.int/web/guest/missions/esa-operational-eo-missions/envisat/instruments/meris/design

Supplementary material

Parameter	Unit
Water body mean water volume	hm^3
Max Depth reservoir/lake	m
Phytoplankton biovolume	mm^3/m^3
Cyanobacteria biovolume	mm^3/m^3
Chrysosporum ovalisporum	biovolume (mm^3/m^3)
Cuspidothrix issatschenkoi	biovolume (mm^3/m^3)
Cylindrospermopsis raciborskii	biovolume (mm^3/m^3)
Dolichospermum lemmermannii	biovolume (mm^3/m^3)
Planktothrix agardhii	biovolume (mm^3/m^3)
Alkalinity	mg $CaCO^3$/l
Ammonia	mg/l
Chlorophyll a	µg/l
Conductivity	µS/cm
Nitrite + nitrate	mg/l
Nitrogen, total	mg/l
Orthophosphate	mg/l
Oxygen content	mg/l
Oxygen sat	%
pH	
Phosphorus, total	µg/l
Secchi depth	m
Surface temperature	ºC

Supplementary material

Parameter	Unit
Accumulated precipitation	mm^3
Days < 0º	Total number
Days>25º	Total number
Highest 24-hour precipitation	mm^3
Hours of light	Total number
Max Atmospheric Temperature	ºC
Average of atmospheric pressure	hPa
Average of Atmospheric Temperature	ºC
Average of cloud cover	%
Parameter	ºC
Average of daily minimum temperature (ºC)	ºC
Min Atmospheric temperature (ºC)	ºC
No of days with snow cover (%) (> 50 % covered)	Total number

Table A1. List of parameters used in the PCA

Supplementary material

OTU #	NCBI BLAST		
	Strain	**Access code**	**Similitude cover (%)**
OTU 1	*Planktothrix agardhii* CHAB643	KC013281	100
OTU 2	*Aphanizomenon gracile* UAM531	JN886011	99
OTU 6	*Limnothrix redekei* CCAP 1459/29	HE974998	99
OTU 26	*Aphanizomenon gracile* PMC631.10	HQ157688	99

Table A2 .NCI BLAST identification of relevant OUT's from Casero et al (2019)