

# **Occurrence and life-cycle strategies of bloom-forming Nostocales (cyanobacteria) in deep lakes in Northern Germany and in Lake Kinneret, Israel**

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**Abstract**

Nostoclean cyanobacteria differentiate heterocysts to fix dissolved nitrogen (N<sub>2</sub>) and dormant cells (akinetes) to survive harsh environmental conditions. The distribution and proliferation of Nostocales and their hibernation strategies in deep stratified lakes of different trophic states and climate zones were investigated. Studies were carried out in the meso-eutrophic Lake Kinneret (Israel) and in the mesotrophic Lake Scharmützelsee and the oligo-mesotrophic Lake Stechlinsee (Germany).

The spatio-temporal distribution of akinetes in sediments was analysed. The abundance in the sediment surface increased with water depth and differed due to basin morphometry. Light and temperature distribution revealed shallow areas of sedimentary akinete pool as a potential inoculum to contribute to the formation of a pelagic population. Hence, a small viable akinete pool was deposited in shallow sediments.

A CARD-FISH protocol was implemented to identify and quantify the akinetes in sediments. Tested Nostocales strains and akinetes in sediment samples were successfully labelled with 16S rRNA targeted probes and microscopically detected by their fluorescence signal. Akinete enumeration in field samples confirmed the suitability of the CARD-FISH approach.

Species- or genus-specific differences in the overwintering strategies of Nostocales were observed. Akinetes were the only overwintering form of *Anabaena* spp. On the other hand, *Aphanizomenon flos-aquae* overwintered as vegetative filaments in the pelagial of Lake Stechlinsee. In the other lakes studied, *Aphanizomenon* spp. performed an intermediate life cycle, with akinetes and a small pelagic population in winter. This suggested that *Aphanizomenon* spp. had another overwintering strategy contrary to the exclusively dormant strategy of *Anabaena* spp.

Putative strains of cylindrospermopsin (CYN)-producing *Aphanizomenon ovalisporum* were isolated from Lake Kinneret. Their morphologic and phylogenetic diversity, as well as the presence of CYN-encoding gene cluster and CYN production, were analysed. Four of the six strains were assigned to *Anabaena bergii*. Although all the isolated strains possess at least fragments of CYN gene cluster, no CYN production was detected. Non-CYN-producing *Aphanizomenon ovalisporum* strains and the presence of non-toxic *Anabaena bergii* in Lake Kinneret have not been reported before. This suggested that toxic and non-toxic Nostocales strains can coexist in lakes.

## Zusammenfassung

Nostocale Cyanobakterien bilden Heterozysten zur Fixierung molekularen Stickstoffs ( $N_2$ ) aus und entwickeln dormante Stadien (Akineten) zur Überdauerung ungünstiger Umweltbedingungen. In dieser Studie wurden die Verteilung sowie die Verbreitung der Nostocales und ihre Strategien zur Überwinterung in tiefen geschichteten Seen unterschiedlicher Trophie und unterschiedlicher Klimazonen untersucht. Die Untersuchungsgewässer waren der meso-eutrophe See Genezareth (Israel), der mesotrophe Scharmützelsee sowie der oligo-mesotrophe Stechlinsee (Deutschland).

Die räumlich-zeitliche Verteilung der Akineten im Seesediment wurde untersucht. Die Abundanz an der Sedimentoberfläche steigt mit zunehmender Wassertiefe und die Artzusammensetzung kann sich aufgrund der Seebeckenmorphometrie unterscheiden obwohl sie im Pelagial sehr ähnlich ist. Die Licht- und Temperaturbedingungen im See ermöglichen nur dem Akineten-Pool der Flachwasserbereiche als Inokulum zur Bildung einer pelagischen Population beizutragen. In den Sedimenten der flachen Bereiche lagert nur ein kleiner Teil des Akineten-Pools.

Ein CARD-FISH-Protokoll wurde zur Identifizierung und Quantifizierung von Akineten im Sediment modifiziert. Mit spezifischen 16S rRNA Sonden wurden Nostocales-Stämme und Akineten in Sedimentproben erfolgreich angefärbt und anhand ihres Fluoreszenzsignals mikroskopisch erfasst. Vergleichszählungen mit herkömmlicher Hellfeldmikroskopie bestätigten die Eignung der CARD-FISH-Methodik.

Weiterhin wurden Unterschiede in den Überwinterungsstrategien der Nostocales beobachtet. *Anabaena* spp. überwintern ausschließlich als Akineten, wohingegen *Aphanizomenon flos-aquae* rein vegetativ im Pelagial des Stechlinsees überdauert. Andere *Aphanizomenon* spp. vollzogen in den anderen untersuchten Seen einen intermediären Lebenszyklus mit Akinetenbildung und einer geringen pelagischen Population im Winter. Dies deutete darauf hin, dass *Aphanizomenon* spp. eine andere Überwinterungsstrategie gegenüber der ausschließlich dormanten Strategie von *Anabaena* spp. verfolgt.

Aus dem See Genezareth wurden mutmaßlich Cyndrospermopsin (CYN)-produzierende Stämme von *Aphanizomenon ovalisporum* isoliert. Neben ihrer CYN-Produktion und dem Vorhandensein des dafür codierenden Genclusters wurden die Stämme morphologisch und phylogenetisch analysiert. Keines der Isolate produzierte CYN obwohl zumindest Fragmente des CYN-Genclusters nachgewiesen wurden.

Bei einem Teil der isolierten Stämme handelte es sich um *Anabaena bergii*. Bislang war weder das Vorkommen der nicht-toxischen *Anabaena bergii* noch das von nicht CYN produzierenden *Aphanizomenon ovalisporum*-Stämmen im See Genezareth bekannt. Dies lässt auf eine Koexistenz von toxischen und nicht-toxischen Nostocales Stämmen in Seen schließen.

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**List of abbreviations**

APHA	probe for <i>Aphanizomenon</i>
CA	catchment area
CARD	catalyzed reporter deposition
Chl a	chlorophyll <i>a</i>
CYL	probe for <i>Cylindrospermopsis raciborskii</i>
CYN	cylindrospermopsin
DIE	Lake Scharmützelsee, Middle Basin, routine sampling
DNA	desoxyribonucleid acid
DW	dry weight
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FISH	fluorescence <i>in situ</i> hybridization
HRP	horseradish peroxidase
INS	Lake Scharmützelsee, Northern Bay, routine sampling
$I_k$	low light saturation parameter
kDA	kilodalton
$K_d$	vertical light attenuation coefficient
LC-MS	liquid chromatography-mass spectrometry
LOI	loss on ignition
MEL	Lake Melangsee
ML	maximum likelihood
N	sample size
NMDS	non-metric multidimensional scaling
NOSTOC	probe for <i>Nostoc</i> , <i>Anabaena</i> and <i>Aphanizomenon</i>
PAR	photosynthetically active radiation
PBS	phosphate buffer saline
PC-IGS	intergenic spacer of phycocyanin operon
PCR	polymerase chain reaction
RIE	Lake Scharmützelsee, Southern Basin, routine sampling
rRNA	ribosomal ribonucleid acid
RT	room temperature
S	subunit
SCH	Lake Scharmützelsee
ST	Lake Stechlinsee
t	theoretical retention time
TSA	tyramid signal amplification
TN	total nitrogen
TP	total phosphorus
$Z_{eu}$	eutrophic zone
$Z_{max}$	maximum depth
$Z_{mean}$	mean depth
$Z_{min}$	minimum depth

## 1. General introduction

### 1.1 Proliferation and potential toxicity of nostocalean cyanobacteria

Cyanobacteria represent the oldest organisms of oxygenic photoautotrophs. Their proliferation during the Precambrian period led to the evolution of higher terrestrial plant and animal life (Paerl and Otten 2013). Cyanobacteria are taxonomically divided into five orders: non-filamentous Chroococcales and Pleurocapsales, and filamentous Oscillatoriales, Stigonematales and Nostocales (Anagnostidis and Komárek 1985; van den Hoek 1995). Taxonomic revision of nostocalean cyanobacteria has been performed in recent years (Komárek 2010; Zapomělova 2009) and the main diacritical features of morphospecies for *Aphanizomenon* (Komárek and Komárkova 2006) and for planktonic *Anabaena* (Komárek and Zapomělova 2007, 2008) have been described. Unless otherwise stated in the following, former generic names are used according to Geitler's (1932) monograph.

Most species of the order Stigonematales and Nostocales, attention in the present work will be drawn to the latter, are able to fix (dissolved) atmospheric nitrogen, N<sub>2</sub>. Thus, Nostocales are enabled to colonize both nutrient-scarce and nutrient-enriched habitats, such as lakes and reservoirs, worldwide. The most visible sign of their ecological success can be expressed by their frequent, partly harmful cyanobacterial blooms occurring in deep stratified lakes (Figure 1.1). "Harmful" in an environmental context means turbidity which can suppress aquatic macrophytes. Bacterial decomposition of dying blooms may lead to oxygen depletion (Paerl and Huisman 2008). Promoting factors, such as high concentrations of phosphorus and dissolved organic matter, low dissolved inorganic and organic nitrogen, and a low nitrogen to phosphorus ratio, can positively influence cyanobacterial blooms (Paerl and Otten 2013). Many bloom-forming species are also known to produce toxic secondary metabolites which can cause intoxication in mammals, e.g. affecting hepatopancreatic, digestive, endocrine, dermal, and nervous systems in humans (Chorus and Bartram 1999). Consequently, the use of water for recreational purposes or drinking can be reduced.



**Figure 1. 1 Northern Bay of Lake Scharmützelsee in September 2013. Notice the cyanobacterial bloom (*Anabaena* spp.) in the foreground.**

Invasive species are of ecological interest due to their competitive potential against native species in an overlapping ecological niche. Most Nostocales species benefit from rising water temperatures as a result of climate change (Wagner and Adrian 2009) due to relatively high temperature optima for their growth rate (Mehnert et al. 2010). Hence, tropical and subtropical species mostly increase in polymictic shallow lakes of temperate regions (Stüken et al. 2006; Wiedner et al. 2007). They coexist with native Nostocales of the genus *Anabaena* and *Aphanizomenon*, and most frequently with *Aphanizomenon gracile* (Rücker et al. 2007). The vegetation period is extended for native temperate Nostocales species, and thus, they can achieve higher growth rates and can build up large populations (Jöhnk et al. 2011; Mehnert et al. 2010). Moreover, nostocalean cyanobacteria are widely distributed in deep oligo- and mesotrophic stratified lakes because of their nitrogen-fixing heterocysts which enable them to be dominant in a phytoplankton community even under low nitrogen concentrations. Hence, species invade stratified lakes in low abundances where mainly *Aphanizomenon flos-aquae* and different *Anabaena* species can be found (Stüken et al. 2006).

The study will focus on the distribution of Nostocales in the three deep stratified lakes of different trophic states and two climatic zones examined in detail: Lake Kinneret (Israel), Lake Scharmützelsee and Lake Stechlinsee (Germany).

The subtropical Lake Kinneret (Sea of Galilee) is located in the north-eastern part of Israel. Lake Kinneret is meso-eutrophic and thermally stratified from April-December. Lake water is mainly used for drinking, irrigation and reuse in the populated coastal areas of Israel, and thus, the water level depends on the

equilibrium of natural inflows and artificial withdrawal for water supply (Ostrovsky et al. 2013). The phytoplankton assemblage has an annual feature of a spring bloom of the dinoflagellate *Peridinium gatunense*, as reported by Pollinger (1986). However, the bloom developed irregularly between 1996 and 2011 only after winters with high inflow from the River Jordan. Fungal epidemics attacked *Peridinium gatunense* (Alster and Zohary 2007) and also the higher summer phytoplankton biomass of Nostocales, and an increase in proportion of cyanobacteria to total biomass is responsible for changes in the species assemblage (Zohary 2004). The first bloom of the invasive cylindrospermopsin (CYN; hepatotoxin)-producing species *Aphanizomenon ovalisporum* occurred in 1994 (Pollinger et al. 1998) and the toxin was occasionally detected (Banker et al. 1997). The local non-toxic species *Cylindrospermopsis raciborskii* (Woloszynská) Seenayya and Subba Raju 1972 was found for the first time in the summer of 2000 (Alster et al. 2010; Zohary and Shlichter 2009). The shift in phytoplankton composition towards N-fixing species was driven by environmental variables, such as high water temperature, low wind-driven turbulence and occasional pulses of phosphorus during the summer (Hadas et al. 1999, 2002).

The temperate mesotrophic Lake Scharmützelsee and oligotrophic Lake Stechlinsee are located in north-eastern Germany. Both are hard-water lakes and are thermally stratified in the summer. Phytoplankton assemblages have changed in recent years due to, among other things, reduced wastewater discharges with a promoted growth of Nostocales. A high summer phytoplankton biomass of Nostocales occurs in both lakes.

*Cylindrospermopsis raciborskii*, the perennial species from the tropical and subtropical region, has spread to temperate regions over the last few decades (Padisák 1997; Wiedner et al. 2007) and is widespread in Germany (Stüken et al. 2006) mostly in lakes with high turbidity (Nixdorf et al. 2003; Stüken et al. 2006). In the tropics, *Cylindrospermopsis raciborskii* is highly abundant in deep stratified lakes (McGreggor and Fabbro 2000). This species is assumed to have a wide physiological tolerance, and it still occurs at low temperatures (Briand et al. 2004; Stüken et al. 2006). A high biovolume of *Cylindrospermopsis raciborskii* occurred regularly in Lake Scharmützelsee from 1995-2002; but the species was not detected in routine phytoplankton samples from 2006-2010, whereas it was found in sediment traps at the deepest point of the lake at this time. *Cylindrospermopsis raciborskii* has been found in routine samples of phytoplankton composition again since 2011.

*Aphanizomenon aphanizomenoides* (Forti) Hortobágyi and Komárek 1979 and *Anabaena bergii* Ostefeld 1908 are also proven to be found in German lakes (Stüken et al. 2006). On the one hand, *Aphanizomenon aphanizomenoides* (currently designated as *Sphaerospermum*) has been observed in Lake Scharmützelsee annually since 2007 (except for 2008); on the other hand, this species was recorded once in Lake Stechlinsee in 2008. However, *Anabaena bergii* has hitherto not been observed in either Lake Scharmützelsee or Lake Stechlinsee.

*Aphanizomenon ovalisporum* shows the lowest growth rate in experiments of competitive native and invasive Nostocales species below 20 °C (Mehnert et al. 2010). This could be one reason why this species has not yet been found in German lakes. *Aphanizomenon aphanizomenoides* in German lakes has very low abundance, but, interestingly, reaches the highest growth rate in experiments. This species is probably at the beginning of its population development (Mehnert et al. 2010). CYN is widely distributed in German lakes (Fastner et al. 2007) but, so far, only in very small proportions produced by *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* (Preußel et al. 2006). An introduction of CYN-producing *Aphanizomenon ovalisporum* in German lakes could cause an increase in cylindrospermopsin concentrations.

## 1.2 Life cycle of Nostocales in lakes of temperate regions

Another special feature of N-fixing heterocystous cyanobacteria is the ability to produce akinetes (“akinetos” – motionless). Akinetes were initially reported by Carter in 1856 (Adams and Duggan 1999). These thick-walled resting cells differentiate from vegetative cells mainly at the end of exponential growth (Sutherland et al. 1979), and are resistant to adverse conditions, such as low temperatures or desiccation (Baker and Bellifemine 2000). Akinetes can survive for long periods in lake sediments (Livingstone and Jaworski 1980) and can even survive for a short period in low Earth orbit (Olsson-Francis et al. 2009), thus, showing its potential of high adaptability. The akinete envelope is composed of polysaccharides and glycolipids (Cardemil and Wolk 1981). Akinetes demonstrate the remaining intact photosynthetic lamellae and the presence of the reserve products glycogen and cyanophycin (Sarma 2012).

Many recent studies consider akinetes to play an important role in the perennial Nostocales life cycle in temperate regions (Hense and Beckmann 2006; Jöhnk et al. 2011; Kaplan-Levy et al. 2010). Akinetes may also play a key role in the invasion of tropical and subtropical species and are the reason why species lacking resting cells are not detected in temperate habitats (Stüken et al. 2006).

Akinetes are formed at the end of the vegetative growth period in the autumn and settle down to the lake bottom, stay dormant but viable at the sediment surface during winter, and germinate in spring, as shown in the scheme of the theoretical concept of Nostocales' life cycle in temperate regions (Figure 1.2). Akinetes can also be subject to loss processes during winter. They can be attacked by viruses or actino-mycetes (Chorus and Bartram 1999), but the complexity of this process is still uncertain.

Germination of viable akinetes is considered as one stage of the "recruitment" process (Karlsson-Elfgren and Brunberg 2004), which is followed by the potential growth phase on the sediment and migration to the pelagic zone. Akinete germination appears to be stimulated by, but not necessarily dependent on, high phosphorus concentrations (Wolk 1966), warm temperatures in the range of 15 to 27 °C, light, pH 7 to 8, and the presence of oxygen and sodium acetate (Yamamoto 1975). The germination of *Anabaena* spp. akinetes requires light, whereas *Nostoc* akinete germination is based on a fixed temporal sequence of cellular events and is not affected by environmental conditions (Adams and Duggan 1999). Laboratory experiments with akinetes indicate that they need a certain time to mature. Even when external conditions are favourable again shortly after the differentiation process, germination will not occur immediately (Karlsson 2003).

There is evidence from field studies that germination may occur in the pelagic zone before akinetes reach the sediment (Baker 1999; Baker and Bellifemine 2000; Rucker et al. 2009). Kravchuk et al. (2006) reveal that *Anabaena flos-aquae* form two types of akinetes in the course of the vegetation period; those of the first phase germinate in the water-column, thus serving for vegetative reproduction, and those which are formed towards the end of the growing season settle down to the sediment and serve as resting stages. Nevertheless, it seems that a certain internal energy and nutrient level has to be reached before germination is initiated again. Positive effects on germination are recorded by enhanced nutrient concentrations, mainly phosphate (Adams and Duggan 1999), and resuspension into the water-column (Verspagen et al. 2004). Other studies indicate that the germination process is furthermore accelerated by higher temperatures (Barbiero 1993). Kim et al. (2005) report that the most active akinete germination occurs from the end of March to early April (8 to 12 °C), but no other environmental conditions tested affect akinete germination in nature.

In a study carried out in Lake Melangsee, an eutrophic polymictic shallow lake in north-eastern Germany (Wiedner et al. 2007), germination is assumed to start at



15 °C. Tingwey (2009) reports similar observations for germination (as low as 13 °C) of *Cylindrospermopsis raciborskii* and *Aphanizomenon* spp. in sediment samples from this lake. All reports on akinete germination are based on different field or culture study approaches, which make it difficult to compare the data and to derive general information about germination triggers for Nostocales.

However, after successful germination, further development of young filaments depends on abiotic conditions. Light and temperature increases in the water-column lead to an increase in the growth rate (Mehnert et al. 2010). Gas vesicles arise in vegetative cells which regulate buoyancy in the pelagic zone. Thus, Nostocales are able to move actively through the water-column and to stratify in regions of favourable environmental conditions. During the growth phase, Nostocales differentiate heterocysts and fix nitrogen in the absence of combined inorganic nitrogen sources (Figure 1.2). Different physiological stress factors contribute to akinete differentiation in late summer or autumn. Phosphorus is required for the full development of akinetes in several Nostocales species (Kaplan-Levy et al. 2010). Controversially, other studies report that phosphorus limitation appears to be a major trigger (Meeks et al. 2002; van Dok and Hart 1996). A recent laboratory study shows external signals such as potassium deprivation, temperature and light triggers akinete development in *Aphanizomenon ovalisporum* (Sukenic et al. 2013); but low akinete numbers are observed under dark conditions and high temperatures.

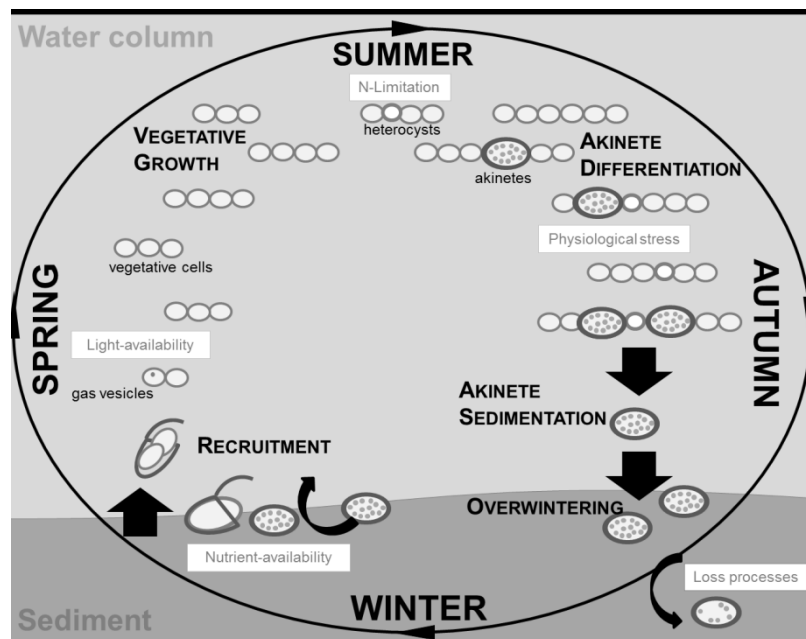


Figure 1. 2 Scheme of the theoretical concept of the Nostocales life cycle in temperate regions. The figure was modified according to Hense and Beckmann (2006).

### 1.3 Scope of the thesis

The work of this thesis was integrated into the Joint German-Israeli BMBF-MOST Project: “Life cycle of Nostocales – An intrinsic dynamic component essential to predict cyanobacterial blooms in lakes and reservoirs”. The overall aim of the project was to elucidate the distribution and regulation of Nostocales in deep stratified lakes of different trophic states and climatic zones. Gaps in knowledge regarding regulation of the life cycle of Nostocales, abundance and spatial heterogeneity of vegetative population in pelagic zones, and akinetes in sediments, from which pelagic population recruits, were determined. Findings promote the prediction of cyanobacterial blooms and potentially estimate further development of Nostocales and the occurrence of their toxins under changing trophic and climatic conditions. Generally, results contribute to a better understanding of freshwater ecosystem functioning. Furthermore, outcomes should help to establish a scientific basis for water management and to assess the risks of cyanotoxins to human health.

Hence, the following main scientific objectives were derived:

- Elucidation of life-cycle strategies of Nostocales in two deep thermally stratified lakes: the mesotrophic Lake Scharmützelsee and oligotrophic Lake Stechlinsee.
  - i) Determination of temporal and spatial variability in akinete abundance in sediments.
  - ii) Overwintering of akinetes in sediments and vegetative cells in the pelagic zone.
- Development of a molecular biological detection and quantification method for akinetes of Nostocales in sediments of all lakes studied.
- Studies on the occurrence of potentially cylindrospermopsin (CYN)-producing Nostocales species in Lake Kinneret.

Research was mainly focused on the regulation of the vegetative growth of Nostocales in the pelagic zone of mostly shallow eutrophic lakes. The vegetative cells in the pelagic zone and overwintering of akinetes in sediments must be considered for a better understanding of the distribution and potential spread of Nostocales and their blooms in deep lakes.

The overwintering success of akinetes was sporadically studied in the sediments of shallow lakes (Karlsson-Elfgren and Brunberg 2004; Kim et al. 2005; Kravchuk et al. 2006; Rucker et al. 2009). However, to the best of our knowledge, no investigations have been made on large thermally stable stratified lakes.

Additionally, the importance of the size of the sedimentary akinete pool for the success of the following pelagic population is not unequivocally clear. Rucker et al. (2009) found that the following population size in spring cannot be estimated by the akinete pool of *Cylindrospermopsis raciborskii*. However, Padisák (2003) showed a correlation between the size of the akinete pool of *Cylindrospermopsis raciborskii* and the following population size in Lake Balaton.

Akinete pool data in sediments are missing for a final clarification, especially for deep thermally stratified lakes, and this led to the following research question:

**Chapter 2 - Do seasonal and spatial variability occur in the akinete abundance in the sediments of deep stratified lakes depending on water depth and lake basin morphometry? Which sediment surface areas can potentially contribute as sedimentary akinete inoculum to pelagic population in spring?**

One reason for the gaps in knowledge regarding overwintering akinetes in sediments is the time-consuming detection and quantification of akinetes in sediment samples. The number of akinetes in sediment samples were currently light microscopically determined mostly by the Utermöhl method (1958). However, the abundance of akinetes in the sediments of deep oligo- and mesotrophic lakes is very low, and sometimes akinetes are difficult to find among numerous sediment particles similar in size and shape. Based on this lack of knowledge the following research question was derived:

**Chapter 3 - Could akinetes be identified and enumerated in deep lake sediments via a developed catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) method? Is this method a useful tool for akinete enumeration and more time advantageous versus the Utermöhl method (1958)?**

Until now, it has been unclear whether different Nostocales species are the same or different regarding their overwintering strategy in the temperate lakes studied. A low abundance of filaments in the pelagic zone cannot be regularly observed in the winter. First studies of the Baltic Sea (Suikkanen et al. 2010) indicate that representatives of the genera *Nodularia*, *Aphanizomenon* and *Anabaena* form different amounts of akinetes and overwinter partly as vegetative cells in the water-column. Thus, the research question arose:

**Chapter 4 - Do species- or genus-specific differences exist in the overwintering strategies of Nostocales in the deep and shallow lakes studied? Can the detection limit of filaments in winter plankton samples be reduced by enrichment?**

Lake Kinneret is the central drinking water reservoir of Israel. However, the invasive Nostocales species *Aphanizomenon ovalisporum* occurring is considered to be toxic (Banker et al. 1997). The local species *Cylindrospermopsis raciborskii* has not hitherto produced CYN in this lake. So far, no other non-toxic Nostocales species have been found in this lake, therefore, the following question was derived:

**Chapter 5 - Do non-CYN-producing strains coexist with toxic strains of *Aphanizomenon ovalisporum* in Lake Kinneret? Can we find any evidence of other CYN-producing Nostocales species in this lake?**

Answers to the questions referred to can be found in the conclusions of **chapter 6**.

#### **1.4 Methodological considerations**

This thesis preferentially aimed to fill remaining gaps in the knowledge regarding sedimentary akinete pools of Nostocales in deep stratified lakes. In order to investigate the distribution of akinetes and their potential ecological role in deep lakes, the sediments of Lake Scharmützelsee and Lake Stechlinsee were sampled along transects and over the course of one year. The focus was to determine differences in akinete abundance in sediments according to temporal development and water depth gradient, and in relation to the basin morphometry of lakes (**Chapter 2**). The akinete pool in lakes and its potential contribution to the pelagic population in spring was estimated based on the akinete abundance calculated in sediments. Additionally, proliferation of Nostocales in Lake Stechlinsee was temporally tracked in a sediment core.

Akinete numbers were mostly microscopically determined by the Utermöhl method (1958) in recent studies and in the present study (see **Chapter 2**). Diluted sediment samples were put into sedimentation chambers and the number of akinetes was counted on the bottom of this chamber using an inverse microscope. Thus, akinetes were hard to find between sediment particles and organisms which could be easily confused with akinetes, such as diatoms or chrysophyceae cysts. Therefore, a CARD-FISH (catalyzed reporter deposition fluorescence *in situ* hybridization) method for identification and enumeration of akinetes was developed in the current thesis (**Chapter 3**). The cells were labelled with genera-

or species-specific 16S rRNA probes and fluorescence dye, which made them much more visible in fluorescence microscopic analysis of sediment samples.

The Nostocales life-cycle strategy of the exclusively dormant overwintering of akinetes in sediments was assumed not to be the only way in lakes. In this thesis, enriching filaments via a plankton net in the winter plankton of Lake Scharmützelsee and Lake Stechlinsee was tried, and they were analysed by light microscopy (**Chapter 4**). Furthermore, akinetes in sediments were detected. The shallow polymictic Lake Melangsee was used for comparison.

It was suggested in this thesis that Nostocales further proliferate in subtropical and temperate lakes. In order to analyse the potentially toxic Nostocales species composition in Lake Kinneret, phytoplankton samples were taken from the pelagic zone and filaments were isolated to strains (**Chapter 5**). These strains were analysed morphologically and phylogenetically (16S rRNA and PC-IGS) and tested for cylindrospermopsin (CYN) production. Gene fragments for CYN synthesis (*cyrJ* only occur in CYN-producing strains) were detected and cyanotoxin analyses of CYN were conducted with ELISA (enzyme linked immunosorbent assay) and LC-MS/MS (liquid chromatography-mass spectrometry) to demonstrate the CYN concentration in strains.

## **1.5 List of publications and author contributions**

The following section represents an overview of the publications which were prepared from the research of this thesis and gives the respective contributions of the authors listed. As this thesis was part of a Joint German-Israeli Project (BMBF FKZ 02WT0985), the extensive studies in the field and at the microscope (for chapters 2 and 4) could only have been carried out with the collaborative work of a colleague, Thomas Gonsiorczyk.

### **1.5.1 List of publications**

This thesis is based on the following publications which were published in scientific journals, as conference articles or prepared as manuscripts for submission, respectively.

#### **Chapter 2**

Ramm, J., Gonsiorczyk, T., Rücker, J., Wiedner, C., Knie, M. & Nixdorf, B: Spatial distribution and seasonal variation of cyanobacterial akinetes in lake sediments, manuscript.

### Chapter 3

Ramm, J., Lupo, A., Hadas, O., Ballot, A., Rücker, J., Wiedner, C. & Sukenik A. (2012) A CARD-FISH protocol for the identification and enumeration of cyanobacterial akinetes in lake sediments. *FEMS Microbiology Ecology* **82**, 23-36.

### Chapter 4

Ramm, J., Rücker, J., Gonsiorczyk, T., Knie, M., Wiedner, C. & Nixdorf B.: Overwintering strategies of Nostocales (cyanobacteria) in lakes of Northern Germany, manuscript.

(published in excerpts in the conference article: \*Ramm et al. 2013)

\* Ramm, J., Rücker, J., Gonsiorczyk, T., Wiedner, C., Knie, M. & Nixdorf, B. (2013) Überwinterungsstrategien nostocaler Cyanobakterien in Seen Norddeutschlands. *Erweiterte Zusammenfassungen der Jahrestagung 2012 in Koblenz*, 134–138. Presented at the Jahrestagung der Deutschen Gesellschaft für Limnologie (DGL), Hardegsen: Eigenverlag der DGL.

### Chapter 5

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

#### 1.5.2 Author contributions

The relative contributions of the authors are listed in the following section.

A. Ballot (AB), A. Lupo (AL), A. Sukenik (AS), B. Nixdorf (BN), C. Wiedner (CW), J. Ramm (JR), J. Rücker (JRü), M. Knie (MK), O. Hadas (OH), R. N. Kaplan-Levy (RNK), T. Gonsiorczyk (TG), T. Rundberget (TR).

### Chapter 2

JRü, CW, JR and TG designed the study; JR and TG performed the research; JR analyzed the data with contributions by TG and MK; JR wrote the manuscript with contributions by MK, JRü and BN.

**Chapter 3**

AS, CW, JR and AL designed the study; JR, AL, and AB performed the research; JR analyzed the data with contributions by AL; JR wrote the article with contributions by AS, AL, CW, JRü, AB and OH.

**Chapter 4**

JR, JRü designed the study; JR, MK and TG performed the research; JR analyzed the data with contributions by MK; JR wrote the manuscript/ conference article with contributions by JRü, BN, MK and CW.

**Chapter 5**

AB and JR designed the study; AB and JR performed the research; AB and JR analyzed the data with contributions by TR; AB wrote the article with contributions by JR, AS, RNK, OH, TR and CW.

## **2. Spatial and seasonal variability in a sedimentary akinete pool of Nostocales and its potential as inoculum size**

### **2.1 Abstract**

Nostocales form thick-walled, resting cells (akinetes) for overwintering in lake sediments of Central Europe. In the late summer, akinetes differentiate from vegetative cells and settle to the lake bottom. These resting stages can remain intact for decades. The pelagic Nostocales population recruits after the germination of akinetes, which mainly takes place in the spring with increasing temperatures and light intensities. The study aimed to i) characterize the sediment surfaces inhabited by viable akinetes that potentially contribute to the formation of a pelagic population, and ii) estimate the inoculum size of the available sedimentary akinete pool. We investigated the horizontal and vertical distribution and seasonal variability of akinetes in sediments and the seasonal course of light and temperature in two deep dimictic lakes (Lake Stechlinsee and Lake Scharmützelsee). The sediment surface was sampled along transects from lake shore to the centre of the lake and in one annual cycle for akinete analysis. One vertical sediment profile was taken to show the ancient distribution of akinetes. The akinete pool was calculated and the potential inoculum was estimated using assumed light and temperature data for akinete germination. The akinete abundance in sediments depended on the basin morphometry and water depth gradient. The temporal development of akinetes in sediments showed an expected increase of akinetes in autumn or winter. The vertical dispersion of akinetes in Lake Stechlinsee's sediment indicated a possible change in trophic state. The akinete pool in Lake Scharmützelsee was larger than in Lake Stechlinsee. Barely 20.5 % of the total akinete pool in 51 % of the total sediment surface in Lake Scharmützelsee can potentially contribute as inoculum to the pelagic population.

### **2.2 Introduction**

Nostocales usually perform a seasonal life cycle in lakes in Central Europe and form akinetes (resting stages) to overwinter in the lake's surface sediments. Akinetes are formed at the end of the exponential growth phase in the late summer (Sutherland et al. 1979) and settle to the bottom of the lake, overwinter there and germinate in the spring (Hense and Beckmann 2006; Jöhnk et al. 2011; Kaplan-Levy et al. 2010). Hellweger et al. (2008) showed in an *in silico* gene knockout experiment with prohibited akinete formation that formation of akinetes is



important for *Anabaena* to survive as a population on an annual basis. There is still a lack of knowledge concerning the number of akinetes formed in natural populations of different Nostocales species and of their loss processes during the winter.

However, intact akinetes can stay viable in sediments for a long period of time (Livingstone and Jaworski 1980). Thus, sedimentary viable akinetes and their empty envelopes can be used for reconstruction of past cyanobacteria abundances (Räsänen et al. 2006) and, in relation with other chemical parameters, potential changes of nutrient limitation in the lake can be estimated (van Geel et al. 1994).

The annual overwintering success of intact akinetes has been well-studied in different shallow lake sediments (Karlsson-Elfgren and Brunberg 2004; Kim et al. 2005; Kravchuk et al. 2006; Rucker et al. 2009). So far, limited results are available on large, thermally stable stratified lakes, even though it is well known that differences exist in the akinete abundance in a depth gradient. One reason for the gaps in knowledge of overwintering akinetes in deep lakes results from the complex and time-consuming detection and quantification of akinetes in sediment samples. Akinete numbers in sediment samples were hitherto mostly microscopically determined by the Utermöhl method (1958). In comparison to shallow lakes, low numbers of akinetes have been detected in surface sediments of deep lakes. Furthermore, some organisms, such as pelagic diatoms, are similar in size and shape and can be confounded with akinetes between numerous sediment particles making quantification difficult.

The resting stages accumulate annually, to a large extent, in sediments of the profundal zone (Baker 1999; Head et al. 1998). However, akinete germination mainly takes place in the littoral zone due to higher temperatures, illumination and sediment mixing (Head et al. 1999; Karlsson-Elfgren and Brunberg 2004; Rengefors et al. 2004). Kravchuk and Anisimova (2012) also stated that the akinetes' hot spots in littoral sediments are potential sites of "bloom" initiation. However, various factors can contribute to the uneven, patchy distribution of akinetes in littoral sediments.

The importance of the size of the sedimentary akinete pool for the success of the population in the following growing season has remained unknown up to now. Rucker et al. (2009) found that the akinete pool of *Cylindrospermopsis raciborskii* could not completely explain the following population size in the spring. The germination conditions were probably more responsible for the population size than the number of akinetes in the sediments. However, Padisák (2003) reported a

correlation between the size of the akinete pool of *Cylindrospermopsis raciborskii* and the following population size in Lake Balaton.

This chapter deals with the spatial distribution and seasonal variability of cyanobacterial akinetes in the sediments of two deep stratified lakes in Northern Germany, Lake Scharmützelsee and Lake Stechlinsee. The aim was, firstly, to observe the spatial heterogeneity of akinetes in sediments due to lake basin morphometry and how far akinete abundance in surface sediments depends on the water depth gradient. Vertical distribution of akinetes in sediments should give information about potential trophic changes in a lake over time, and the temporal development of akinetes in surface sediments was investigated over one annual cycle. Secondly, it was assumed that a limited akinete pool in a distinct sediment spot contributes as a potential inoculum to planktonic Nostocales population in deep stratified lakes.

## **2.3 Materials and Methods**

### **2.3.1 Study sites**

The deep oligotrophic Lake Stechlinsee is located in the north-eastern part and the mesotrophic Lake Scharmützelsee in the south-eastern part of Brandenburg (NE Germany). Both lakes were created during the last ice age, about 12,000 years ago. Lake Stechlinsee emerged from a melted dead ice-block and erosion of meltwater channels, while Lake Scharmützelsee was formed by meltwater runoff and is shaped like a glacial channel lake. Therefore, both lakes differ substantially in their basin morphometry (Figure 2.1 and Table 2.1).

Lake Scharmützelsee is elongated and divided into the relatively flat, polymictic Northern Bay (maximum depth of 7 m; routine sampling point INS = point T3 in Figure 2.1-2A) and the thermally stable stratified Southern Basin (maximum depth of 29.5 m; routine sampling point RIE = point 20 in Figure 2.1-2B). A central basin is located in between, which is stratified in summer phases and has a maximum depth of 21 m at the routine sampling point DIE (= point 14 in Figure 2.1-2B; Christen 2006; Grüneberg et al. 2011). Lake Scharmützelsee has been highly eutrophicated since the 1960s. A direct discharge of inadequately treated wastewater and diffuse discharges led to a deterioration of water quality (Grüneberg et al. 2011) and was reflected mainly by a high phytoplankton biomass (Nixdorf et al. 2003). The catchment area was improved due to reduced sources of pollution in the course of political changes around 1990. The phytoplankton biomass and composition responded with a lag of 13 years on reducing external load. Since 2003, the lake has been classified as mesotrophic

after LAWA (1999). The reduction in the trophic state resulted in a number of changes in the biological communities of the lake, such as the recolonization of submerged macrophytes (Hilt et al. 2010). Between 2004 and 2006, the phytoplankton biomass developed differently in the Northern Bay, with up to threefold higher values than in the Southern Basin ( $2.8 \pm 0.9$  vs.  $1.2 \pm 0.3$  mg L<sup>-1</sup>) with a high proportion of cyanobacteria (40 %).

Lake Stechlinsee is significantly deeper with a maximum water depth of 69 m, but its water surface area is only about one-third the size of Lake Scharmützelsee. The lake is divided into four basins with a relatively small water surface area (Casper 1985). It is one of the few, still oligotrophic lakes in Northern Germany, which makes it particularly attractive for tourism. Its unique character led to an increasing number of tourists, especially from 1959 to 1997. The lake was part of the cooling circuit of a nuclear plant from 1965 to 1990. With the closure of the plant, cooling water circulation stopped. Another source of stress was the water inflow of a nearby, highly eutrophic lake from 1969 to 1985. Although Lake Stechlinsee is still oligotrophic, there are indices of increasing eutrophication, such as the decline of submerged macrophytes (Van de Weyer et al. 2009) or the growth of nostocalean cyanobacteria.

**Table 2. 1 Limnological characteristics of the lakes studied.**

Lakes	Abbr.	Geographical Position	Z <sub>max</sub> (m)	Area (km <sup>2</sup> )	t (year)	CA (km <sup>2</sup> )	Mixis	Trophic state
Stechlinsee	ST	53°10' N; 13°02' E	69.5	4.25	> 40 <sup>a</sup>	26	mo <sup>b</sup> - di	oligo- mesotrophic
Scharmützel-see	SCH	52°14' N; 14°02' E	29.5	12.09	16	110	di	mesotrophic

Abbreviations: Z<sub>max</sub>: maximum depth, Z<sub>mean</sub>: mean depth, t: theoretical retention time, CA: catchment area, mo: monomictic, di: dimictic

<sup>a</sup> according to Casper (1985)

<sup>b</sup> in some warm years monomictic (Koschel et al. 2002)

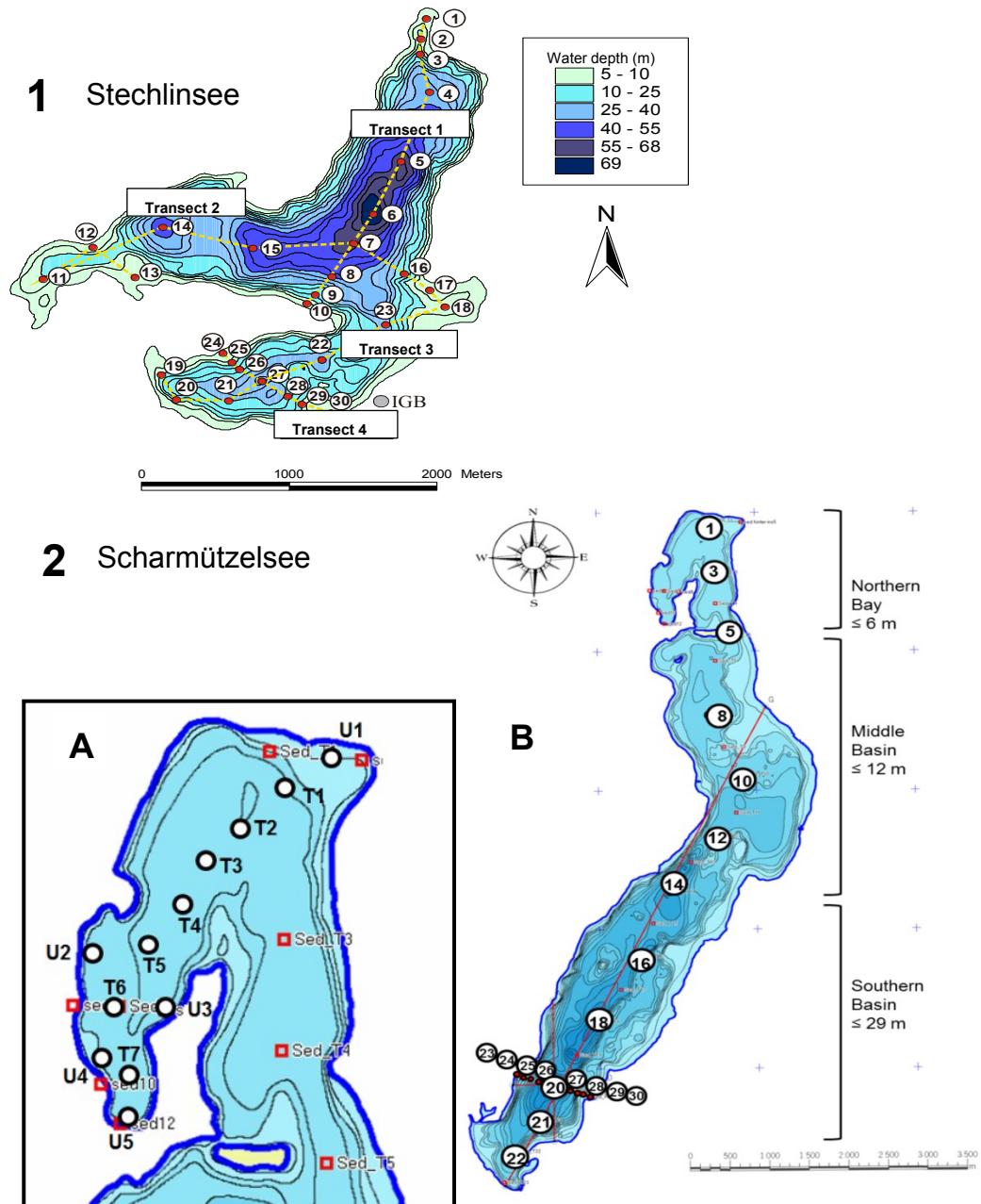


Figure 2. 1 Bathymetric maps of the lakes studied with sediment sampling points (0-1 cm). 1) Lake Stechlinsee with four flexible transects and 2) Lake Scharmützelsee with A - transect in the Northern Bay and B - transects of longitudinal axis (points 1-22) and cross axis above the deepest point of the lake (points 23-30). Routine sampling points: INS = point T3, DIE = point 14, RIE = point 20.

### 2.3.2 Light and temperature conditions

Vertical profiles of water temperature and underwater photosynthetically active radiation (PAR) were determined in half-metre intervals above routine sampling points INS, DIE and RIE monthly to fortnightly from 2009-2011 (see Appendix, Figures 1 and 2). A multi-parameter probe (Hydrolab H20, Austin, TX, USA) was used for temperature measurement and the underwater light intensity was measured with two spherical quantum sensors (SA 193, LiCor, Lincoln, NE, USA) to determine the vertical light attenuation coefficient  $K_d$ , which was interpolated daily. Global radiation data ( $\text{J cm}^{-2} \text{d}^{-1}$ ) were provided by the Potsdam Institute for Climate Impact Research (PIK, Germany). The daily sum of global radiation was converted by a factor of 0.045 to photon flux density ( $\mu\text{E m}^{-2} \text{s}^{-1}$ ), in accordance with Hilt et al. (2010), considering the average of sunlight hours per month in the years 2009-2011. The euphotic zone ( $Z_{eu}$ ) and light supply at the sediment surface of RIE was calculated on the basis of the Lambert-Beer law.  $Z_{eu}$  is defined as the depth where light intensity falls to 1 % of that at the surface and photosynthesis still occurs. Monthly averages of temperature and light values were used in this study.

### 2.3.3 Temporal and spatial sediment sampling

The sediments of Lake Stechlinsee were sampled at 30 sampling points in September 2009. The sampling points chosen were positioned along four flexible transects across the lake basins, according to Mothes (1980), to analyse horizontal differences in akinete abundances (Figure 2.1). The seasonal variability of akinetes at the deepest sampling point was studied (Figure 2.1-1, station 6) in 2010, and a whole sediment core was taken at this point to show the ancient distribution of akinetes in this lake.

The spatial heterogeneity of akinetes in the sediments of Lake Scharmützelsee was firstly studied in the Northern Bay in February 2009. A transect of 7 open stations and 5 stations close to the lakeshore were examined (Figure 2.1-2A). In March 2010, two transects of 12 stations along the lake's longitudinal axis were sampled in a distance of 1 km, and across the longitudinal axis above the deepest point of the lake in a distance of 100 m (8 sampling points; Figure 2.1-2B). The seasonal dynamics of akinetes in the sediments of Lake Scharmützelsee were studied in 2010 in the shallow polymictic eutrophic Northern Bay (INS) and in the deep dimictic mesotrophic Southern Basin (RIE).

### 2.3.4 Extraction and processing of sediments

The sampling of sediments was performed with an Uwitec sediment corer (Mondsee, Austria; diameter 50 mm). Immediately after sampling, the upper first centimetre of the undisturbed sediment surface was taken and placed in sealable polypropylene cups. Three sediment cores were sampled and combined into a mixed sample at one site in order to minimize the heterogeneity of a single sampling point. Another undisturbed sediment core of 20 cm length was taken from the deepest point of Lake Stechlinsee and cut at centimetre intervals.

Subsamples were taken for the determination of dry weight (DW; 8 h at 105 °C) and loss on ignition (LOI; 3 h at 500 °C). The calcium carbonate content of sediments in Lake Stechlinsee was also determined (CaCO<sub>3</sub> 3 h at 900 °C). The remaining original samples were stored until microscopical akinete analysis in the dark at 4 °C or fixed with formalin (4 % final concentration).

### 2.3.5 Microscopical analysis

Sediment samples were fixed with Lugol's solution and diluted 1:100 for Lake Scharmützelsee and 1:225 for Lake Stechlinsee with degassed tap-water before analysis. Diluted samples were analysed microscopically according to the Utermöhl method (1958) using sedimentation chambers (HYDRO-BIOS, Kiel, Germany) and a Nikon Diaphot300 inverted light microscope. The akinetes were defined as "intact" when cytoplasm was clearly visible. A classification of akinetes was only possible for *Cylindrospermopsis raciborskii* to species level and *Aphanizomenon* spp. to genus level. The genus *Anabaena* forms differently shaped akinetes, so that principally cylindrical and oval shapes were distinguished and, in most cases, the akinetes were grouped together as *Anabaena* spp. *Anabaena lemmermannii* constituted an exception and forms bean-shaped akinetes which usually stick together in clusters. These nests remain in the sediment but are difficult to quantify adequately due to the formation of lumps. Therefore, they were counted separately from other *Anabaena* species. Nostocales species with their akinete types potentially occurring in Lake Stechlinsee and Lake Scharmützelsee are listed in Table 2.2 and shown in Figure 2.2.

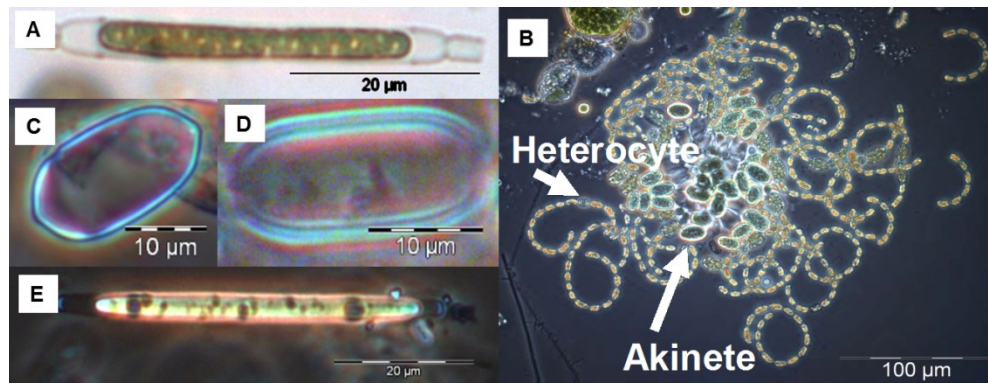


Figure 2. 2 Akinete types of Nostocales species potentially occurring in studied lakes (see Table 2.2). A – *Aphanizomenon*, B – *Anabaena lemmermannii*, C – *Anabaena oval*, D – *Anabaena cylindric*, E – *Cylindrospermopsis*. Photographs provided by M. Knie.

### 2.3.6 Estimation of inoculum size

We defined the akinete germination requirements in order to estimate the potential inoculum size of akinetes in the sediments for both lakes. Temperature above 8 °C (Rücker et al. 2012) and light intensity of more than 6  $\mu\text{E m}^{-2} \text{s}^{-1}$ , adapted from Agrawal and Singh (2000), was found to be sufficient for akinete germination.

Additionally, a scenario of different temperatures and light intensities was applied for Lake Scharmützelsee to show the impact of the akinete pool as a potential inoculum to the planktonic population under changing germination conditions. Temperatures of 8, 10 and 15 °C and light intensities of 6, 15 and 30  $\mu\text{E m}^{-2} \text{s}^{-1}$  corresponded to the average values typically observed at mean water depth in the spring (April-June 2009-2011). Due to the relatively similarly tested  $K_d$  and temperature values in the Northern Bay and Southern Basin for this investigation period, the inoculum size was analysed from samples taken at the deepest sampling point RIE.

The averaged abundance of intact akinetes in the sediments of those water depths in which defined germination conditions can be achieved was multiplied with corresponding sediment surface areas. Please note that the sediment areas were calculated according to two-dimensional isobath maps of the lakes studied.

Table 2. 2 List of occurring Nostocales species as vegetative cells and akinetes in pelagic zone and types of akinetes (Figure 2.2) in pelagic zone and sediments of lakes studied.

Nostocales species	L. Scharmützelsee		L. Stechlinsee		akinete type
	veg. cells	akinetes	veg. cells	akinetes	
<b><i>Anabaena</i></b>					
<i>Ana. affinis</i>	+	+	+	+	Anabaena oval
<i>Ana. circinalis</i>	+	+	+	+	Anabaena oval
<i>Ana. crassa</i>	+	+	+	+	Anabaena oval
<i>Ana. flos-aquae</i>	+	+	+	+	Anabaena oval
<i>Ana. lemmermannii</i>	+	+	+	+	Anabaena lemmermannii
<i>Ana. macrospora</i>	+	+	+	+	Anabaena oval
<i>Ana. mendotea</i>	+	+	+	-	Anabaena cylindric
<i>Ana. planctonica</i>	+	+	+	+	Anabaena oval
<i>Ana. solitaria</i>	+	+	+	+	Anabaena cylindric
<i>Ana. viguieri</i>	+	+	+	+	Anabaena oval
<b><i>Aphanizomenon</i></b>					
<i>Aph. aphanizomenoides</i>	-	-	+	-	Aphanizomenon
<i>Aph. flos-aquae</i>	+	+	+	-	Aphanizomenon
<i>Aph. gracile</i>	+	+	-	-	Aphanizomenon
<i>Aph. issatschenkoi</i>	+	+	-	-	Aphanizomenon
<b><i>Cylindrospermopsis</i></b>					
<i>Cyl. raciborskii</i>	+	+	-	-	Cylindrospermopsis

+ present, - not present



### 2.3.7 Statistical analysis

Linear regression analysis between akinete abundance, sediment characteristics (DW and LOI) and water depth were carried out with square root or reciprocal transformed data. The final regression model uses square root transformed data of depth and akinete abundance (Eq. 2.1).

$$\text{Equation 2.1} \quad \sqrt{\text{akinete abundance}} = a + b\sqrt{\text{depth}}$$

Nostocales species composition in Lake Scharmützelsee was examined using non-metric multidimensional scaling (NMDS). The biovolume concentration of vegetative cells and akinetes in the pelagic zone and the sedimentary akinete abundance of the polymictic Northern Bay and the dimictic Southern Basin were analysed. Input data of sedimentary akinete abundance corresponds to Figure 2.1. Biovolume data (annual totals of the years 2005-2010), which were provided by A. Tworeck (LBH Hoehn, Freiburg), have been grouped according to Table 2.2.

All statistical analyses were conducted with the software R using the vegan package for NMDS (R Core Team 2012).

## 2.4 Results

### 2.4.1 Vertical distribution of akinetes in sediments

Akinete abundances and sediment composition were examined in a vertical profile at the deepest point of Lake Stechlinsee in 2010 (Figure 2.3). An increase in intact *Anabaena* akinete concentration was shown in the uppermost sediment layers. Whereas high concentrations of empty akinete envelopes were found in the deeper sediment layers, suggesting that *Anabaena flos-aquae* and *Anabaena lemmermannii* were possibly already present 60 to 100 years previously (sediment horizon 8-14 cm). The sediment composition showed drastic changes (Figure 2.3 right). In the upper sediment layers, CaCO<sub>3</sub> content increased, probably because of more intensive pelagic calcite precipitation due to eutrophication (higher primary production leads to higher pH values and higher supersaturation with respect to the precipitation of CaCO<sub>3</sub>).

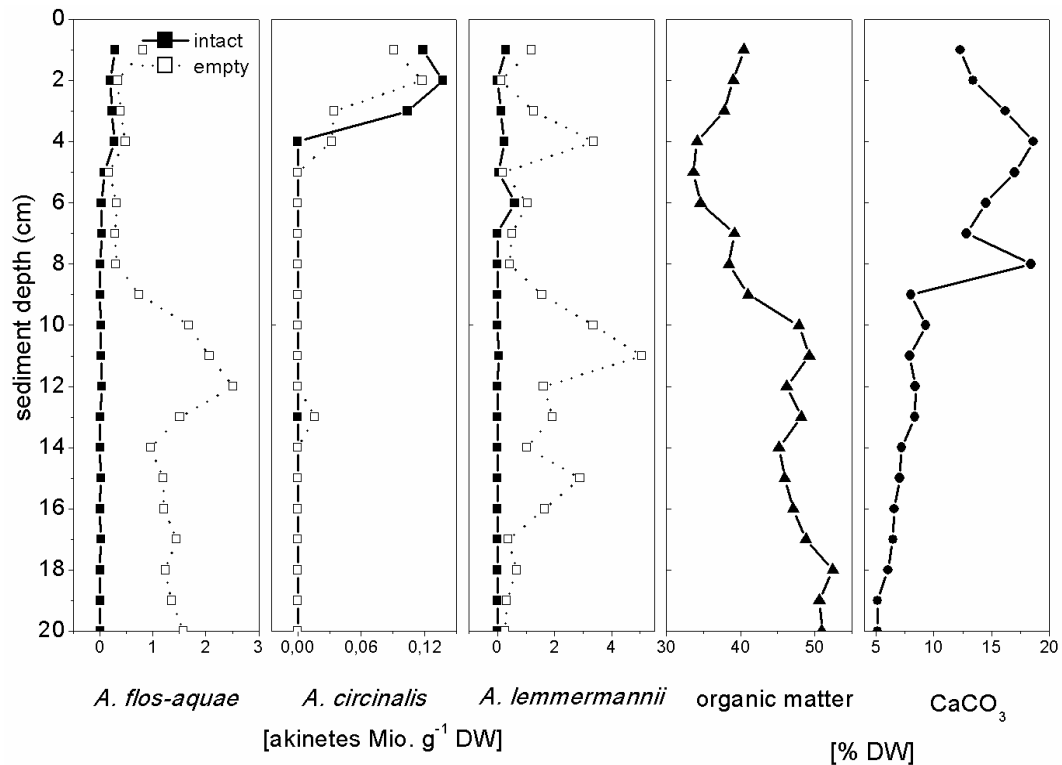
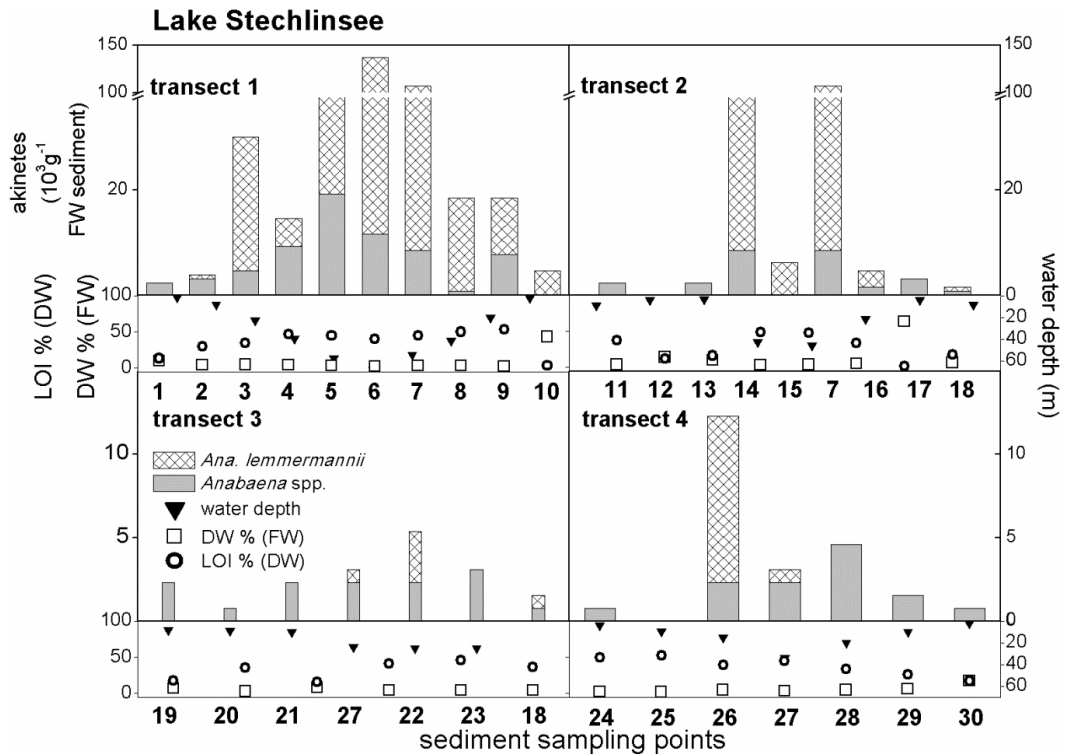


Figure 2. 3 Cyanobacterial akinete abundances, proportion of organic matter and content of calcium carbonate in a vertical sediment profile (0-20 cm) taken at the deepest point of Lake Stechlinsee.

#### 2.4.2 Horizontal distribution of akinetes in sediments

The intact akinete abundance in all four transects of Lake Stechlinsee increased in the sediments with increasing water depth (Figure 2.4). The highest number of akinetes were found in the sediments of the northernmost basin over the deepest point of the lake (transect 1). The species *Anabaena flos-aquae* and *Anabaena lemmermannii* constituted about 95 % of all Nostocales akinetes at the sediment sampling points of Lake Stechlinsee. Akinetes of *Aphanizomenon flos-aquae* were not found (compare Table 2.1). Barely 22 % of all akinetes observed in Lake Stechlinsee's sediment were intact cells with visible protoplast and were presumably viable. *Anabaena lemmermannii* demonstrated their akinete patchiness in sediments by the formation of nests, spots of up to 200 akinetes, which were not detected at sampling points lower than 8 m water depth. The highest sediment dry weight proportion of 63 % was found at point 17 in the Eastern Basin of Lake Stechlinsee (Figure 2.4, transect 2, lower panel, 3.5 m water depth). The closest shore sites in the littoral showed, with few exceptions, higher DW proportions than the profundal sites with lower LOI of DW.



**Figure 2. 4** Akinete abundances of Nostocales, LOI and DW in surface sediments (0-1 cm) of Lake Stechlinsee. Locations of sampling points are given in Figure 2.1.

The number of intact akinetes in Lake Scharmützelsee increased with water depth as in Lake Stechlinsee (Figure 2.5). The viable akinete number was at its lowest on average with 2,000 intact cells  $\text{g}^{-1}$  fresh sediment in the shallow Northern Bay and at its highest near the deepest point in the Southern Basin of the lake (Figure 2.5A). The akinete number was slightly higher at station U1 (approximately 1 m water depth) than at other lake shore sites (U2-U5) in the littoral part of the Northern Bay. The proportion of intact cells as part of the total sum of akinetes was 27 % in the lake. Intact akinetes of *Anabaena lemmermannii* and *Cylindrospermopsis raciborskii* were not present in the Northern Bay, whereas they occurred, although not frequently, in all transect points in the Middle and Southern Basin. The highest proportion of intact akinetes as part of the total akinete abundance of *Anabaena lemmermannii* was found at point 2 (Figure 2.5C). Surprisingly, the deepest point in Lake Scharmützelsee (Figure 2.5B, point 20; 19,136 total intact akinetes  $\text{g}^{-1}$  fresh sediment) was not the place of the highest akinete deposition as suggested. The surrounding sites showed higher abundances of as many as 25,200 total akinetes  $\text{g}^{-1}$  fresh sediment.

Barely viable akinetes of *Anabaena* spp. and *Aphanizomenon* spp. were found in the riparian areas of the Southern Basin. All lake shore sites consisted of sandy sediments with a high DW proportion of FW and low LOI of DW (Figure 2.5A-C, lower panels).

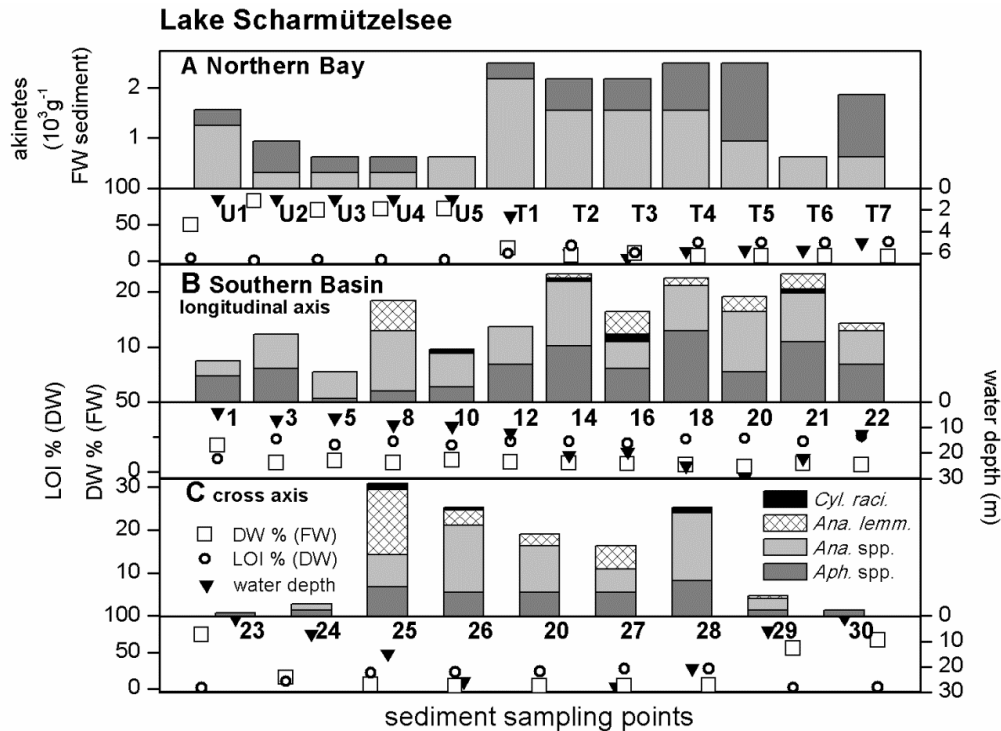
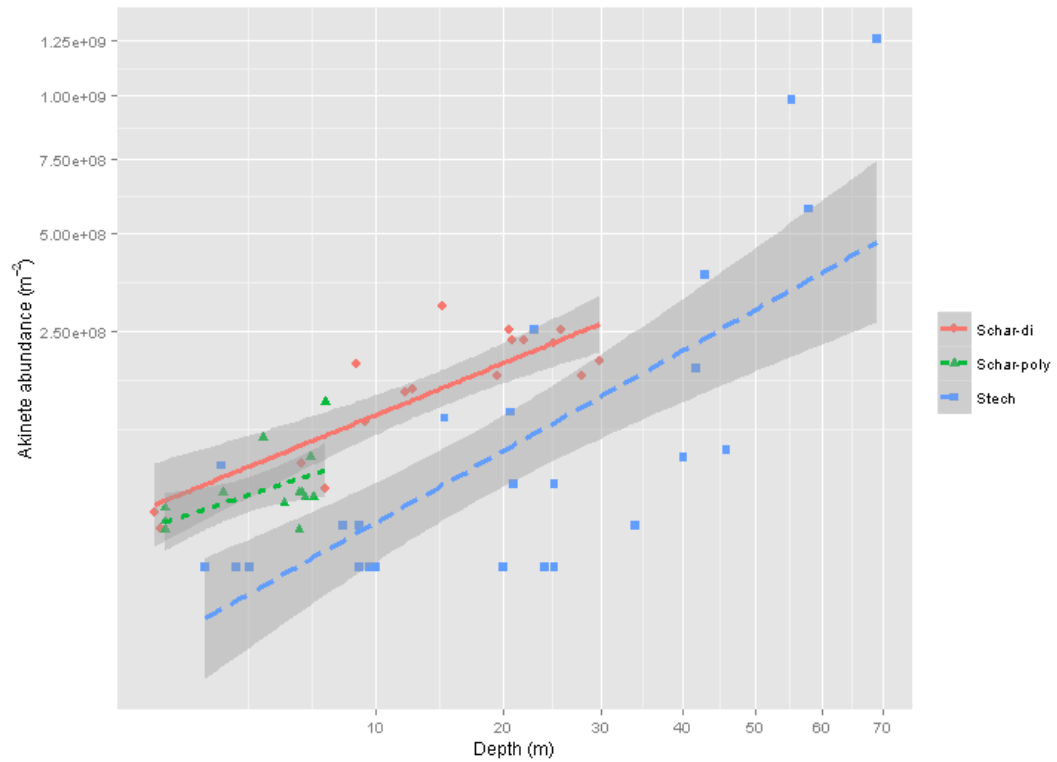


Figure 2. 5 Akinete concentration of Nostocales, LOI and DW in the surface sediments (0-1 cm) of Lake Scharmützensee along transects in the Northern Bay (A), in a longitudinal axis over the lake (B) and cross axis (C) in the Southern Basin at the deepest point of the lake. Sampling sites are shown in Figure 2.1

The abundance of viable akinetes in the sediments of both lakes was significantly correlated with water depth (Figure 2.6). Since sediment characteristics, such as dry matter content and loss on ignition, are also correlated with water depth, the regression models were not improved by their addition (Table 2.3). However, the fit for data of the polymictic Northern Bay is weak, as indicated by a low  $R^2$  value.

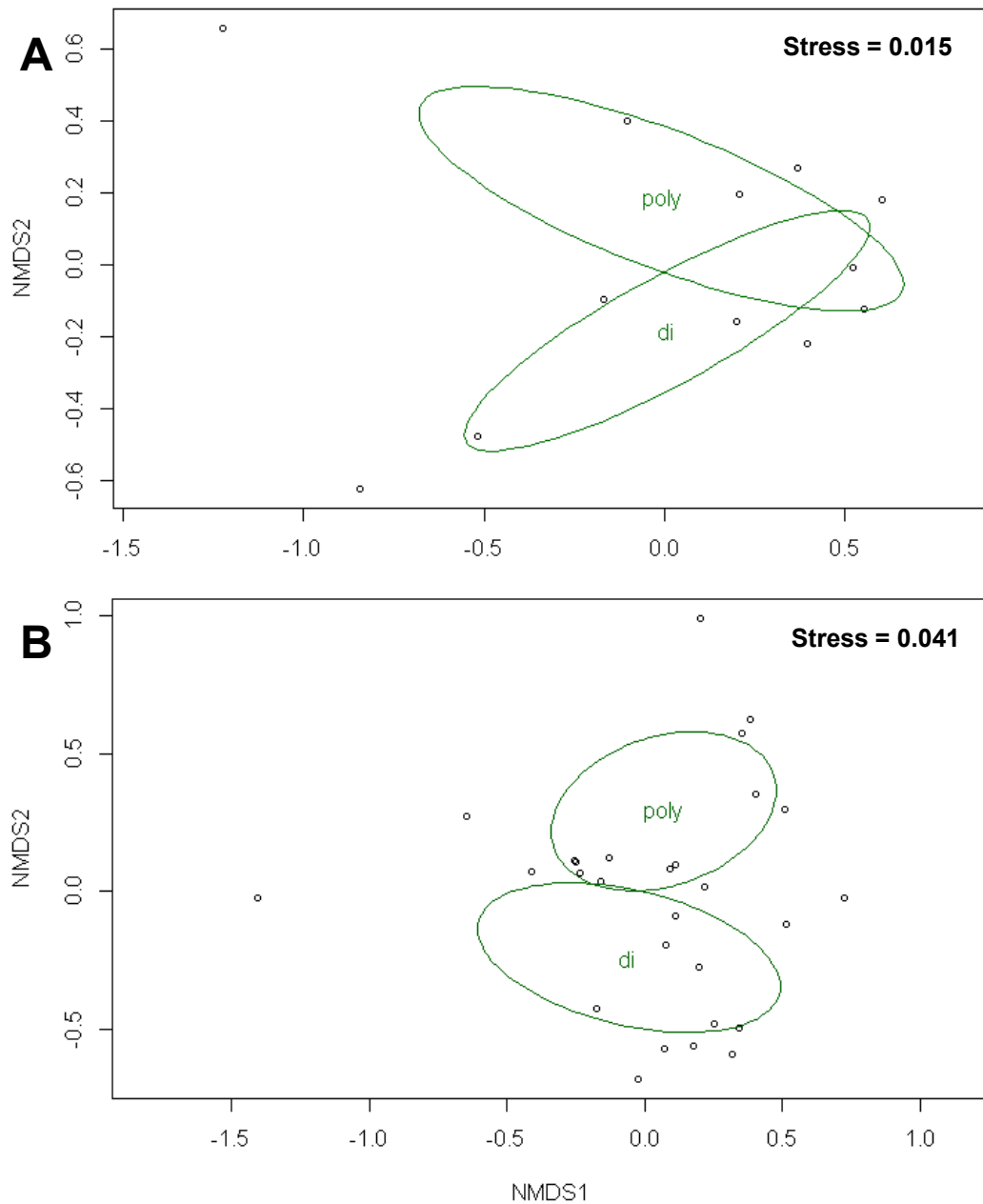
The structure of the pelagic population of Nostocales grouped according to their akinete morphology showed little difference between lake basins, as revealed by NMDS (Fig 2.7A, two of three axes shown, stress = 0.015). The ordination spaces of the two basins overlapped to a large extent. On the other hand, the composition of the sedimentary akinete pool differed between both basins. The sampling points separated clearly in the ordination by NMDS (Figure 2.7B, two of four axes shown, stress = 0.041).



**Figure 2. 6** Linear regression models and confidence bands of intact akinete abundances in relation to increasing water depth of Lake Scharmützelsee (Schar) and Lake Stechlinsee (Stech). Transect points studied are given in Figure 2.1. Values were square root transformed. Schar-di = dimictic Southern Basin, Schar-poly = polymictic Northern Bay.

Table 2. 3 Parameters, confidence intervals and goodness of fit estimated for linear regressions.

Lake	Independ. variable	Intercept (CI)	Slope (CI)	R <sup>2</sup>
	$\sqrt[2]{depth}$	-8602.6 (-14079.6 – 3125.7)	3662.6 (2481.3 – 4843.9)	0.58
Stechlin- see	$\frac{1}{DW}$	821.7 (-6783.2 - 8426.6)	24788.5 (-3613.8 - 53190.9)	0.07
	$LOI^2$	826.3 (-5428.8 - 7081.3)	4.3 (0.5 - 8.1)	0.13
	$\sqrt[2]{depth}$	1786.7 (-1750.2 - 5323.6)	2657.2 (1781.0 - 3533.3)	0.70
Schar- mützelsee dimictic	$\frac{1}{DW}$	4986.0 (2297.1 - 7675.0)	40939.0 (26776.3 - 55100.8)	0.68
	$LOI^2$	5231.8 (2734.7 - 7728.8)	15.8 (10.6 - 21.0)	0.70
	$\sqrt[2]{depth}$	879.2 (-2627.7 - 4386.1)	2121.6 (357.3 - 3886.0)	0.29
Schar- mützelsee polymictic	$\frac{1}{DW}$	3373.0 (1102.6 - 5644.0)	16376.0 (-4198.6 - 36950.4)	0.12
	$LOI^2$	4088.4 (2104.3 - 6072.5)	2.7 (-2.3 - 7.7)	0.02



**Figure 2. 7** NMDS ordination of *Nostocales* species composition in the polymictic Northern Bay (poly) and dimictic Southern Basin (di) of Lake Scharmützensee. A) Pelagic *Nostocales* biovolume (annual totals of 2005-2010) and B) akinete abundance in sediments. Species composition according to akinete types in Table 2.2.

### 2.4.3 Seasonal variation of akinetes in sediments

The temporal development of akinete abundance in the sediment surfaces of Lake Scharmützensee and Lake Stechlinsee showed a seasonal variation. The proportion of intact akinetes as part of the total amount increased in the autumn (Figure 2.8).

Intact akinetes of *Anabaena lemmermannii* increased sixty-fold and *Anabaena* akinete numbers were nine-fold higher in the Northern Bay, or five-fold higher in the Southern Basin in autumn compared to summer. At the beginning of winter, the number of intact akinetes of *Anabaena* spp. declined again in Lake Scharmützelsee. Whereas in Lake Stechlinsee, an additional two-fold increase in intact *Anabaena* akinetes was recorded in November, showing that akinetes of this genus were still in the settling process to the deepest point in September.

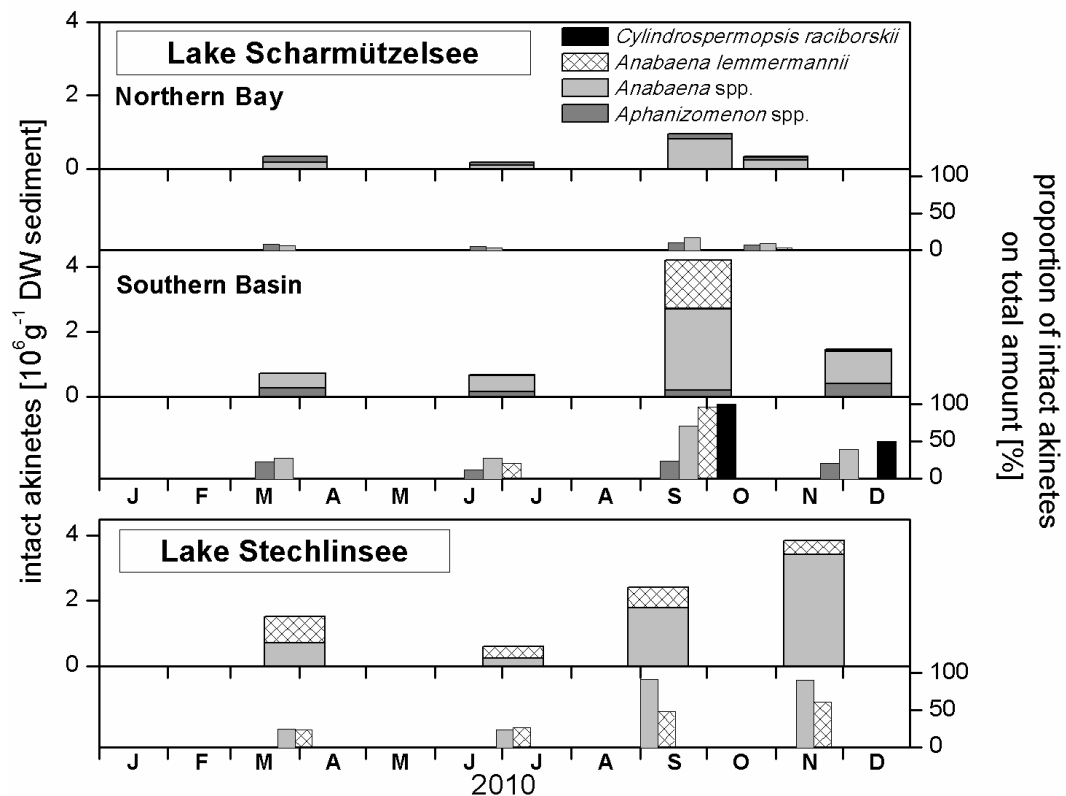


Figure 2. 8 Seasonal variation of viable akinetes and proportion of intact cells on total akinete abundance in surface sediments (0 - 1 cm) of Lake Scharmützelsee and Lake Stechlinsee. Sampling points see Figure 2.1: Lake Scharmützelsee Northern Bay – point T3 in 6 m, Southern Basin - point 20 in 29.5 m and Lake Stechlinsee - point 6 in 69 m water depth.

#### 2.4.4 Estimation of inoculum size of akinetes in sediments depending on underwater light and temperature

Based on the horizontal distribution of akinetes in surface sediments, the potential theoretical inoculum size for Nostocales populations developing next year was estimated. The averaged abundance of intact akinetes in sediments of different water depths multiplied with corresponding sediment surface areas is given in Figure 2.9.



The sediment area increased in the deeper water depths of the Northern Bay (7 m) and Middle Basin (9-10 m) of Lake Scharmützelsee and led to a high amount of deposited akinetes due to the basin morphometry (Figure 2.9, left). Envelopes but no viable akinetes of *Anabaena lemmermannii* and *Cylindrospermopsis raciborskii* were found in the Northern Bay, whereas resting stages of these species were found in the Middle Basin at water depths of more than 9 m. The largest sediment zone was captured at 13-14 m water depth in the Southern Basin with a high average of sedimentary akinete pool. The akinete amount in deeper regions decreased with the size of the sediment area.

The sediment areas of the four Lake Stechlinsee basins were presented together and showed a decrease with increasing depth of the water (Figure 2.9, right). Intact akinetes of *Anabaena lemmermannii* occurred irregularly down to a water depth of 8 m. The highest *Anabaena lemmermannii* sedimentary pool was found from 43 m profundity (maximum average of over  $3.7 \cdot 10^{13}$  akinetes in 52 m water depth). The size of *Anabaena flos-aquae* akinetes in the sediment bed source was scattered along the water depth and reached a maximum average value of  $5 \cdot 10^{12}$  akinetes.

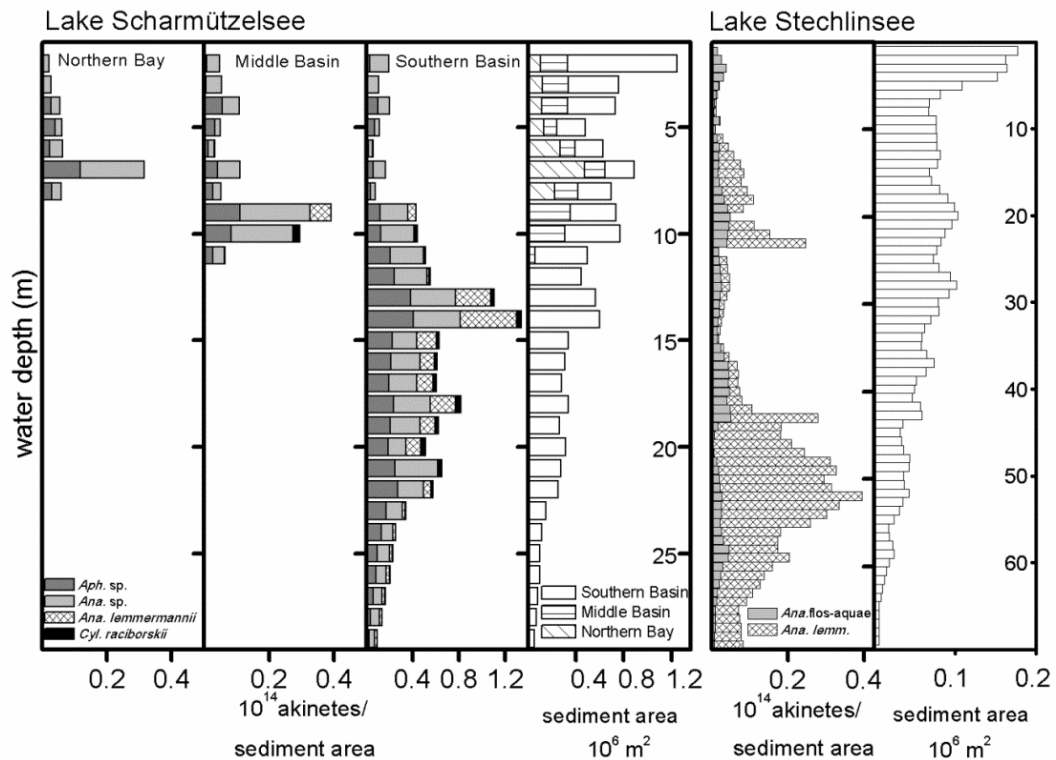


Figure 2. 9 Akinetes per sediment areas and sediment area at different water depths of the Northern Bay, Middle Basin and Southern Basin of Lake Scharmützelsee (left) and Lake Stechlinsee (right).

The assumed minimum temperature of 8 °C and light intensity of 6  $\mu\text{E m}^{-2} \text{s}^{-1}$  for akinete germination involved for Lake Stechlinsee that only 10 % of total akinetes was deposited in sediment regions in which temperature is likely for germination (12 m water depth is achieved up to the beginning of summer in June). Therefore, deeper sediment zones were regarded as a sink of akinetes. The akinetes deposited here may not germinate because of low temperatures and lack of light. This could also explain the increase of intact (non-germinated) akinetes in sediments with increasing water depth.

The light intensity of more than 6  $\mu\text{E m}^{-2} \text{s}^{-1}$  defined was closely linked to euphotic depth ( $Z_{\text{eu}}$ ) in approximately 9 m water depth above the deepest point of Lake Scharmützelsee (Figure 2.10), where akinetes were theoretically still photosynthetically active. The light supply constituted the limiting factor for akinete germination compared with the temperature of 8 °C.

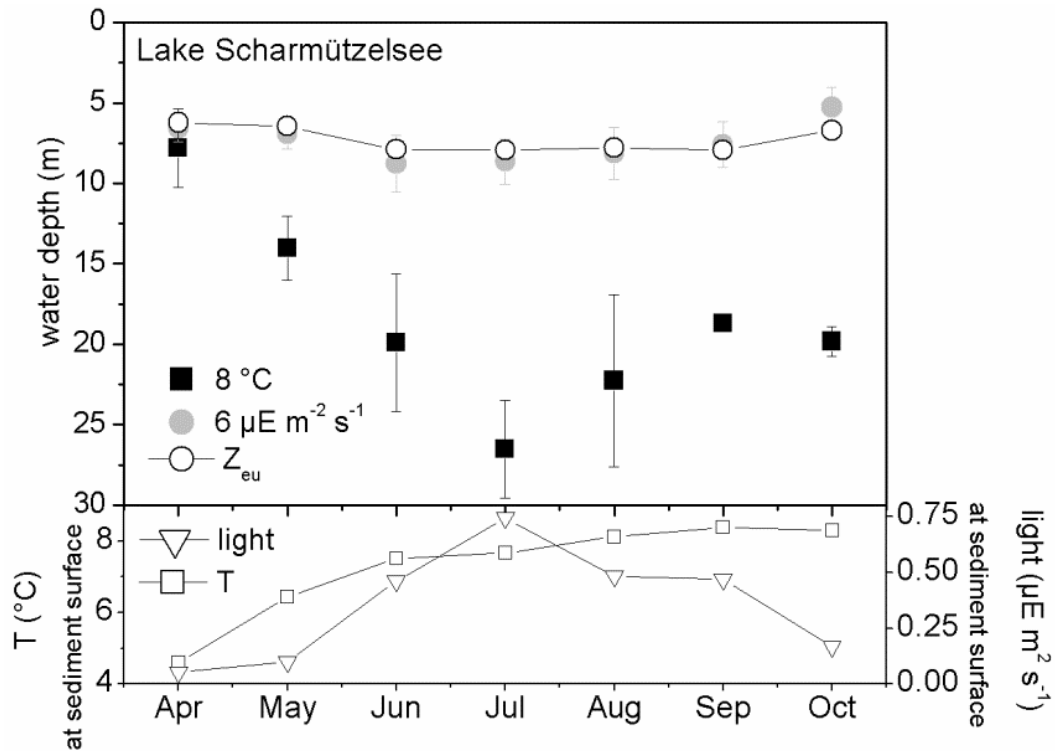


Figure 2. 10 Temporal characterization of light and temperature conditions in Lake Scharmützelsee sufficient for akinete germination (upper panel) and light and temperature conditions at the sediment surface (lower panel; annual means 2009-2011).

The potential inoculum size of akinetes in surface sediments calculated is shown in Table 2.4. The hypothesized light intensity and temperature as described above led to a potential inoculum size of  $2.8 \cdot 10^{14}$  akinetes; which means that 20.5 % of the total akinetes in 51 % of sediment surface in Lake Scharmützelsee were

viable. On the other hand, only 30 % of sediment with a pool of  $2.6 \cdot 10^{13}$  akinetes is available as the inoculum size for Lake Stechlinsee. To summarize the results for both lakes, a higher proportion of shallow water areas and a larger akinete pool resulted in a higher inoculum size in Lake Scharmützelsee than in Lake Stechlinsee.

In the scenario, a reduced theoretical germination of 6 % of total akinetes in 22 % of total sediment area in Lake Scharmützelsee would be obtained under the changed germination conditions required for the Nostocales species of  $30 \mu\text{E m}^{-2} \text{s}^{-1}$  light supply and a temperature of  $15 \text{ }^\circ\text{C}$ .

**Table 2. 4 Scenario of potential inoculum size of akinetes in sediments (0-1 cm) of Lake Scharmützelsee under variable light and temperature conditions.**

germination conditions		potential inoculum size							
temperature ( $^\circ\text{C}$ )	light ( $\mu\text{E m}^{-2} \text{s}^{-1}$ )	Akinetes ( $10^{14}$ )				total aki.	sediment area ( $10^6 \text{ m}^2$ )	water depth (m)	proportion on total akinete pool (%)
		<i>Aph.</i> sp.	<i>Ana.</i> sp.	<i>Ana.</i> <i>lemm.</i>	<i>Cyl.</i> <i>raci.</i>				
8	6	1.0	1.7	0.1	0.0	2.8	6.2	0 - 9	20.5
10	6	0.7	1.1	0.0	0.0	1.8	4.7	0 - 7	13.1
10	16	0.4	0.7	0.0	0.0	1.0	3.2	0 - 5	7.6
15	30	0.2	0.6	0.0	0.0	0.8	2.7	0 - 4	6.0
		4.9	6.2	2.1	0.4	13.7	12.1	0 - 29.5	total

## 2.5 Discussion

### 2.5.1 Spatial distribution of akinetes in sediments

Horizontal differences in akinete abundances could be detected in both water bodies studied. They were particularly strongly pronounced in Lake Scharmützelsee. This is mainly due to the different morphometry of each lake basin and different degree of anthropogenic pollution in the past and present (Grüneberg et al. 2011). A higher trophic state is presented in the shallow Northern Bay of the lake (Christen 2006). Accordingly, the phytoplankton

biovolume is higher, especially during the summer months. The total biovolume in the pelagic zone is two to three times higher and more dominated by cyanobacteria in the Northern Bay than in the Southern Basin, where the species composition of Nostocales does not differ (Rücker et al. 2012). By contrast, higher abundances of akinetes were observed in the sediments of the deep Southern Basin than in the shallow Northern Bay.

These horizontal differences obviously play a role in the development of Nostocales. Vegetative cells and akinetes can be exchanged between the lake basins through the water-column. However, it is assumed that akinetes are differently incorporated into Lake Scharmützelsee's sediments in each lake basin due to various ratios of sediment to surface water body which affects the recruitment. This ratio is about three times higher in the shallower Northern Bay than in the deep, steeply furrowed Southern Basin. The settling of the cyanobacterial population increases with larger volume in the deeper water-column. Akinetes have a specific gravity of more than  $1.16 \text{ g cm}^3$  and are the heaviest cells among vegetative cells and heterocysts (Hori et al. 2002). The accumulation of resting stages in the sediment regions of deeper water zones in the Southern Basin occurs due to "sediment focusing" or natural redistribution of fine particles from shallow to deep areas in lakes, as described previously (Cirés et al. 2013; Kravchuk et al. 2006). The depositional zone of Lake Scharmützelsee was assumed to have an autochthonous organic sediment layer (Gyttja) thicker than 20 cm by Grüneberg et al. (2011). According to this, almost 50 % of the lake's sediment area was covered with > 20 cm Gyttja (Grüneberg B, personal communication). The eastern part of the Southern Basin lake shore was mainly observed as a sediment erosion zone and the western part as an accumulation zone presented in the akinete abundance of the cross axis transect above the deepest point of the lake (Figure 2.5C, sampling points 23-30) due to the main wind direction west to northwest.

The akinete abundance in the sediments of the shallow Northern Bay was slightly higher at station U1 (Figure 2.1A), located near the reed belt and covered with macrophytes in the vegetation period, compared to other lake shore sites. The Northern Bay is generally exposed to wind and surface blooms of cyanobacteria are frequently observed in the late summer near station U1. Thus, cyanobacteria aggregate near the shore in the littoral during a "surface bloom-event", and akinetes are formed and deposited in the sediments. Akinetes in littoral sediments are stressed by wave impact, so that they will be transported into deeper water layers. Kravchuk and Anisimova (2012) found that macrophytes can play a positive role in the accumulation of akinetes in the littoral part due to the affect of

hydrodynamic conditions. Moreover, macrophytes can prevent the sediment from mixing with their root systems, and thus, can stop transport towards deeper layers.

On the other hand, a comparatively low akinete number was found at some sediment sampling points where macrophytes occur in Lake Scharmützelsee (Hilt et al. 2010, Figure 1), which could also be caused by the sediment type. All sediment sampling points in the clear littoral of Lake Scharmützelsee were of a less than 2-4 cm deep Gytjtja layer (Grüneberg et al. 2011, Figure 1). Sandy soils with high filtration capacity and mobility in contrast to silts could lead to an akinete concentration in deeper layers (Vladimirova 1968).

By contrast, Gunkel et al. (2009) stated for Lake Tegel that vertical transport of particulate organic matter deeper than the topmost 2-3 cm in sandy littoral sediments was hindered by, for example, epipsammic algae producing extra-cellular polymeric substances (EPS) which can form a dense three-dimensional structure. However, akinetes in vertical deeper sediment regions were not considered in this study to be able to germinate in the following spring, and thus, could not serve as inoculum for the next year's planktonic population.

The vertical distribution of akinete abundance in the sediment core of Lake Stechlinsee showed the largest number of *Anabaena* akinete envelopes in sediment layers that were deposited about 100 years ago (data based on sediment accumulation per year; no sediment core dating). This suggests that the present still oligotrophic Lake Stechlinsee had a phase of higher trophic level in the past, presumably during the phase of industrialization and deforestation.

*Aphanizomenon flos-aquae* has not produced akinetes recently or in the past in Lake Stechlinsee. Thus, no dormant overwintering was assumed for this species (Suikkanen et al. 2010; chapter 4 in this study). The species was observed in winter directly under the ice down to 2 m water depth (Üveges et al. 2012).

The temperature in winter is an important factor for Nostocales to overwinter vegetatively in the pelagic zone (Cirés et al. 2013; *Aphanizomenon flos-aquae* 9.8 °C in February and *Aphanizomenon gracile* 7.5 °C in January).

### 2.5.2 Seasonal variation of akinetes in sediments

The seasonal analysis of akinetes in sediments confirmed that the input of akinetes in sediments is greatest in the autumn after akinete formation. Akinete abundance decreased again in the winter months up to 70 %, as also observed in the shallow polymictic Lake Melangsee by Rucker et al. (2009). The reasons for this may be loss processes, grazing by zooplankton organisms or potential infections of fungi or virus. Furthermore, freshly sedimented particles with associated akinetes can be

transported into deeper sediment layers by bioturbation of meiofauna or macrozoobenthos. Small spring inocula of akinetes are able to build important pelagic populations (Karlsson-Elfgren and Brunberg 2004).

### 2.5.3 Inoculum size of akinetes in sediments

Both the pool of viable akinetes in sediments and the pool of vegetative cells in the pelagic zone as starting populations need to be quantified in order to estimate the potential bloom formation of the Nostocales population. In this study, we considered the spatial distribution of akinetes in the sediments of different water depths and lake basin morphometry.

The highest akinete numbers in both lakes were presented in the sediment areas of the deeper water zones of each lake basin. In the shallow parts of the lakes, akinete abundances were lower but varied very strongly in magnitude. We assumed for deep stratified lakes that akinetes deposited in zones of unsuitable light and temperature for akinete germination and subsequent vegetative growth could not provide an inoculum for performing the next year's planktonic Nostocales population.

The scenario of the potential inoculum size of akinetes in sediments showed that light intensity in the water-column is crucial and temperature is of minor importance for akinete germination. The higher the germination requirements of the Nostocales species on high light intensities and temperature, the lower the inoculum size of the sedimentary akinete pool which could contribute to the pelagic population and vice versa. Kim et al. (2005) showed under laboratory conditions that akinete germination rates at low temperatures (5 and 10 °C) were high, and germination decreased with increasing temperature.

Sedimentary akinetes in regions deeper than approximately 9 m water depth were not used in calculating the potential inoculum size due to confined photosynthetic activity in the euphotic zone. Nevertheless, these akinetes deposited in deeper areas could contribute to the reconstruction of the Nostocales population or they can be used for early identification of the potential future population of new or invasive species. Akinetes of the invasive species *Cylindrospermopsis raciborskii*, for example, were detected in Lake Scharmützelsee in the deeper sediment regions (Figure 2.8, from 9 m water depth) and in sediment traps (located at 9 m and 27 m water depth above the deepest point RIE), while filaments of this species were so scarce in water samples that they were not found in the routinely determined phytoplankton.

Typically, vegetative cells of Nostocales are differentiating to akinetes as an overwintering strategy. However, holoplanktonic non-akinete forming species, such as *Aphanizomenon flos-aquae* in Lake Stechlinsee, have been previously described (see following chapter 4; Üveges et al. 2012) which have the advantage of being present in the pelagic zone throughout the year. However, these species can be easily replaced by other competitive species when growth conditions diminish in the pelagic zone.

In summary, in this study, we presented the spatial and seasonal distribution of akinetes in the sediments of two deep dimictic lakes affected by physical factors, for example, the morphometry of lake basins, sediment type and wind waves. The number of intact akinetes in the surface sediments of Lake Stechlinsee and Lake Scharmützelsee increased with water depth. The vertical sediment profile of Lake Stechlinsee led to the assumption that *Anabaena flos-aquae* and *Anabaena lemmermannii* were possibly already present 60 to 100 years previously. Akinetes of *Aphanizomenon flos-aquae* were hitherto not found in the sediments of Lake Stechlinsee. Viable akinetes of *Anabaena lemmermannii* and *Cylindrospermopsis raciborskii* were not present in the shallow polymictic Northern Bay of Lake Scharmützelsee, whereas they occurred, although not frequently, at all sediment sampling points in the deep dimictic Southern Basin. By contrast, the pelagic Nostocales biovolume showed no clear separation in either lake basin. A higher proportion of shallow water depth areas and a larger akinete pool were found in Lake Scharmützelsee. This resulted in a higher potential inoculum size in Lake Scharmützelsee than in Lake Stechlinsee.

Further studies on sediments in the littoral part are required to obtain a better overview of the influence of, for example, sediment type or macrophyte beds on akinete distribution in the sediments of deep lakes, and thus, to the potential inoculum. However, one main problem is the time-consuming counting of these resting stages in sediments with light-microscopy methods. Therefore, a new quantification technique was developed (chapter 3).

### 3. A CARD-FISH protocol for the identification and enumeration of cyanobacterial akinetes in lake sediments<sup>1</sup>

#### 3.1 Abstract

Akinetes are the dormant cells of Nostocales (cyanobacteria) that enable the organisms to survive harsh environmental conditions while resting in bottom sediments. The germination of akinetes assists the dispersal and persistence of the species. The assessment of the akinete pool in lake sediments is essential to predict the bloom formation of the Nostocales population. We present here the implementation of an improved Catalyzed Reporter Deposition (CARD) Fluorescence *in situ* Hybridization (FISH) protocol to assist and improve the identification and quantification of akinetes in sediment samples. Several 16S rRNA oligonucleotide probes were evaluated for labelling akinetes of various species of *Anabaena*, *Aphanizomenon* and *Cylindrospermopsis*. Akinetes of all the taxa studied were successfully labelled and could be easily detected by their bright fluorescence signal. The probes' specificity was tested with 32 strains of different taxa. All six *Cylindrospermopsis raciborskii* strains were labelled with a specific probe for its 16S rRNA. A more general probe labelled 73 % of the *Anabaena* and *Aphanizomenon* strains. The counting data of field samples obtained with CARD-FISH and the regular light microscopy approach did not differ significantly, confirming the suitability of both methods. The CARD-FISH approach was found to be less time-consuming due to a better visibility of akinetes.

#### 3.2 Introduction

Akinetes, which are differentiated resting cells produced by many species of filamentous, heterocystous cyanobacteria, enable the organism to survive adverse conditions, such as cold winters and dry seasons, and to maintain germination capabilities until the onset of suitable conditions for vegetative growth. Akinetes maintain a low level of metabolic activity, including photosynthesis, respiration and *de novo* synthesis of proteins and lipids (Rai et al. 1985; Sarma and Ghai 1998; Thiel and Wolk 1983), and can stay viable for decades (Livingstone and Jaworski 1980; Stockner and Lund 1970). They germinate in response to

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<sup>1</sup> Ramm, J., Lupo, A., Hadas, O., Ballot, A., Rucker, J., Wiedner, C. & Sukenik A. (2012) A CARD-FISH protocol for the identification and enumeration of cyanobacterial akinetes in lake sediments. *FEMS Microbiology Ecology* **82**, 23-36.



resuspension and improved environmental conditions, mainly light and temperature (Baker and Bellifemine 2000; Huber 1985; Kaplan-Levy et al. 2010; Karlsson-Elfgren et al. 2004).

Thus, akinetes provide a potential inoculum for Nostocales growth (Baker 1999). Baker (1999) monitored akinetes in the Murray River, Australia, to determine the extent of sporulation and contribution of recruitment from sediments. Rucker et al. (2009) showed for a shallow lake in north-eastern Germany, that the akinete “seed bank” in the sediment built up after the pelagic population maximum was reached, but unknown loss processes reduced the number of viable akinetes by 34-50 % during winter. Similar observations on seasonal patterns of akinete abundances in sediments were also reported from a small Korean reservoir by Kim et al. (2005). Apart from these few studies, we lack the quantitative knowledge required to evaluate and model akinete germination in the context of the Nostocales life cycle and successive bloom formation (Hense and Beckmann 2006, 2010; Jöhnk et al. 2011).

So far, the enumeration of akinetes in lake sediments has been performed using light microscopy mainly according to the method of Utermöhl (1958). Sedimentation chambers were filled with diluted sediment samples and the akinetes were counted with an inverse microscope (Head et al. 1999; Rucker et al. 2009; Suikkanen et al. 2010). Other counting methods using Fuchs-Rosenthal, Sedgewick-Rafter or a haemocytometer have been reported (Hori et al. 2003; Kravchuk et al. 2006; Tsujimura 2004). However, these methods have several shortcomings. Firstly, it is highly time-consuming and laborious to spot akinetes in the sediment sample because of their low abundance relative to sediment particles of various sizes and shapes. Secondly, in many cases, it is difficult to identify akinetes unambiguously, because they are very similar in size and shape to some benthic diatoms (e.g. *Navicula*) or to cysts of chrysophytes.

The overall aim of this study was to validate and implement a Catalyzed Reporter Deposition Fluorescence *in situ* Hybridization (CARD-FISH) technique to assist in the identification and enumeration of akinetes in sediment samples from lakes. Several oligonucleotide probes targeted at the 16S rRNA of Nostocales were applied and their specificity was examined with isolated akinetes and trichomes from 32 culture strains. Finally, CARD-FISH-assisted enumerations of akinetes in sediment samples were validated by comparative counts using the Utermöhl (1958) method.

### 3.3 Materials and methods

#### 3.3.1 Culture cultivation and sediment sampling

Various species of Nostocales were cultivated in BG11 (Stanier et al. 1971) or Z8 (Kotai 1972) medium in a 12:12 h light/dark cycle ( $80 \mu\text{E m}^{-2} \text{s}^{-1}$ ) at 20 °C. All the Nostocales species and strains used in this study are listed in Table 2. Isolated akinetes were prepared from three cultures of Nostocales species (*Aphanizomenon ovalisporum*, *Anabaena planctonica* and *Cylindrospermopsis raciborskii*). For the isolation of akinetes, 100 mM HCl was used to lower the pH of the cultures to 4.0 to destroy vegetative cells. Acidified samples were kept in darkness for 24 h at 4 °C until the akinetes were harvested. The remaining floating filaments and disintegrated cell debris were removed by aspiration or low-speed centrifugation. The akinetes gathered were washed and resuspended in fresh medium. Alternatively, akinetes were harvested from *Aphanizomenon ovalisporum* culture cultivated for three weeks in BG11 lacking potassium, as previously described (Sukenik et al. 2007).

Sediment samples were collected from Lake Kinneret (Sea of Galilee 32°47'N; 35°34'E), Israel, and from two lakes in NE Germany: Lake Scharmützelsee (52°14' N; 14°02' E) and Lake Stechlinsee (53°10' N; 13°02' E). Sampling was carried out during 2008 and 2009. At each site studied, sediment cores, were collected from the deepest point of the lake with Perspex tubes (50 mm diameter) using a Tessenow sampler (Tessenow et al. 1977) or an Uwitec corer (Uwitec, Mondsee, Austria). The sediment cores were sliced into 1 cm sections and corresponding layers of three cores were pooled and mixed to minimize heterogeneity at the sampling site. The samples were transported in dark boxes to the laboratory and then stored at 4 °C until further analysis. In addition, particulate material settling in the water column was collected by sediment traps installed 2-3 m above the bottom in Lake Kinneret. The trap consisted of four PVC tubes, 10 cm in diameter and 60 cm long. A bottle was screwed to the bottom of each tube in order to collect the settling sediment (Koren and Klein 2000). Sediment traps were replaced every 21-30 days by a diver.

#### 3.3.2 CARD-FISH method modification and optimization

The CARD-FISH method (Schönhuber et al. 1999) was adjusted to label akinetes of the Nostocales species using horse-radish peroxidase (HRP) conjugated primers designed for the short domain of the 16S rRNA of the target cyanobacteria. The method calls for five sequential steps: i) fixation, ii) permeabilization, iii) hybridization, iv) tyramid signal amplification (TSA), and v) visualization under fluorescence microscopy.

The steps of the protocol and various modifications examined during the method verification process are described in detail below and are schematically shown in Figure 3.1.

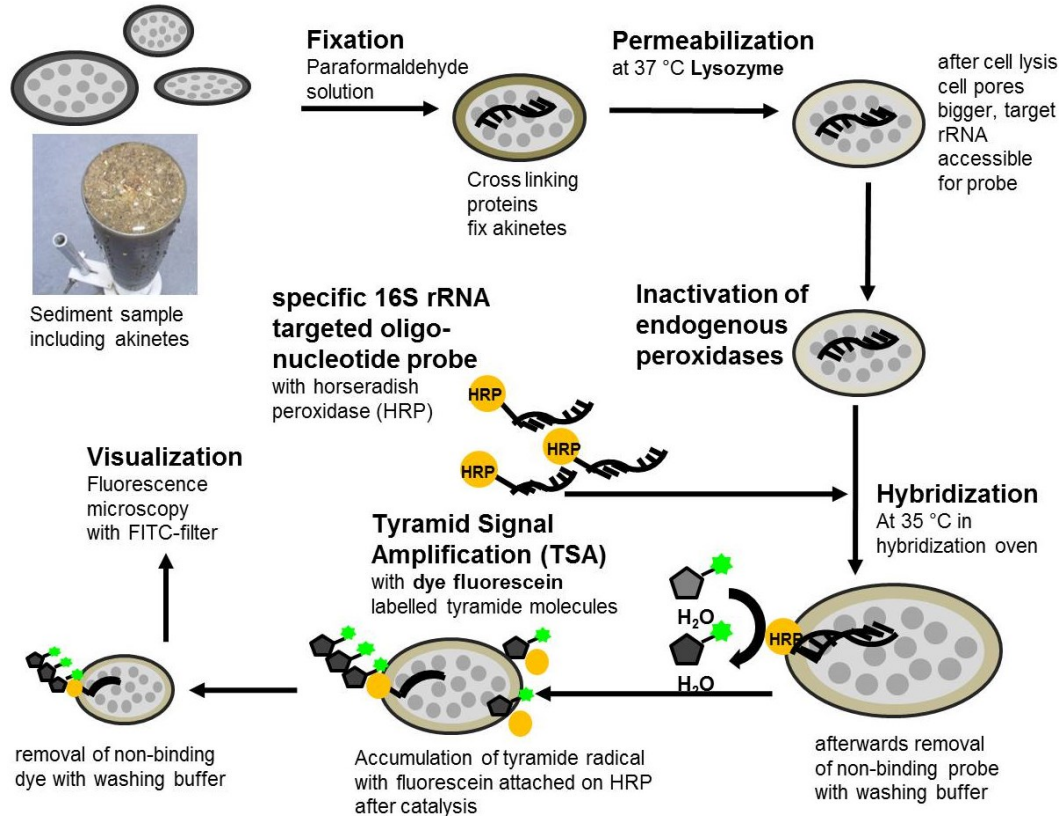


Figure 3. 1 A schematic presentation of the CARD-FISH-procedure and its adjustment for the enumeration of akinetes in sediments (see text for more details).

i) *Fixation* – A volume of 200  $\mu\text{l}$  of laboratory culture strain (ca.  $0.1\text{--}0.5 \mu\text{g chl mL}^{-1}$ ), an isolated akinetes suspension ( $\sim 250,000$  akinetes in 200  $\mu\text{l}$ ) or sediment sample diluted 1:1 in distilled  $\text{H}_2\text{O}$  (MQ; Millipore) was mixed with 600  $\mu\text{l}$  paraformaldehyde (4 % in PBS - Phosphate Buffer Saline; 130 mM NaCl, 10 mM sodium phosphate buffer, pH 8.4) and incubated at 4 °C for 2 h. After incubation, the formaldehyde solution was removed by centrifugation at 16,000 g for 5 min and the pellet was washed twice in PBS. Subsamples were finally suspended in 1 ml of 1:1 PBS:Ethanol mix and stored at -20 °C until further processing. The PBS:Ethanol fixed samples were sonicated in a Branson 5510 sonication bath (Branson, CT, USA) for 4 cycles of 10 min each. The samples were filtered with sterilized MQ water onto white polycarbonate membrane filters (pore size 0.2  $\mu\text{m}$ ; Whatman International Ltd, Maidstone, UK) supported by a GF/C filter (Whatman) applying gentle vacuum.

Dry filters were embedded in 35-40 °C low melting point agarose (Lonza, ME, USA or MetaPhor Bioproducts, Rockland, Maine, USA; 0.1 % (wt/vol)) in MQ water to avoid cell loss during cell wall permeabilization. Alternatively, an aliquot of a fixed suspension of filaments or isolated akinetes was spotted on a microscope slide and air-dried for at least 2 h. The slides or the air-dried agar-coated filters were subsequently dehydrated with a series of ethanol washes (50 %, 80 % and 96 % (vol/vol), 3 min each wash) and air-dried.

ii) *Permeabilization* – The filters/slides were incubated either in lysozyme solution or in proteinase K solution (10 mg lysozyme ml<sup>-1</sup> or proteinase K ml<sup>-1</sup>, 100 mM Tris-HCl pH 7.5 and 50 mM EDTA) at 37 °C for at least 60 min under low continuous shaking in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany). The incubation time was adjusted for the lysozyme treatment, as proposed for CARD-FISH of marine picoplankton (Pernthaler et al. 2002) and freshwater actinobacteria (Sekar et al. 2003). Afterwards, the lysozyme solution was removed by ethanol washing series, as described above, or proteinase K solution was removed by three washing steps with MQ water for 1 min, respectively. The samples were then treated for inactivation of endogenous peroxidases with either 10 mM HCl or 0.01 % H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature (RT) to reduce potential noise in TSA originating from internal peroxidases (Medina-Sanchez et al. 2005). Subsequently, the samples were washed twice in MQ water for 1 min and dehydrated with 96 % ethanol. Dried slides were kept at RT and dried filters were kept in -20 °C until further use.

iii) *Hybridization* – Samples were hybridized with HRP conjugated 16S rRNA targeted probe purchased from Thermo Fisher Scientific or Biomersnet (Ulm, Germany). Three oligonucleotide probes (Table 3.1) were used: a) NOSTOC probe for the genera *Nostoc*, *Aphanizomenon* and *Anabaena* (Rudi et al. 2000), b) APHA probe for *Aphanizomenon* (Rudi et al. 2000) and c) CYL probe for *Cylindrospermopsis raciborskii* (designed for this study). All probes are available at probeBase, <http://www.microbial-ecology.net/probebase/> (Loy et al. 2007). The HRP probe (either NOSTOC, APHA or CYL; 2 µl of 500 ng µl<sup>-1</sup>) was added to 2 ml of hybridization solution (900 mM NaCl, 20 mM Tris-HCl pH 7.5, 20 % (vol/vol) formamide, 0.01 % SDS (sodium dodecyl sulfate), MQ water) and applied to samples on slides or filters. The samples were placed in Petri dishes containing blotting paper dipped in hybridization buffer without the probe or filters, and were placed in a reaction tube with hybridization solution and incubated at 35 °C for at least 2.5 h (a higher hybridization temperature might harm the probe conjugated HRP; Pernthaler et al. 2001).

Samples were removed from the hybridization mixture and washed twice for 20 min each at 35 °C in pre-warmed washing buffer containing 145 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA, and 0.01 % SDS. The samples were exposed to hybridization buffer that did not contain any probe for control experiments.

iv) *Tyramide signal amplification (TSA)* – Hybridized samples were incubated in TNT buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Tween 20) at RT for 15 min to equilibrate the probe-delivered HRP. Subsequently, the samples were dabbed onto blotting paper to remove excess buffer and fluorophore tyramide working solution was applied onto each sample, following the manufacturer’s instructions, and incubated at RT for at least 10 min in the dark. The fluorophore working solution of the TSA<sup>TM</sup> Plus fluorescence system kit (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA) consisted of 1 part fluorophore tyramide stock solution and 50 parts 1x Plus amplification diluent. Samples were consecutively washed three or four times with TNT-buffer for 5 min and were air-dried. Samples were immediately stored at -20 °C for up to several days without any loss of fluorescence signal.

**Table 3. 1 Sequences of oligonucleotide probes used in this study.**

Probe	Accession no. <sup>a</sup>	Target organism(s)	Sequence of probe (5'-3')	Target <sup>b</sup> site (rRNA positions)	Reference
NOSTOC	pB-00244	<i>Nostoc</i> , <i>Aphanizomenon</i> , <i>Anabaena</i>	GCTCAACCAR <sup>1</sup> ATM <sup>2</sup> ARAGCA GTGGAAACTA	16S (616-644)	Rudi et al. (2000)
APHA	pB-00025	<i>Aphanizomenon</i>	CCCCTAGCTTT CGTCCCTCAGT GTCAGT	16S (748-775)	Rudi et al. (2000)
CYL	pB-02627	<i>Cylindrospermopsis</i> <i>raciborskii</i>	TAACAGCAGA CTTTCAGTTC ACCTGC	16S (580-606)	This study

<sup>a</sup> Oligonucleotide probe sequences from probeBase: <http://www.microbial-ecology.net/probebase/> (Loy et al. 2007)

<sup>b</sup> *Escherichia coli* numbering (Brosius et al. 1978)

<sup>1</sup> Sequence in IUPAC code R=G/A; <sup>2</sup> M=A/C

v) *Visualization under Fluorescence Microscopy* – Samples were embedded with Citifluor mountant (Citifluor Ltd., London, UK) and visualized using either a Zeiss Axioskop or a Zeiss Axioimager 2 epifluorescence microscope. The following filter sets were applied for a tyramide fluorescence signal: either Zeiss filter set 05 and GFP filter (Zeiss filter set 10) or FITC filter (Zeiss filter set 09). A HBO 100-W Hg vapour lamp, a 40x Plan Apochromat objective and an AxioCam for digital photos (Carl Zeiss, Jena, Germany) were used. Image analyses were processed with Zeiss software AxioVision. In terms of the evaluation of images, critical values were constituted for the intensity of the fluorescence signal: The signal was defined as “strong fluorescence” when akinetes were visible at an exposure time (ex-t) of 100 ms, “weak fluorescence” at 300 ms ex-t and “non-fluorescent” if no cells were observable at 600 ms ex-t.

### 3.3.3 Probe design and test of probe specificity with cultured strains

The CYL probe was designed for *Cylindrospermopsis raciborskii* based on the 16S rRNA sequence information of several Nostocales strains. The similarity of the NOSTOC and APHA probes with various *Anabaena* and *Aphanizomenon* strains was checked by sequence alignment. The specificity of the three probes (Table 3.1) was firstly examined using isolated akinetes of *Aphanizomenon ovalisporum*, *Anabaena planctonica*, and *Cylindrospermopsis raciborskii*.

In a second step, the specificity of the NOSTOC and CYL probes was tested with 32 different cyanobacterial strains. The success of labelling was investigated by image analysis using three different exposure times (as described above) to evaluate the intensity of the fluorescence signal.

### 3.3.4 Impact of sediment dilution on akinete enumeration

The samples were diluted with sterile distilled water before filtering on polycarbonate filters for the counting of akinetes in sediment. The dilution was aimed to improve the separation of akinetes from the bulk of sediment particles without affecting the estimation of akinete concentration in the original undiluted sample. Five dilution rates were tested: 1:10, 1:20, 1:50, 1:100, and 1:200 (vol/vol). Dilution experiments were carried out with a sediment sample taken from Lake Scharmützelsee (sampling procedure as described above). Akinetes were labelled with the NOSTOC probe and counted in triplicates always covering the same area of the filter.

### 3.3.5 Standard addition of akinetes into sediment samples

A standard addition (spiking) experiment was performed in order to verify the reliability of the enumeration of CARD-FISH labelled akinetes in sediments. Aliquots of 50 µl sediment sample (diluted 1:100) were mixed, prior to filtration, with different quantities of akinetes collected from laboratory cultures of *Aphanizomenon ovalisporum*. The number of akinetes that were mixed with the sediment aliquots ranged between 4,000 and 23,500. All samples were subjected to hybridization with the APHA probe and staining procedure. The number of CARD-FISH positive akinetes was determined by epifluorescence microscopy, and finally, the original number of akinetes added to each sample was plotted against the number of CARD-FISH positive akinetes.

### 3.3.6 Comparative counts with the CARD-FISH and Utermöhl method

We used sediment and sediment trap material from three lakes for the comparative counting of akinetes (as described above). For the CARD-FISH enumeration, three aliquots of each sediment sample were diluted 1:100 and filtered. Filters were further processed by the modified CARD-FISH protocol using the three probes. The akinetes were counted under a epifluorescence microscope at 400x magnification and covering a known area of the filter.

The CARD-FISH signal in samples from sediment traps was differentiated into akinetes with high-intensity fluorescence and akinetes with normal medium-low fluorescence. It is postulated that the high fluorescence akinetes are those with high functionality and those with a better ability to germinate, assuming that all the akinetes are permeable to the 16 S rRNA targeted oligonucleotide probe to the same extent.

For the enumeration according to Utermöhl (1958), aliquots of the sediment samples were diluted 1:100 with degassed tap water, fixed with Lugol's solution and put into the sedimentation chamber (HYDRO-BIOS, Kiel, Germany). The akinetes were counted under a Wild M40 or a Nikon Diaphot300 inverted light microscope at 400x magnification and covering a known area of the sedimentation chamber. Intact akinetes in which the cytoplasm was well labelled and visualized could be distinguished from akinetes in which only the envelope was slightly labelled but no cell content was observed. The akinetes of *Anabaena* and *Aphanizomenon* occurring in natural sediment samples could only be identified on a genus level, except for akinetes of *Anabaena lemmermannii* which are bean-shaped and appear in nests of up to 200 akinetes. Due to its high spatial variability, this species was counted separately.

### 3.3.7 Statistical analysis

Data was statistically analysed using unpaired two-sample t-test (two-tailed) for the comparisons of mean values obtained in the differently treated samples. A *P*-value of < 0.05 was considered as statistically significant.

## 3.4 Results

### 3.4.1 Method modification and optimization

The CARD-FISH method (Schönhuber et al. 1999) was modified and adjusted for the labelling of Nostocales cyanobacteria and their akinetes, firstly, qualitatively for laboratory cultures and then, quantitatively for field samples. The following modifications were examined using NOSTOC probe on sediment samples: Application of proteinase K versus lysozyme to enhance cell permeabilization and use of 0.01 mM HCl versus 0.01 % H<sub>2</sub>O<sub>2</sub> treatment to inactivate endogenous peroxidases. These modifications were compared quantitatively to negative controls of non-hybridization and non-dye samples. The permeabilization by proteinase K yielded a lower number of visible akinetes compared to the lysozyme treatment (Figure 3.2A) which was used for further analysis. A marked difference in akinetes counted was observed between hydrochloric- and peroxide-treated samples in an effort to inactivate endogenous peroxidases. Peroxide treatment led to the highest number of akinetes in sediment, but high variability among replicates was recorded (Figure 3.2B) and, therefore, not further used. All negative control experiments, those without hybridization process and those without TSA, showed very weak negligible unspecific fluorescence (data not shown).

Hybridization buffer was optimized by using different formamide concentrations and the final washing step was optimized (up to four washes). The concentration of 20 % formamide was found to be ideal for all probes applied in this study and three final washing post-TSA stages were adequate to remove excess fluorescein (not shown).



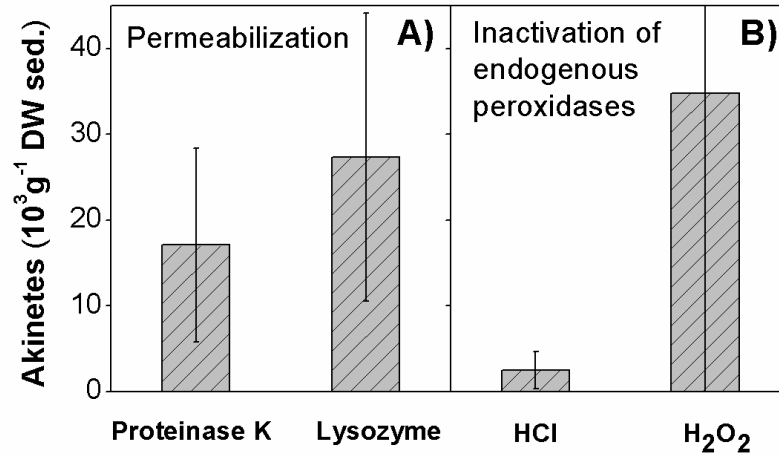


Figure 3. 2 Akinete yield after different modifications of the CARD-FISH protocol. (A) permeabilization with proteinase K or lysozyme and (B) inactivation of endogenous peroxidases with HCl or H<sub>2</sub>O<sub>2</sub>. Error bars indicate standard deviation (N = 3).

### 3.4.2 Probe design and test of probe specificity with culture strains

The alignment of the 16S rRNA sequences of various Nostocales strains and the three complementary oligonucleotide probes are shown in Figure 3.3. The CYL probe designed matches only to *Cylindrospermopsis raciborskii* sequences. The other two probes, NOSTOC and APHA, targeted other regions in the 16S rRNA and showed substantial homology with various *Aphanizomenon* and *Anabaena* species. Examples of CARD-FISH labelled isolated akinetes from cultures of *Aphanizomenon ovalisporum* (using APHA probe), *Anabaena planctonica* (using NOSTOC probe) and *Cylindrospermopsis raciborskii* (using CYL probe) are presented in Figure 3.4.

All akinetes showed easily observed strong fluorescence signals. The NOSTOC and CYL probes were tested for their specificity with a collection of Nostocales strains and some other non-Nostocales species (Table 3.2). The NOSTOC probe positively labelled most of the *Anabaena* and *Aphanizomenon* strains, but not the strains of *Cylindrospermopsis raciborskii*; 73 % out of the 22 *Anabaena* and *Aphanizomenon* strains tested were easily detected with that probe.

probes	580	590	600	610	620	630	640	650				
NOSTOC	GCTCAACCARATMARAGCAGTGGAAACTA											
APHA	GCAGGTGGAAGTCTGCTGTGTTA											
CYL	GCAGGTGGAAGTCTGCTGTGTTA											
<b>Anabaena</b>												
AB2008/45	GGTCT	CTA	T	AAGATCTA	T	TG		CAGGGCTA				
AB2010/02	GGTCT	CTA	T	AAGATCTA	T	TG		CAGGGCTG				
AB2010/03	GGTCT	CTA	T	AAGATCTA	T	TG		CAGGGCTG				
AB2008/56	GGTCC	C	T	AAGATTTG				CAGAGCTA				
AB2008/17	GGTCC	C	T	AAGATTTG				CAGAGCTA				
AB2008/22	GGTCC	C	T	AAGATTTG				CAGAGCTA				
AB2008/84	GGTCC	C	T	AAGATTTG				CAGAGCTA				
ST07-182	GGTCC	C	T	AAGATTTG				CAGAGCTA				
<b>Aphanizomenon</b>												
AB2008/16	GGTCC	C	T	AAGATCTA	GT			CAGAGCTA				
AB2008/18	GGTCC	C	T	AAGATCTA	GT			CAGAGCTA				
AB2008/19	GGTCC	C	T	AAGATCTA	GT			CAGAGCTA				
AB2008/21	GGTCC	C	T	AAGATTTG				CAGAGCTA				
AB2008/25	GGTCC	C	T	AAGATTTG				CAGAGCTA				
AB2008/31	GGTCC	C	T	AAGATTTG				CAGAGCTA				
AB2008/62	GGTCC	C	T	AAGATTTG				CAGAGCTA				
AB2008/65	GGTCC	C	T	AAGATTTG				CAGAGCTA				
SP08-33	GGTCC	T	G	AAGATCTA	GT			CACAACTA				
SP08-34	GGTCC	T	G	AAGATCTA	GT			CACAACTA				
ST07-115	GGTCC	C	T	AAGATTTG				CAGAGCTA				
AB2010/05	GGTCT	CCG	T	AAGATCTG	T			CAGGGCTA				
AB2010/06	GGTCT	CCG	T	AAGATCTG	T			CAGGGCTA				
<b>Cylindrospermopsis</b>												
AB2010/01	GGTCT			AAGATTTG	T		G	CAGAACTA				
AB2010/04	GGTCT			AAGATTTG	T		G	CAGAACTA				
<b>Anabaenopsis</b>												
AB2008/61	GGTCC	CCA	T	AAGATGAG	T	TC	G	CAGAGCTA				
<b>Others</b>												
DQ279771	CGTCC	T	CTG	TC	C	AAGACAGTG	T	CTG	G	GAAACAGCTA		
AY620239	CGTCC	T	TTTGTG			AAGACTGGA	T	TCCGG	G	GAAAACCTA		
DQ279771	CGTCC	T	CTG	TC	C	AAGACAGTG	T	CTG	G	GAAACAGCTA		
AF132790	CGTAC	T	C	CCAGCC	GG	C	AAGCCCGCA	CT	TGCGG	G	CC	GTTGGCTA
AY172834	CGTCC	T	C	CC	TC	AAAAGTGA	T	TCC	TG		GTTGGCTT	

probes	710	720	730	740	750	760	770	780		
APHA	ACTGACACTGAGGGACGAAAGCTAGGGG									
CYL	ACTGACACTGAGGGACGAAAGCTAGGGG									
<b>Anabaena</b>										
AB2008/45	SGAAGAACACCAGAGGCGAAAGCGCTCTACTAGGCCGCA							AGCGAATGGGA		
AB2010/02	SGAAGAACACCAGAGGCGAAAGCGCTCTACTAGGCCGCA							AGCGAATGGGA		
AB2010/03	SGAAGAACACCAGAGGCGAAAGCGCTCTACTAGGCCGCA							AGCGAATGGGA		
AB2008/56	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCGAG							AGCGAATGGGA		
AB2008/17	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
AB2008/22	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
AB2008/84	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
ST07-182	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCGAG							AGCGAATGGGA		
<b>Aphanizomenon</b>										
AB2008/16	SGAAGAACATCGGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
AB2008/18	SGAAGAACATCGGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
AB2008/19	SGAAGAACATCGGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
AB2008/21	SGAAGAACATCGGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
AB2008/25	SGAAGAACATCGGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
AB2008/31	SGAAGAACATCGGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
AB2008/62	SGAAGAACATCGGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
AB2008/65	SGAAGAACATCGGTGGCGAAGGCGCTCTGCTAGGCCAAG							AGCGAATGGGA		
SP08-33	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGACTGCT							AGCGAATGGGA		
SP08-34	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGACTGCT							AGCGAATGGGA		
ST07-115	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCGAG							AGCGAATGGGA		
AB2010/05	SGAAGAACACCAGTGGCGAAGGCGCTCTACTAGGCCGCA							AGCGAATGGGA		
AB2010/06	SGAAGAACACCAGTGGCGAAGGCGCTCTACTAGGCCGCA							AGCGAATGGGA		
<b>Cylindrospermopsis</b>										
AB2010/01	SGAAGAACACCAGTGGCGAAGGCGTTTTGCTAGACCCTA							AGCGAATGGGA		
AB2010/04	SGAAGAACACCAGTGGCGAAGGCGTTTTGCTAGACCCTA							AGCGAATGGGA		
<b>Anabaenopsis</b>										
AB2008/61	SGAAGAACACCAGTGGCGAAGGCGCTCTACTAGACCATA							AGCGAATGGGA		
<b>Others</b>										
DQ279771	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCGCA							AGCGAATGGGA		
AY620239	SGAAGAACATCGGTGGCGAAGGCGCTACTGGACCGAG					C		AGCGAAGGGA		
DQ279771	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCGCA							AGCGAATGGGA		
AF132790	SGAAGAACACCAGCGGCGAAGGCGCTTGTGGACCGCA					G	T	C	AGCAATGGGA	
AY172834	SGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCATC					G	C	T	C	AGCGAAGGGA

Figure 3. 3 Alignment of nucleotide sequences of the 16S rRNA in the binding region of probes NOSTOC (616-644 bp), APHA (748-775 bp) and CYL (580-606 bp) from *Anabaena*, *Aphanizomenon* and *Cylindrospermopsis raciborskii* strains used in this study (compare to Table 3.2). Dots represent the same nucleotides as in the probe.

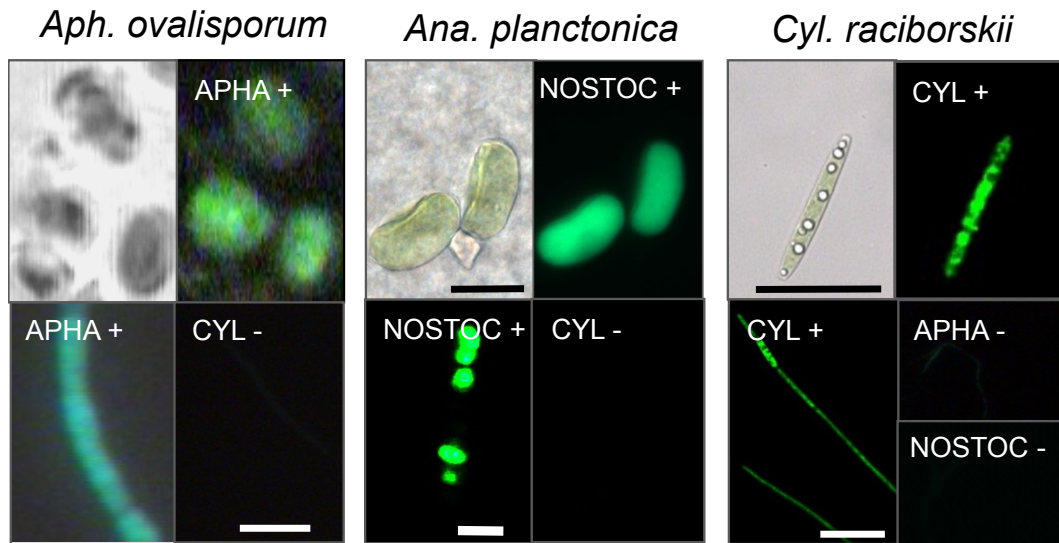


Figure 3. 4 Bright field and fluorescence micrographs of akinetes (upper panels) and fluorescence micrographs of filaments (lower panels) of *Aphanizomenon ovalisporum* (left), *Anabaena planctonica* (middle) and *Cylindrospermopsis raciborskii* (right) labelled with either APHA, NOSTOC or CYL probes (Labelled white letters). *Aphanizomenon ovalisporum* was not labelled with NOSTOC probe and *Anabaena planctonica* was not labelled with APHA probe. Pictures taken at magnification 400x with a light microscopy (pictures at top left) or epifluorescence microscopy and an exposure time of 100 ms; scale bar: 10  $\mu$ m.

No fluorescence signals were observed for two *Anabaena flos-aquae* strains and four *Aphanizomenon gracile* strains. The NOSTOC probe did not hybridize with any of the six *Cylindrospermopsis raciborskii* strains as they showed no fluorescence signals. The CYL probe hybridized with all *Cylindrospermopsis raciborskii* strains studied as an enhanced fluorescence signal was observed (Figure 3.4). The CYL probe also labelled 8 of 22 *Anabaena* and *Aphanizomenon* strains tested. The APHA probe was only tested with one strain of *Aphanizomenon ovalisporum* and one strain of *Cylindrospermopsis raciborskii*. The probe labelled *Aphanizomenon ovalisporum* but not *Cylindrospermopsis raciborskii* (Figure 3.4).

**Table 3. 2** Specificity of probes NOSTOC and CYL for various Nostocales strains. Two species of the order Chroococcales were used as controls.

Taxon	Strain	Geographic origin	NOSTOC <sup>a</sup>			CYL <sup>a</sup>		
			no <sup>b</sup>	weak	strong	no	weak	strong
<b><i>Anabaena</i></b>								
<i>A. bergii</i>	AB2008/45	Melangsee, GER	-	+	-	-	-	+
	AB2010/02	Lake Kinneret, ISR	-	+	-	-	-	+
	AB2010/03	Lake Kinneret, ISR	-	+	-	-	-	+
	ZIE26AB	Zierker See, GER	-	+	-	-	-	+
<i>A. circinalis</i>	ST08_15	Stechlinsee, GER	-	+	-	+	-	-
<i>A. crassa</i>	AB2008/56	Nehmitzsee, GER	-	+	-	-	-	+
<i>A. flos-aquae</i>	AB2008/22	Scharmützelsee, GER	+	-	-	+	-	-
	AB2008/84	Nehmitzsee, GER	+	-	-	+	-	-
<i>A. planctonica</i>	AB2008/24	Scharmützelsee, GER	-	-	+	+	-	-
	ST07_195	Stechlinsee, GER	-	-	+	+	-	-
<i>A. spiroides</i>	AB2008/55	Langer See, GER	-	+	-	-	-	+
<b><i>Aphanizomenon</i></b>								
<i>A. aphanizomenoides</i> <sup>c</sup>	22C4-9	Heiliger See, GER	-	-	+	-	-	+
<i>A. flos-aquae</i>	ST07_115	Stechlinsee, GER	-	-	+	+	-	-
	ST07_42	Stechlinsee, GER	-	-	+	-	-	+

**Table 3. 2 (continued) Specificity of probes NOSTOC and CYL for various Nostocales strains. Two species of the order Chroococcales were used as controls.**

Taxon	Strain	Geographic origin	NOSTOC <sup>a</sup>			CYL <sup>a</sup>		
			no <sup>b</sup>	weak	strong	no	weak	strong
<i>Aphanizomenon</i>								
<i>A. gracile</i>	AB2008/16	Scharmützelsee, GER	+	-	-	+	-	-
	AB2008/31	Scharmützelsee, GER	+	-	-	+	-	-
	AB2008/62	Langer See, GER	+	-	-	+	-	-
	AB2008/65	Melangsee, GER	+	-	-	+	-	-
<i>A. issatschenkoi</i>	SP08_33	Stolpsee, GER	-	+	-	+	-	-
	SP08_34	Stolpsee, GER	-	+	-	+	-	-
<i>A. ovalisporum</i>	AB2010/05	Lake Kinneret, ISR	-	+	-	+	-	-
	AB2010/06	Lake Kinneret, ISR	-	+	-	+	-	-
<i>Cylindrospermopsis</i>								
<i>C. raciborskii</i>	24G7	Kl. Werbellinsee, GER	+	-	-	-	-	+
	AB2010/01	Lake Kinneret, ISR	+	-	-	-	-	+
	AB2010/04	Lake Kinneret, ISR	+	-	-	-	-	+
	19F6	Melangsee, GER	+	-	-	-	-	+
	26D9	Rangsdorfer See, GER	+	-	-	-	-	+
	ZIE11CR	Zierker See, GER	+	-	-	-	-	+
<i>Anabaenopsis</i>								
<i>A. elenkinii</i>	AB2008/61	Langer See, GER	+	-	-	-	-	+

**Table 3. 2 (continued) Specificity of probes NOSTOC and CYL for various Nostocales strains. Two species of the order Chroococcales were used as controls.**

Taxon	Strain	Geographic origin	NOSTOC <sup>a</sup>			CYL <sup>a</sup>		
			no <sup>b</sup>	weak	strong	no	weak	strong
<b>Others</b>								
<i>Chroococcus sp.</i>	AB2008/113	Scharmützelsee, GER	+	-	-	-	+	-
<i>Microcystis aeruginosa</i>	49_018	unknown	+	-	-	-	+	-
	14_524	unknown	+	-	-	-	+	-

<sup>a</sup> probe (see sequences in Table 3.1)

<sup>b</sup> fluorescence signals - the signal was defined as “strong fluorescence” when akinetes were visible at an exposure time (ex-t) of 100 ms, “weak fluorescence” as akinetes were visible at 300 ms ex-t and “non fluorescent” if no cells were only observable at 600 ms ex-t or not observed at all.

<sup>c</sup> recently was reclassification to the genus *Sphaerospermum*: *Sphaerospermum aphanizomenoides* (Forti) Zapomělová, Jezberová, Hrouzek, Hisem, Rěháková et Komárková, comb. nov. (Zapomělová et al. 2009)

### 3.4.3 Impact of sediment dilution on akinete enumeration

The sediment samples were diluted in order to reduce the interference of sediment particles in the observation and counting of akinetes. Dilution factors, ranged between 10 and 200, were implied (Figure 3.5). The number of akinetes estimated was rather constant at dilution rates 1:100 and 1:200, but about two times higher than in lower dilution rates. High variability in the akinete number estimated in the original sediment sample was recorded at high dilutions, as the same area of the filter which presented only a fraction of the total filter area was counted in all dilutions. Increasing the area covered during the enumeration decreased the variability, as more akinetes were included in the counting. For further analysis of samples, a dilution of 1:100 was used and the filter area covered during the enumeration was adjusted to count between 100 and 200 intact akinetes.

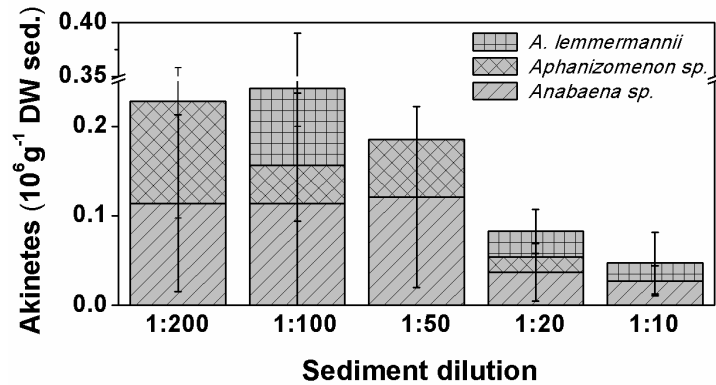


Figure 3. 5 The effect of sediment dilution on CARD-FISH akinete enumeration. A sediment sample taken from Lake Scharmützensee was diluted before filtering on polycarbonate filters. Akinetes were labelled with the NOSTOC probe followed by the CARD-FISH protocol and counted in triplicates covering always the same area of the filter. The estimated number of intact akinetes in each filtrated sample is presented as a function of the dilution rate. Error bars indicate standard deviation of triplicate counting.

#### 3.4.4 Verification of CARD-FISH enumeration by standard addition

The number of CARD-FISH positive akinetes in sediment samples amended with a known number was determined and plotted as a function of the number of akinetes added (Figure 3.6). The intercept of the line with the y-axis represents the number of akinetes in the original sediment sample. Sediment samples free of externally added akinetes (control) were labelled and counted using the same procedure and yielded very similar numbers of akinetes compared to the standard addition extrapolated value (5,500 and 5,100 akinetes, respectively).

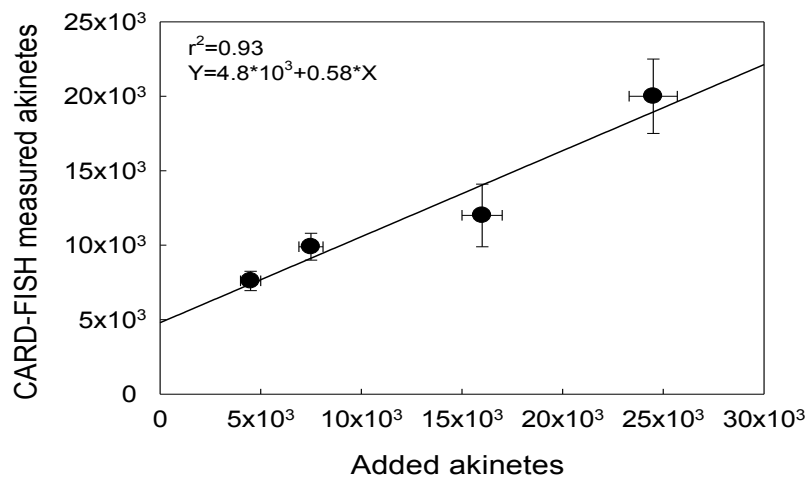


Figure 3. 6 Enumeration of *Aphanizomenon ovalisporum* akinetes in 1:100 diluted sediment samples of Lake Kinneret to which known amounts of isolated akinetes were added ("standard addition" or "spiking" experiment). All samples were subjected to hybridization with the APHA probe and staining procedure prior to enumeration under fluorescence microscope. A linear relationship between the number of added akinetes and counted akinetes is assumed ( $r^2=0.812$ ) and the intercept of the line with Y-axis represents the number of akinetes initially presented in the sample.

### 3.4.5 Comparative counts by the CARD-FISH and Utermöhl method

In comparison to the light microscopy approach, the application of CARD-FISH improves the visibility of akinetes in sediment samples due to their fluorescence, as demonstrated in Figure 3.7 for *Anabaena lemmermannii* and *Cylindrospermopsis raciborskii*.

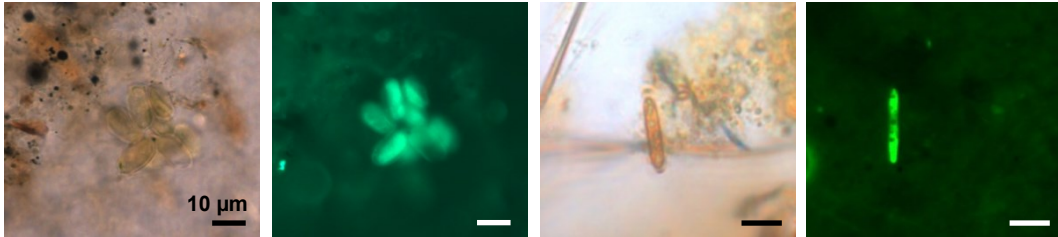


Figure 3. 7 Akinetes in lake sediments: (A) *Anabaena lemmermannii* of Lake Stechlinsee hybridized by NOSTOC probe under light microscopy and (B) the same object observed by fluorescence microscopy (C) *Cylindrospermopsis raciborskii* akinete in Lake Scharmützelsee labelled by CYL probe and observed using light microscope (D) the same object observed by fluorescence microscopy. Scale bar: 10 µm.

The CARD-FISH labelled akinetes are clearly observed with a defined fluorescence signal over a dark, homogeneous background. However, under light microscopy, the akinetes in sediment samples must be of an appropriate contrast in order to differentiate them from detritus particles, diatoms and chrysophyceae cysts over a bright heterogeneous background.

The numbers of akinetes determined with CARD-FISH and Utermöhl are in the same order of magnitude for all sediment samples (Figures 3.8 and 3.9). There is no general trend indicating higher or lower akinete numbers obtained with the CARD-FISH or Utermöhl method, as the following examples demonstrate. Counting the *Aphanizomenon ovalisporum* akinetes in sediments of Lake Kinneret by CARD-FISH protocol using APHA probe (Figure 3.8, upper panel) yielded a smaller (statistically insignificant  $P < 0.05$ ) mean value of total akinetes relative to the Utermöhl method ( $20 \cdot 10^6$  and  $36 \cdot 10^6$   $\text{g}^{-1}$  DW sediment, respectively). In sediment samples collected in Lake Stechlinsee, comparable numbers of total akinetes (average  $4 \cdot 10^6$   $\text{g}^{-1}$  DW sediment) were counted using both methods (Figure 3.8, middle panel), although we found considerable differences in the numbers of *Anabaena* sp. and *Anabaena lemmermannii* akinetes and envelopes (Figure 3.8). In the sediments of Lake Scharmützelsee, the mean total number of akinetes identified by CARD-FISH with NOSTOC and CYL probes was nearly equal to the values determined by the Utermöhl method (Figure 3.8, lower panel). *Anabaena* sp. akinetes represented the largest proportion of akinetes in Lake Scharmützelsee sediments and their abundance was clearly comparable using both



methods (an average of  $0.35 \cdot 10^6 \text{ g}^{-1}$  DW sediment). While no akinetes of *Cylindrospermopsis raciborskii* were detected in Lake Scharmützelsee sediments by the Utermöhl method, ca.  $0.25 \cdot 10^6$  akinetes  $\text{g}^{-1}$  DW sediment were detected by CARD-FISH.

Suspended material collected in sediment traps placed in Lake Kinneret was examined for the presence of akinetes of *Aphanizomenon ovalisporum* using the CARD-FISH protocol with APHA probe in comparison to the Utermöhl light microscopy method (Figure 3.9). In these samples, akinetes could be differentiated into cells with high fluorescence intensity (labelled with an asterisk in Figure 3.9) and akinetes with medium-low fluorescence (Figure 3.9). A similar seasonal trend was obtained by both CARD-FISH and Utermöhl methods, but no clear difference in the number of akinetes estimated by either method was found. In addition, empty envelopes of akinetes were observed in sediments as well as in sediment trap samples (Figures 3.8 and 3.9).

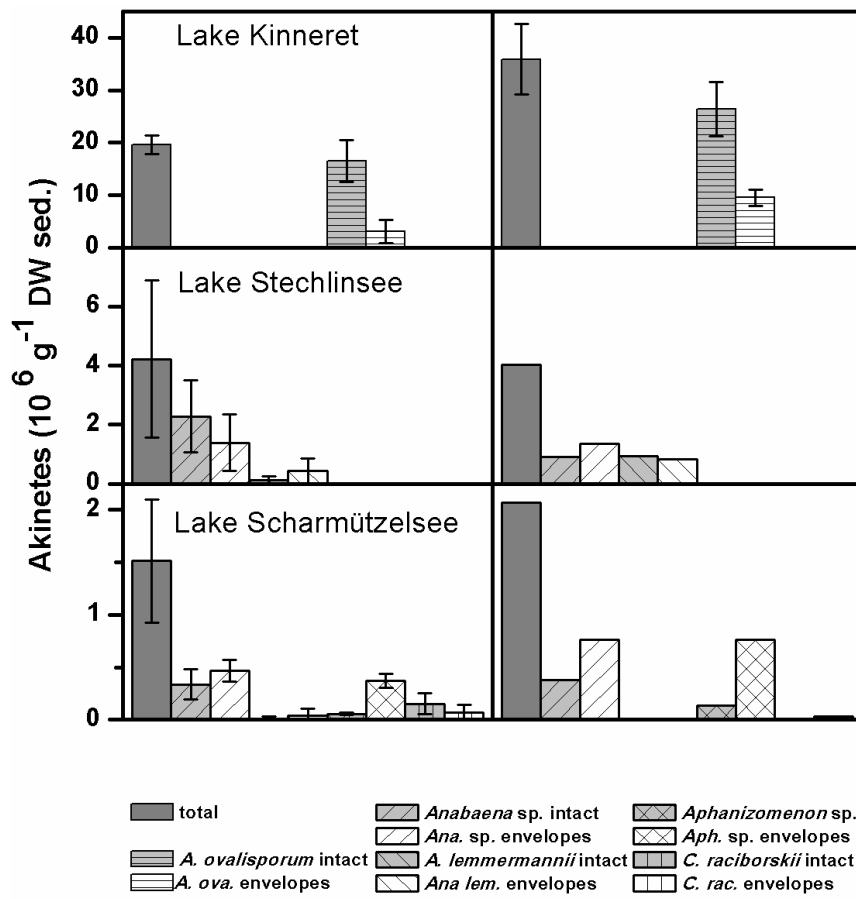


Figure 3. 8 Comparison of akinete numbers determined by the CARD-FISH (left) and the Utermöhl (right) method in sediment samples of the three studied lakes. Error bars indicate standard deviation of triplicate counting.

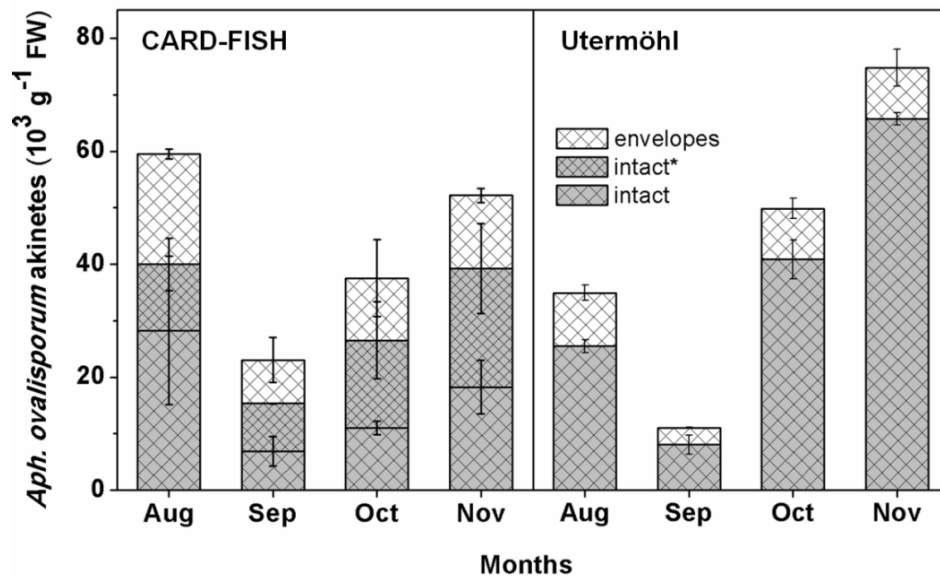


Figure 3. 9 Comparison of akinete numbers determined by the CARD-FISH (left) and the Utermöhl (right) method in sediment traps deposited in Lake Kinneret from August – November 2008. Asterisk represents high intensity fluorescence. Error bars indicate standard deviation (N = 3)

The time and costs comparison of CARD-FISH versus the Utermöhl method showed that CARD-FISH is time-consuming concerning the handling time of protocol steps and more cost intensive (Table 3.3). However, the advantage of the protocol was to process simultaneously a batch of 24 samples in less than 10 h and the microscopic counting of 1.5 h was less in time than Utermöhl (4 h). In total, the working time of 2 h for one CARD-FISH-applied sediment sample was two times lower than for one sample worked with the Utermöhl method.

**Table 3. 3 Time and cost expenditure of CARD-FISH and Utermöhl method used for visualization and enumeration of Nostocales akinetes in Lake's sediment samples**

<b>CARD-FISH protocol (for a batch of 24 samples)</b>		
Steps	Handling time	Reaction time
i) Fixation	4 h	2 h
ii) Permeabilization	1 h	1.5 h
iii) Hybridization	3.5 h	2.5 h
iv) Tyramide signal amplification	1 h	25 min
v) Microscopical counting (for 24 filters)	36 h	
Total working time (for 24 samples)	45.5 h	
(for 1 sample)	~ 2 h	
Costs	~10 € for 24 samples or 0.42 € per sample	
	Lower cost in manpower due to shorter observation time	
<b>Utermöhl (for one sample)</b>		
Steps	Handling time	Sedimentation time
Sample preparation	5 min	3 h or longer depending on volume
Microscopical counting (for one sedimentation chamber)	4 h	
Total working time (for 1 sample)	~ 4 h	
Costs	Low cost in expendables but high cost in manpower	

### **3.5 Discussion**

In this study, we successfully validated and implemented CARD-FISH to identify and enumerate cyanobacterial akinetes in sediment samples.

#### **3.5.1 Method modification**

The outer cell wall of cyanobacteria consists of a peptidoglycan layer, which is in most cases thicker than in other gram-negative bacteria (Stanier and Cohen-Bazire 1977). The cell wall of matured akinetes is very robust and comprises different layers with portions of electron-dense substance (Jensen and Clark 1969). Therefore, a successful fixation and permeabilization of akinetes was required to make cells accessible for the large marker molecule HRP of 40 kDa, which increases with linked molecules such as oligonucleotides (Pernthaler et al. 2002 Schönhuber et al. 1999). Samples that were not treated with lysozyme showed a lower detection level, as indicated by Pernthaler et al. (2002). No positive effect of proteinase K on cell permeability was observed in our study, as also previously reported by Schönhuber et al. (1999). Negative controls without probe yielded negligible fluorescence signals. The embedding of sediment subsamples in low melting point agarose on polycarbonate filters was found to be crucial to avoid cell loss during the permeabilization procedure. Unspecific binding of excess probes and dye to sediment particles were overcome by additional washing steps after hybridization and TSA.

The potential advantage of the CARD-FISH application could arise from the intense labelling of ribosomes. The reporter deposition procedure assures that even relatively old akinetes from deeper sediment layers, in which the ribosome content has presumably decreased, will be visualized and detected. It is important to note that in some cases, fluorescence signals of envelopes of empty akinete were observed, presumably due to nonspecific adsorption of residues of 16S rRNA to the inner cell wall. However, only viable, intact akinetes (as opposed to empty envelopes) are important for the estimation of Nostocales recruitment and bloom formation and the CARD-FISH is an appropriate detection method.

#### **3.5.2 Probe specificity**

The NOSTOC probe and the CYL probe designed are located in the variable region V7, which is the most informative sequence in the 16S rRNA region (Rudi et al. 2000). Thereby, the NOSTOC probe includes a few wobble bases to hybridize with different species of *Nostoc*, *Anabaena* and *Aphanizomenon* spp. The APHA probe is located in a conserved region of the 16S rRNA gene, which we assumed not to be specific enough to distinguish between *Aphanizomenon* and

*Anabaena* spp. Since no *Anabaena* sp. was detected in Lake Kinneret, we concluded that the APHA probe could be useful for the detection of *Aphanizomenon* akinetes in the sediments of Lake Kinneret.

The CYL probe was suitable to label all *Cylindrospermopsis raciborskii* strains studied, nevertheless, false positive fluorescence signals were also observed in two *Aphanizomenon* strains and six *Anabaena* strains from a total of 22 strains. However, false positive counts in field samples can be excluded due to the clearly different morphology of *Cylindrospermopsis raciborskii* akinetes. Moreover, according to the specificity test made on a *Cylindrospermopsis raciborskii* strain from Lake Kinneret with APHA probe, no false positive signal was observed.

With the NOSTOC probe, 73 % of the *Anabaena* and *Aphanizomenon* strains studied were correctly labelled. Therefore, the NOSTOC probe bears the risk of underestimating akinete abundance by 27 %. However, the problem of underestimation also applies when using the light microscopy approach, as discussed below. The lower specificity of the probe might be due to wobble bases that were needed to cover a wide range of different taxa.

### **3.5.3 Method validation**

The CARD-FISH enumeration approach was validated by a standard addition experiment that showed a good recovery rate of known amounts of added akinetes in sediment samples. Additionally, the comparative counts with CARD-FISH and Utermöhl clearly showed akinete numbers in the same order of magnitude, which proves that CARD-FISH is a reliable approach for akinete enumeration.

### **3.5.4 Potentials and Limitations of CARD-FISH versus the Utermöhl method**

Akinetes are hard to distinguish from their background under light microscopy and the enumeration process is rather slow. The clear advantage of the CARD-FISH method is the excellent visibility of akinetes due to their bright fluorescence. This simplifies the detection and allows for much faster counting of akinetes.

The specific fluorescent labelling of akinetes also prevents false positive counts of other sediment particles or organisms of similar size and shape (e.g. diatoms), which can lead to overestimation when using ordinary light microscopy. False positive counts of *Cylindrospermopsis raciborskii* akinetes that are theoretically possible, as the CYL probe also labelled some of the *Anabaena* and *Aphanizomenon* strains studied, can be excluded because of the clear morphological differences between the species' akinetes. The CARD-FISH

method bears the risk of underestimation of *Anabaena* and *Aphanizomenon* akinetes because the NOSTOC probe did not hybridize with 27 % of the strains studied. However, the comparative counting with CARD-FISH and Utermöhl did not reflect a general trend of 27 % lower numbers of *Anabaena* and *Aphanizomenon* akinetes detected with CARD-FISH.

Therefore, it might be possible that the number of akinetes were underestimated with Utermöhl as well due to overlapping sediment clumps and distracting particles which can obscure the view of akinetes. Nevertheless, the comparison between the Utermöhl and CARD-FISH methods does not show a clear trend. In some cases, the Utermöhl method yielded higher values, whereas in other samples, the CARD-FISH assisted enumeration gave a higher akinete number. In all cases, these differences appeared to be statistically insignificant by t-test.

The counting error of both methods was found to be relatively high. High counting errors were also found in other studies using different light microscopy approaches (e.g. Kravchuk et al. 2006; Rucker et al. 2009). The reason for this is the relatively low abundance of akinetes (“the signal”) compared with the highly abundant sediment particles of various shapes and sizes (“the noise”). Of course, the counting error in both methods could be reduced by enlarging the counting area on the filter or in the chamber, though this would also become more time-consuming, especially when using the Utermöhl method.

In summary, we successfully adjusted a CARD-FISH protocol that allows for an easier detection and a less time-consuming enumeration of akinetes in sediments in comparison to the Utermöhl method. The time expenditure for akinete enumeration per sample is twice as long with Utermöhl (4 h) compared to CARD-FISH (2 h) when including all procedure steps (Table 3). The shorter handling time of the CARD-FISH procedure is attributed to the clear fluorescence signal, which undoubtedly makes the akinete identification step easier and faster than in the regular light microscopy approach with untreated samples.

## 4. Overwintering strategies of Nostocales (cyanobacteria) in lakes in Northern Germany

### 4.1 Abstract

Nostoclean cyanobacteria form akinetes (differentiated, thick-walled cells) that arise from vegetative cells and function as resting cells. In temperate regions, akinetes are dormant forms that ensure overwintering in sediments and germinate under favourable environmental conditions in spring, while in the Baltic Sea some Nostocales species overwinter as vegetative filaments. The aim of this study was to observe genus- or species-specific differences in the overwintering strategies of Nostocales in two deep dimictic lakes (oligo-mesotrophic Lake Stechlinsee and mesotrophic Scharmützelsee) and one shallow polymictic lake (eutrophic Lake Melangsee) in Northern Germany. Due to the low abundance of vegetative filaments in the pelagic zone in winter, we used “*standard samples*” and additionally “*enriched samples*” by plankton net to enumerate filaments. Akinete abundance was detected in the “*sediment samples*” of the benthic zone. Filaments of *Anabaena* spp. degraded in the pelagic zone in autumn and higher akinete abundances were detected at the sediment surface in all the lakes. The first planktonic filaments of *Anabaena* spp. were observed in the spring. *Aphanizomenon flos-aquae* filaments were noticed all year round in the pelagic zone of Lake Stechlinsee, but akinete formation was not observed. By contrast, *Aphanizomenon* spp. formed akinetes in Lake Scharmützelsee and Lake Melangsee and no or very few filaments were observed in winter. Consequently, *Aphanizomenon* spp. showed another overwintering strategy contrary to the exclusively dormant overwintering strategy of *Anabaena* spp.

### 4.2 Introduction

Cyanobacteria of the order Nostocales are able to produce two types of specialized cells i) heterocysts to fix dissolved atmospheric nitrogen, and ii) akinetes, differentiated, thick-walled cells that arise from vegetative cells and function as resting cells to resist adverse conditions. The most common and best documented form of the life cycle of Nostocales in lakes of temperate regions is the dormant overwintering by akinetes on the sediment surface with akinete germination in spring under favourable light and temperature conditions and subsequent pelagic growth (Hense and Beckmann 2006; Kaplan-Levy et al. 2010).

In comparison to the dormant strategy, Suikkanen et al. (2010) observed different overwintering strategies of Nostocales in the Baltic Sea: *Aphanizomenon flos-aquae* overwintered as a holoplanktonic taxon exclusively vegetatively as

filaments in the water-column despite the presence of akinetes in sediments. The genus *Anabaena* spp. overwintered as akinetes on the sediment surface and was planktonic exclusively during the warmest season in summer. Both akinete formation and overwintering filaments were found for *Nodularia spumigena*. Furthermore, *Cylindrospermopsis raciborskii*, an invasive species of tropical origin, is known to be found the whole year round in the tropics (Padisák 2003), but contrarily forms akinetes for overwintering and as potential for the following planktonic population growth in spring in the temperate zone (Rücker et al. 2009).

Limited results are hitherto available on the vegetative overwintering of Nostocales in the pelagic zone of lakes (Üveges et al. 2012; Wildman et al. 1975). One reason for the lack of knowledge could be the low abundance or sporadic incidence of Nostocales in winter, indicating that routine enrichment of phytoplankton samples according to the Utermöhl method (1958) in sedimentation chambers (Nixdorf et al. 2010) may not suffice for detecting Nostocales filaments in winter.

The aim of this study was to clarify if species- or genus-specific differences in the overwintering strategy of nostocalean cyanobacteria occur in the lakes studied. We studied the occurrence and temporal development of vegetative cells and resting stages of the genera *Anabaena* and *Aphanizomenon* spp. in the pelagic zone and in the sediments of two deep lakes and one shallow lake in Northern Germany. Additionally, we used a more efficient enrichment of winter plankton samples which could contribute to a better detection of overwintering Nostocales filaments in the pelagic zone.

### **4.3 Materials and Methods**

#### **4.3.1 Study sites**

We chose three substantially different natural lakes in the lowlands of north-eastern Germany: the deep, dimictic oligo-mesotrophic Lake Stechlinsee (ST), the deep, dimictic mesotrophic Lake Scharmützelsee (SCH) and the shallow, polymictic eutrophic Lake Melangsee (MEL). All three are surrounded by forest mainly composed of pine and beech trees and the lakes are partly ice- and snow-covered in the winter. Their locations, dimensions, mixing regime, and trophic states are given in Table 4.1 and bathymetric maps are shown in Figure 4.1.

Furthermore, the morphometric and trophic specifications of Lake Scharmützelsee and Lake Stechlinsee are described in chapter 2.3.



More detailed information of Lake Stechlinsee is presented in Casper (1985) and Koschel et al. (2002), of Lake Scharmützelsee in Christen et al. (2007) and Grüneberg et al. (2011), and of Lake Melangsee in Nixdorf and Deneke (1997), Schmitt and Nixdorf (1999), Wiedner et al. (2007), and Rucker et al. (2009).

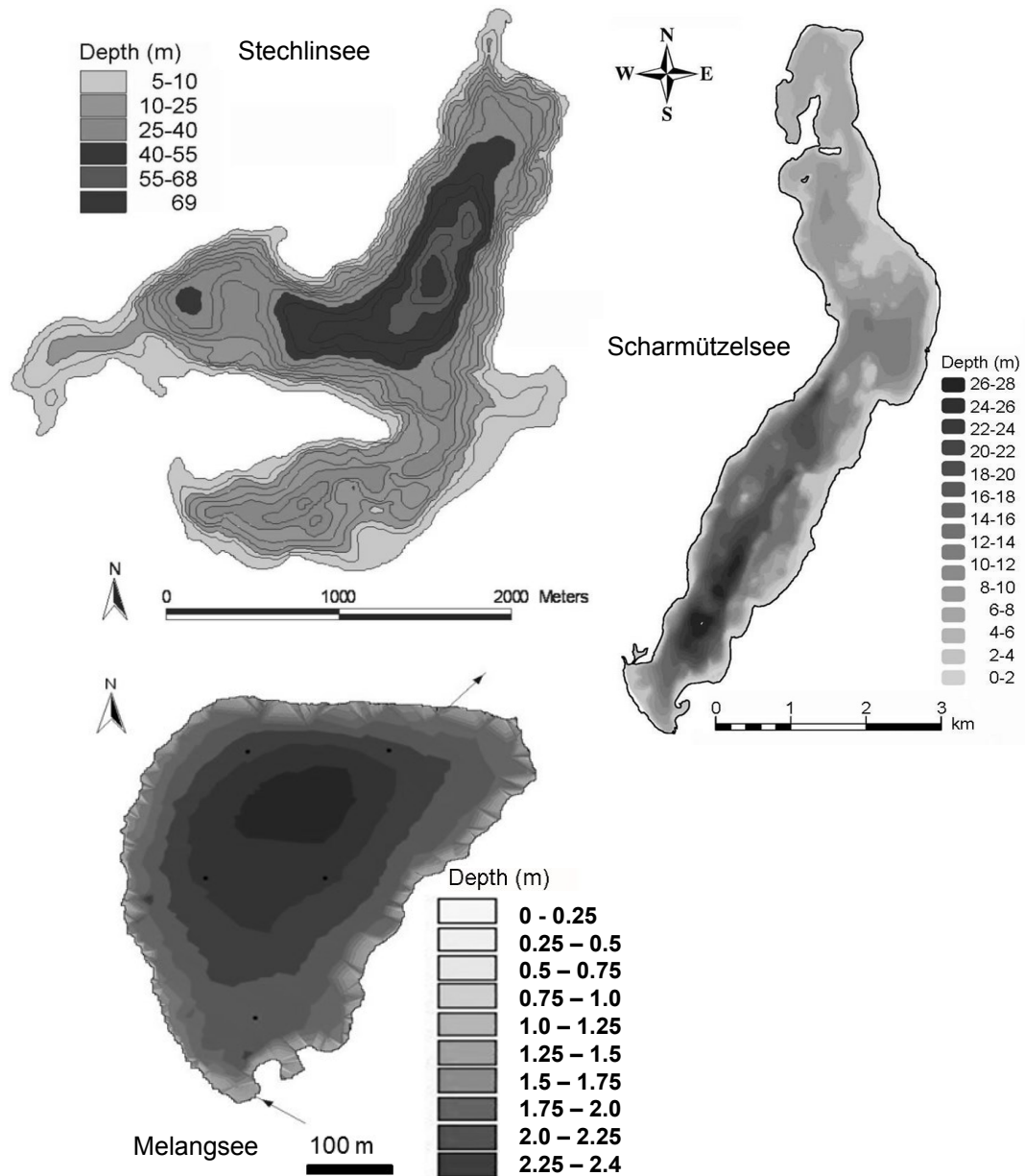


Figure 4. 1 Bathymetric maps of the lakes studied. Sampling sites were located at the deepest points of the lakes ( $Z_{\max}$  in Table 4.1).

**Table 4.1** Limnological characteristics of the lakes studied.

Lake	Geographical Position	Z <sub>max</sub> (m)	Z <sub>mean</sub> (m)	Area (km <sup>2</sup> )	t	CA (km <sup>2</sup> )	Mixis	Trophic state
Stechlinsee	53°10' N; 13°02' E	69.5	22.8	4.25	> 40 y <sup>a</sup>	26	mo <sup>b</sup> -di	oligo- mesotrophic
Scharmützelsee	52°14' N; 14°02' E	29.5	9	12.09	16 y	110	di	mesotrophic
Melangsee	52°09' N, 13°59' E	2.4	1.6	0.12	67 d	4	poly	eutrophic

Abbreviations: Z<sub>max</sub>: maximum depth, Z<sub>mean</sub>: mean depth, t: theoretical retention time in days (d) or years (y), CA: catchment area, mo: monomictic, di: dimictic, poly: polymictic

<sup>a</sup> according to Casper (1985)

<sup>b</sup> in some warm years monomictic (Koschel et al. 2002)

The most abundant Nostocales species in the pelagic zone of Lake Stechlinsee are *Aphanizomenon flos-aquae*, *Anabaena flos-aquae* and *Anabaena lemmermannii* (Salmaso and Padisak 2007; Üveges et al. 2012). *Aphanizomenon gracile*, *Aphanizomenon flos-aquae* and various *Anabaena* spp. are regularly observed in Lake Scharmützelsee (Nixdorf et al. 2003). The Nostocales community of Lake Melangsee is dominated by *Aphanizomenon gracile*, *Anabaena macrospora* and *Cylindrospermopsis raciborskii* (Rücker et al. 2009).

### 4.3.2 Sampling

#### Pelagic zone

The lakes were sampled monthly or fortnightly at the deepest point between 2005 and 2011. The transparency of the lakes was estimated with a Secchi disc. The water in Lake Melangsee was sampled with a 2.3 L Limnos sampler (Turku, Finland) at 0.5 m intervals (0-2 m), while Lake Scharmützelsee and Lake Stechlinsee were sampled every 1 m (epilimnion at thermal stratification and pelagic zone at full circulation; SCH 0-27 m, ST 0-20 m) and mixed in a barrel. Subsamples were fixed with Lugol's solution for phytoplankton analysis ("standard samples"). Other subsamples were taken to analyse the total nutrients (TN, TP) spectro-photometrically according to standard methods (DEV, 1976-2013). Chlorophyll *a* fluorescence in the water-column was measured by using the

FluoroProbe (bbe-Moldaenke, Kiel, Germany). The multi-channel probe gives the proportion of five different spectral algae groups based on their content of chlorophyll.

From November 2009 to April 2010, and in Lake Scharmützelsee until April 2011, additional water samples (total quantity MEL 8 L, SCH 30 L and ST 20 L) were taken and Nostocales filaments were enriched by filtration over a plankton net (25 µm) yielding 100 ml sample volume (*“enriched samples”*). Cyanobacteria were fixed with Lugol’s solution or formaldehyde (final concentration 4 %). Vertical profiles of temperature and light were recorded (details in chapter 2.3, paragraph light and temperature conditions), and mean temperature and mean light availability were calculated according to Hilt et al. (2010).

### **Benthic zone**

The benthic zone of Lake Melangsee was sampled once in 2009, Lake Scharmützelsee quarterly and Lake Stechlinsee monthly in 2010. Sediments were collected using a gravity corer (Uwitec, Mondsee, Austria) with Perspex tubes (60 mm Ø) at the deepest points of the lakes. Undisturbed surface sediments (0-1 cm) of three cores were pooled and homogenized (*“sediment samples”*). Aliquots for akinete enumeration were fixed with formaldehyde (final concentration 4 %). Dry weight (DW) and loss on ignition (LOI) were estimated after drying at 105 °C for 8 h and combustion at 500 °C for 3 h, respectively.

#### **4.3.3 Microscopic analysis**

The Utermöhl technique (1958) was applied for microscopic estimation of Nostocales abundances and biovolume. Magnification was chosen appropriately regarding the guidelines of CEN TC 230 (2003).

A 10 ml sample volume of *“enriched samples”* were used to determine the number of Nostocales filaments as distribution units and filament-attached akinetes in the pelagic zone during winter months, while biovolume was estimated in a 10 ml sample volume of *“standard samples”*. *Aphanizomenon flos-aquae* regularly occurred in Lake Stechlinsee as filaments in bundles, contrarily to Lake Scharmützelsee (own observation). Single filaments were counted due to disintegrated aggregation with Lugol’s solution.

The benthic akinete pool was assessed from *“sediment samples”* after dilution in 3 ml sample volume. *“Sediment samples”* from Lake Scharmützelsee and Lake Melangsee were diluted 100-fold with degassed tap-water and samples from Lake Stechlinsee 225-fold. An akinete was microscopically assigned as *“intact”* when cytoplasm was clearly visible.

#### 4.3.4 Data analysis

The detection limit was calculated according to CEN TC 230 (2003) and was essentially reduced by “*enriched samples*”, as described in the following section. Square root transformation of biovolume data in “*standard samples*” was applied to reduce the weight of the overwhelming importance of the Nostocales population in summer. The akinete abundance in “*sediment samples*” was calculated per g DW sediment.

#### 4.3.5 Detection limit of overwintering filaments

We intended to concentrate the planktonic filaments and attached akinetes in “*enriched samples*” due to their low abundance in winter. Water was passed through a plankton net of 25 µm mesh size for “*enriched samples*”. This sample-handling reduced the detection limit essentially from more than 5,600 filaments L<sup>-1</sup> in “*standard samples*” to 22 distribution units L<sup>-1</sup> in Lake Scharmützelsee, 26 distribution units L<sup>-1</sup> in Lake Stechlinsee and 53 distribution units L<sup>-1</sup> in Lake Melangsee in “*enriched samples*”.

Various lake detection limits are caused by different degrees of enriching the samples. The 80-fold or 300-fold “*enriched samples*” of winter plankton made it possible to detect filament or attached akinete abundance of 4 units L<sup>-1</sup> which cannot be detected by observing “*standard samples*”. Karlsson-Elfgren and Brunberg (2004) suggested a minimum of 1,000 cells L<sup>-1</sup> to gain reliable results for abundance estimates in pelagic sampling. Thus, occasionally occurring filaments were registered with our method.

Nevertheless, we are also dealing in this method, with problems of the ability to capture and to consider objectively all the filaments in the water-column. Free intact akinetes could not be analysed in the “*enriched samples*” due to the similar size of the akinetes and the mesh openings. In a further study, a sedimentation method with high water volume enrichment could be used to take all filaments and akinetes into consideration.

### 4.4 Results

#### 4.4.1 Characteristics and ice cover of lakes

The lakes studied showed relevant differences in their morphology and hydrology (Figure 4.1 and Table 4.1). The ice cover of lakes was observed in the years 2005-2011 (Figure 4.2 to 4.7). Lake Melangsee demonstrated an annual ice cover and Lake Scharmützelsee had no ice cover in the winters of 2006-2007 and 2007-2008. The ice cover of Lake Stechlinsee was first investigated in 2009.

During the “*enriched sample*” programme in the winter of 2009-2010, all the lakes studied had a closed ice cover until March, although the duration varied slightly between the lakes. The ice cover of Lake Stechlinsee lasted continuously for 63 days (January-March) and Lake Scharmützelsee for 74 days (January-March). Lake Melangsee was completely covered by ice for 88 days from December till March. In the winter of 2010-2011, the ice cover of Lake Melangsee lasted continuously for 100 days and Lake Scharmützelsee was covered in two phases for a total of 70 days (both lakes, December-March) and Lake Stechlinsee was covered for 14 days (February-March).

The Secchi depth, chlorophyll *a* and nutrient concentrations are given as annual means and means of the vegetation period (April-October) of the years 2005-2011 in Table 4.2. Lake Stechlinsee presented the highest light availability with a mean Secchi depth of 7.5 m and showed the lowest chlorophyll *a* concentration of 3  $\mu\text{g L}^{-1}$ . The Secchi depth in Lake Scharmützelsee reached 3.2 m with corresponding chlorophyll *a* concentrations of 10  $\mu\text{g L}^{-1}$ , whereas in Lake Melangsee, light penetrated up to 1.2 m and the annual mean chlorophyll *a* concentration was 43  $\mu\text{g L}^{-1}$ . The development of phytoplankton was reflected in the concentrations of total phosphorus (TP) and total nitrogen (TN).

**Table 4. 2 Annual means (left column) and means of vegetation period (April-October; right column) of physical and chemical variables of the lakes studied in the sampling years 2005-2011.**

Lakes	SD (m)		Chl a ( $\mu\text{g l}^{-1}$ )		TP ( $\mu\text{g l}^{-1}$ )		TN ( $\mu\text{g l}^{-1}$ )	
Stechlinsee <sup>a</sup>	7.5	6.6	3.2	3.1	13.9	13.0	400.8	407.1
Scharmützelsee	3.2	2.8	10.0	11.1	36.1	23.4	657.1	601.5
Melangsee	1.0	0.8	42.6	48.9	42.0	47.6	811.2	839.6

Abbreviations: SD: mean Secchi depths, Chl*a*: chlorophyll-*a*, TP: total phosphorus, TN: total nitrogen, annual mean – left column, mean of vegetation period – right column, <sup>a</sup> Means of the years 2007-2011.

#### 4.4.2 Overwintering population of Nostocales in the pelagic and benthic zone

*Anabaena flos-aquae* and *Aphanizomenon flos-aquae* were the main representative species of Nostocales in Lake Scharmützelsee and Lake Stechlinsee during the investigation period, whereas in Lake Melangsee, *Anabaena macrospora* dominated the Nostocales community. *Aphanizomenon gracile* and *Cylindrospermopsis raciborskii* were frequently observed in all lakes. Therefore, lake comparison was focused on the genera *Anabaena* and *Aphanizomenon* which occurred in all lakes.

In Lake Melangsee, the biovolume of Nostocales showed relatively uniform seasonal dynamics from 2005-2011 with an annual summer maximum of *Aphanizomenon gracile* (Figure 4.2). *Anabaena macrospora* showed a population peak in July 2007 ( $8.4 \text{ mm}^3$  vegetative cells  $\text{L}^{-1}$ ) in comparison to other study years, with a high akinete biovolume ( $1.7 \text{ mm}^3 \text{ L}^{-1}$ ). *Anabaena* and *Aphanizomenon* were not detected in “standard samples” in the winter period from December to March. The results of “enriched samples” indicated that the number of *Aphanizomenon* filaments decreased from 40,000 filaments  $\text{L}^{-1}$  in November to below the detection limit under ice cover under low light conditions in February 2010 (Figure 4.3A and B; see definition of detection limit in section 4.3). However, filaments were rediscovered in the pelagic zone in March. *Anabaena* was not detectable as filaments in the “enriched samples” in November 2009 and filaments were not found until April after the break-up of the ice sheet (end of March).

Both *Anabaena* and *Aphanizomenon* akinetes were detected in the surface sediments (“sediment samples”) in Lake Melangsee, reaching densities of  $0.8 \cdot 10^6$  akinetes  $\text{g}^{-1}$  DW sediment and more than  $3 \cdot 10^6$  akinetes  $\text{g}^{-1}$  DW sediment in November 2009, respectively (Figure 4.3C).

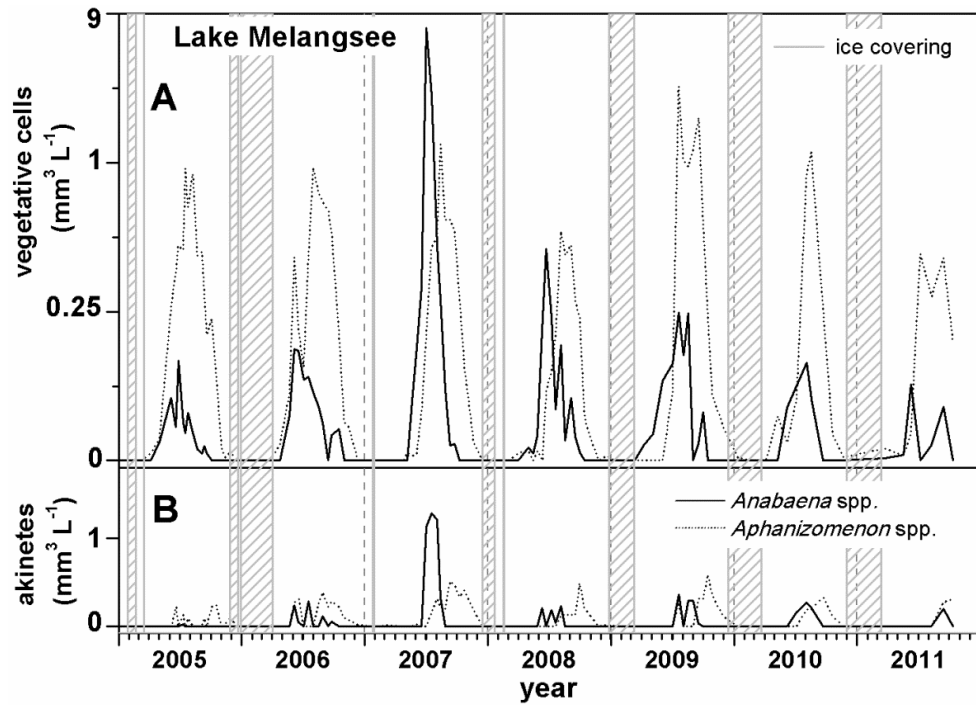


Figure 4. 2 Biovolume of *Anabaena* and *Aphanizomenon* spp. vegetative cells (A) and akinetes (B) in the pelagic zone of Lake Melangsee (“standard samples”) and ice cover periods for the years 2005-2011. Values were square root transformed.

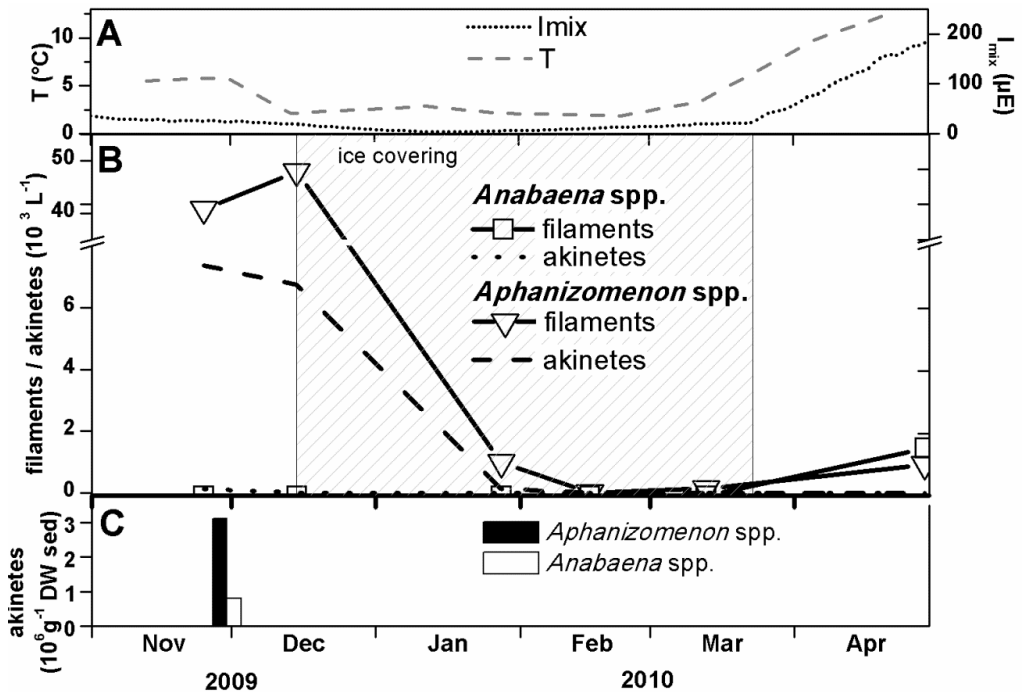


Figure 4. 3 Temporal development of *Anabaena* and *Aphanizomenon* spp. in Lake Melangsee. A – Mean epilimnion temperature and underwater light supply (Imix), B – Filaments and viable akinetes in the pelagic zone (“enriched samples”) and C – akinetes in sediments (“sediment samples”).

*Anabaena* spp. was the most abundant Nostocales genus in Lake Scharmützelsee in the years studied. In summer 2007, *Anabaena* spp. showed the highest maximum biovolume peak of  $2.2 \text{ mm}^3 \text{ L}^{-1}$  in Lake Scharmützelsee compared to the other years (Figure 4.4). Here, akinete production was decoupled from vegetative cell biovolume. *Anabaena* and *Aphanizomenon* were not detected or were very scarce in the “standard samples” from December to February. The “enriched samples” indicated that *Anabaena* filaments decreased at the beginning of the winter period 2009-2010 in Lake Scharmützelsee. No *Anabaena* filaments were detected from February to April 2010. *Aphanizomenon* filaments were not found in the “enriched samples” from November 2009 to August 2010, except in February, with four filaments  $\text{L}^{-1}$  conspicuously below the detection limit (Figure 4.5B).

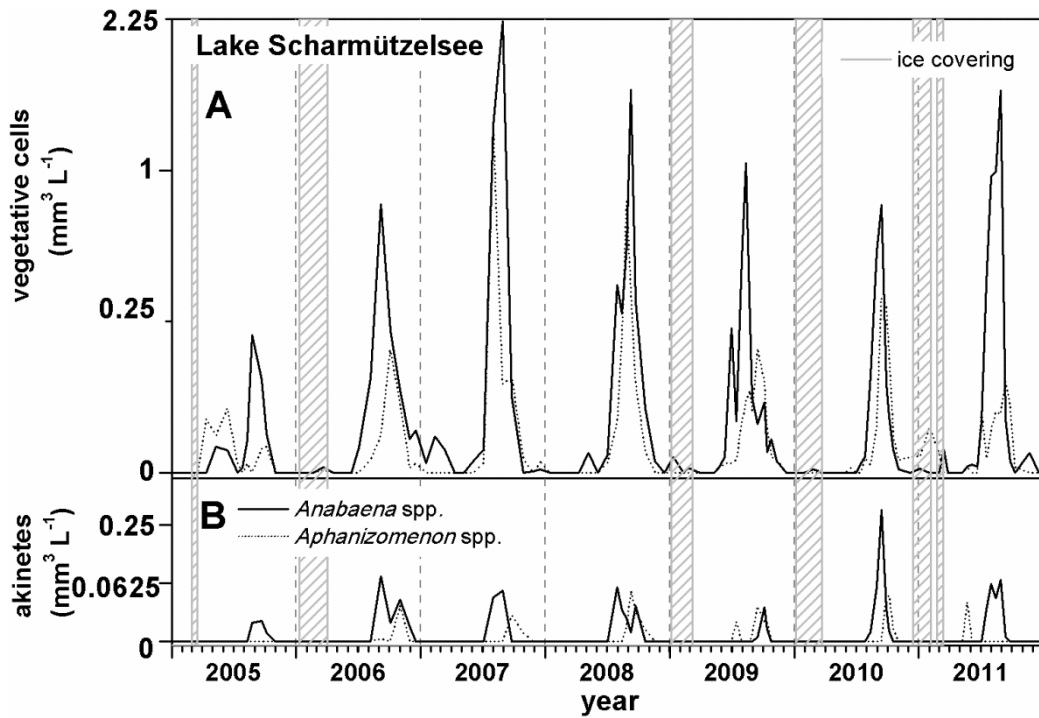


Figure 4. 4 Biovolume of *Anabaena* and *Aphanizomenon* spp. vegetative cells (A) and akinetes (B) in the pelagic zone of Lake Scharmützelsee (“standard samples”) and ice cover periods for the years 2005-2011. Values were square root transformed.

In comparison to 2009, the biovolume of *Anabaena* akinetes in the “standard samples” was ten times higher in autumn 2010, although the biovolume of vegetative cells was low in this year in Lake Scharmützelsee. The highest abundance of *Anabaena* filaments ( $7,500 \text{ filaments L}^{-1}$ ) and akinetes ( $5,600 \text{ akinetes L}^{-1}$ ) in the “enriched samples” of the pelagic zone was found in September 2010 after the annual light and temperature maximum (Figure 4.4A),



contemporary with a high number of *Anabaena* akinetes ( $0.57 \cdot 10^9$  m<sup>-2</sup> surface sediment) in the topmost benthic zone (Figure 4.5C, “sediment samples” app.  $2.5 \cdot 10^6$  akinetes g<sup>-1</sup> DW 0-1 cm sediment). On the other hand, an increase in akinete abundance of *Aphanizomenon* in the surface sediments was first recorded in December 2010. At this time, intact akinetes of *Anabaena* decreased in the top layer of the benthic zone by half, most likely due to degradation or bioturbation.

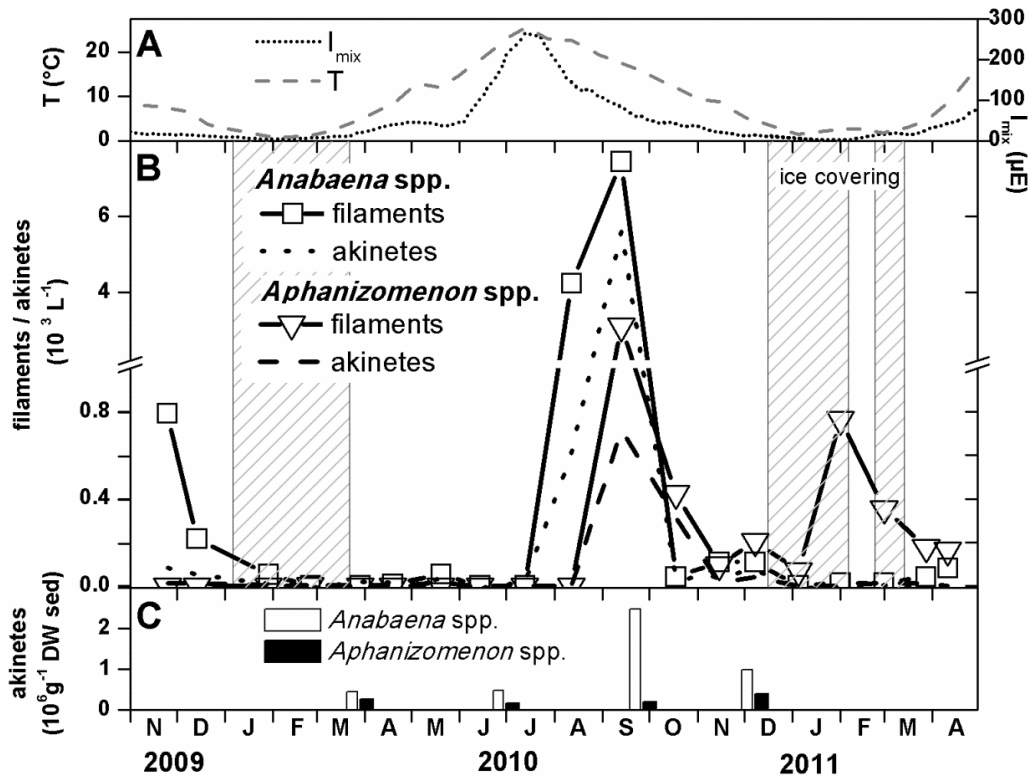


Figure 4.5 Temporal development of *Anabaena* and *Aphanizomenon* spp. in Lake Scharmützelsee. A – Mean epilimnion temperature and underwater light supply ( $I_{mix}$ ), B – Filaments and viable akinetes in the pelagic zone (“enriched samples”) and C – akinetes in sediments (“sediment samples”).

The “standard sample” data of Nostocales in Lake Stechlinsee were available from 2007 onwards (Figure 4.6). However, light and temperature conditions were not measured. *Aphanizomenon flos-aquae* was present from the beginning of the observation period with minimal biovolume and formed a summer peak for the first time in 2009. The species overwintered in the water-column with relatively high biomass ( $0.3$  mm<sup>3</sup> L<sup>-1</sup>) and reached a summer biovolume of  $1.2$  mm<sup>3</sup> L<sup>-1</sup> in May 2010.

*Aphanizomenon flos-aquae* vegetative cells decreased in the “standard samples” in summer 2010 and dropped down similarly to *Anabaena* spp. (Figure 4.7B). In contrast to winter 2009-2010, few filaments were detected in the pelagic zone in winter 2010-2011, and community composition shifted from the dominance of *Aphanizomenon flos-aquae* to the dominance of *Anabaena* spp. in summer 2011 ( $2.5 \text{ mm}^3 \text{ L}^{-1}$ ). “Enriched samples” from Lake Stechlinsee were only examined in winter 2009-2010 (Figure 4.7A), when *Aphanizomenon* filaments were detected but no *Anabaena* filaments. *Aphanizomenon flos-aquae* akinetes were not found either in “standard or enriched samples” or in “sediment samples” throughout the observation period. Conversely, akinetes of *Anabaena flos-aquae*, *Anabaena circinalis* and *Anabaena lemmermannii* were detected in the “sediment samples” of the benthic zone (Figure 4.7C). The lowest akinete abundance was recorded in June 2010, corresponding to the first detection of filaments in the water-column of this year. During the course of phytoplankton development, the total number of akinetes increased in Lake Stechlinsee’s sediment to up to  $4 \cdot 10^6$  akinetes  $\text{g}^{-1}$  DW sediment in November. The *Anabaena lemmermannii* akinete abundance in the benthic zone varied in magnitude due to the formation of very patchily occurring nests with up to 200 cells (Figure 4.7C; October 2010).

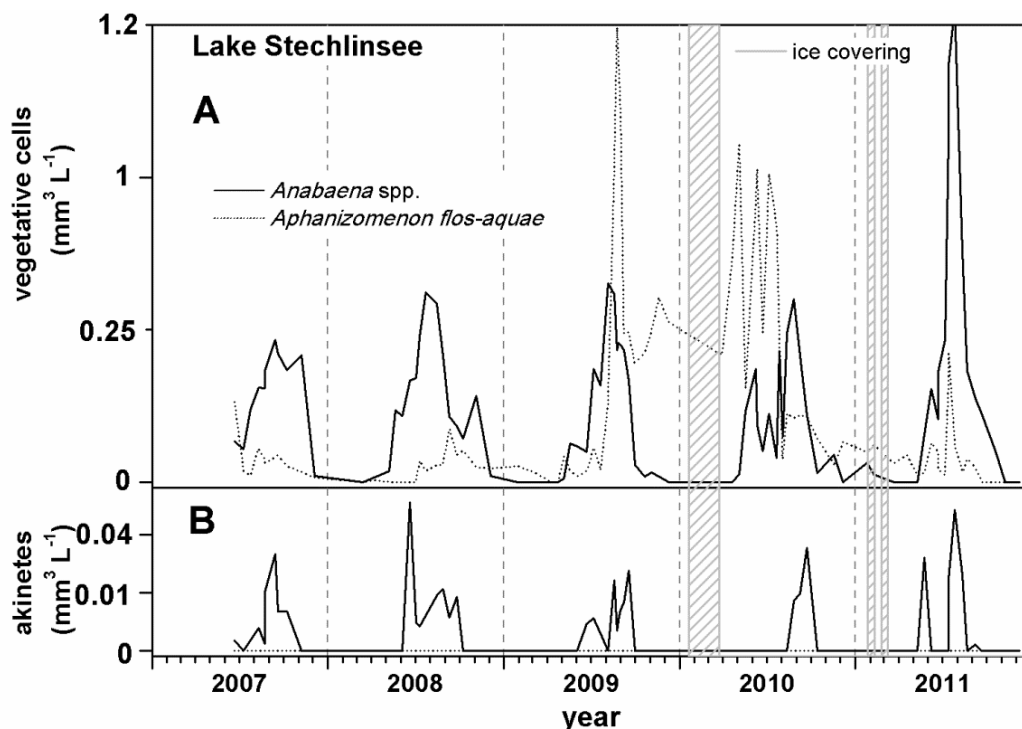


Figure 4. 6 Biovolume of *Anabaena* spp. and *Aphanizomenon flos-aquae*. vegetative cells (A) and akinetes (B) in the pelagic zone of Lake Stechlinsee (“standard samples”) and ice cover periods (2009-2011) for the years 2007-2011. Values were square root transformed.

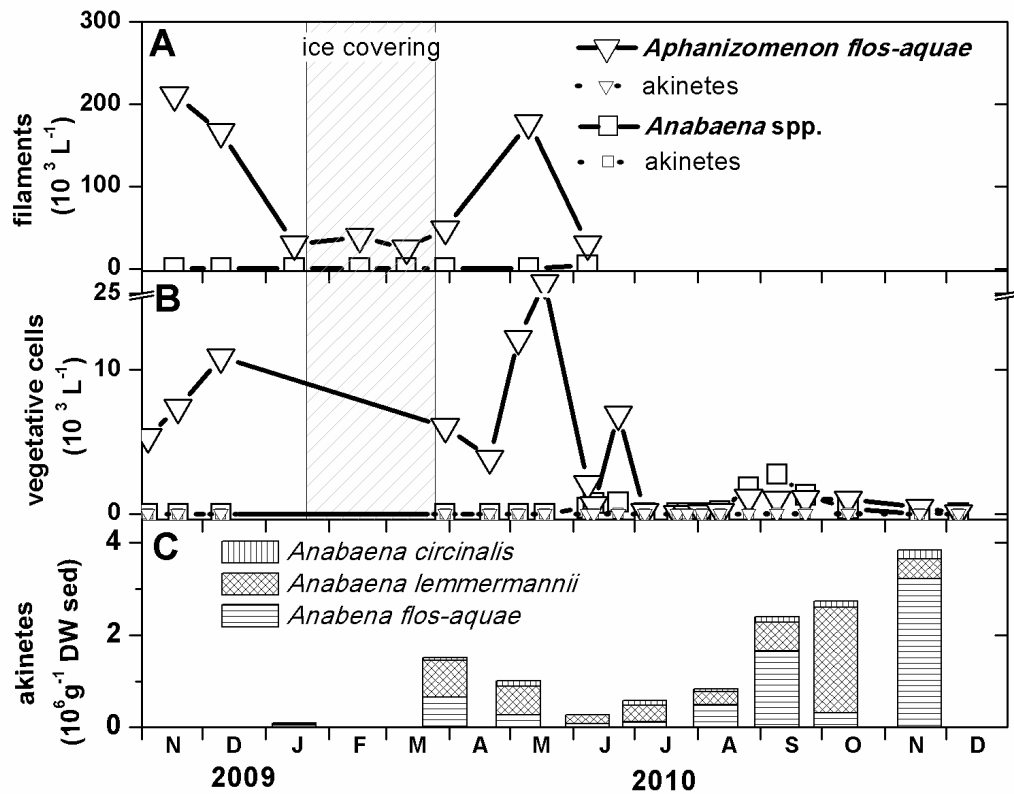


Figure 4. 7 Temporal development of *Anabaena* spp. and *Aphanizomenon flos-aquae* in Lake Stechlinsee. A – Filaments and viable akinetes in the pelagic zone (“enriched samples”) B – vegetative cells in the epilimnion (“standard samples”) and C – akinetes in sediments (“sediment samples”).

## 4.5 Discussion

In this study, we characterized different strategies of Nostocales to overwinter vegetatively in lakes despite the presence of akinetes in sediments as resting stages or without akinete formation. We were also able to reduce the detection limit by enriching the winter plankton in samples.

### 4.5.1 Ice cover and overwintering population of Nostocales

The number of overwintering filaments was strongly influenced by climatic factors in the autumn and winter. Previous studies of lakes in the region of Lake Scharmützelsee showed that winters with closed ice cover and stable temperature stratification were more favourable for the vegetative forms of cyanobacteria than winters without ice cover (Wiedner and Nixdorf 1998). Up to 80 % of the irradiation went through the ice cover depending on the quality of the ice, while solid snow layers caused a reduction of 20 % (Rücker and Henschke 2004). The

low-turbulence conditions under ice enabled Nostocales to stratify with their gas vesicles under the ice cover. Absent ice cover led to stronger wind mixing in the pelagic zone and phytoplankton species could be transferred into deeper water depths.

*Aphanizomenon flos-aquae* showed a winter bloom at the water-ice interface ( $\leq 2$  m water depth) in the deep stratified oligo-mesotrophic Lake Stechlinsee in winter 2009-2010, amounting to more than 99 % of phytoplankton (1 % *Stephanodiscus neoastraea*) in February 2010 (Üveges et al. 2012). In summer 2010, the population peaked during thermal stratification in water depths of  $> 20$  m where irradiation varied between 5 and 20  $\mu\text{E m}^{-2} \text{s}^{-1}$  (not shown).

The population collapsed in winter 2010-2011 due to two short periods of 14-days ice cover and declined in summer 2011 (Figure 4.2). The reconstruction of the *Aphanizomenon flos-aquae* population in the vegetation period in 2011 resulted from a few remaining vegetative cells, which were, however, not sufficient to reach the same population size as in 2010. Conditions in 2011 were more favourable for a major part of akinete-forming Nostocales species. The growth of vegetative cells of Nostocales was inhibited in the spring due to other competitive phytoplankton species which already dominated the water-column, as also observed by Yamamoto and Nakahara (2009), and suggests that *Aphanizomenon flos-aquae* tolerated only a small ecological and physiological optimum, explaining the switch of dominance from *Aphanizomenon* to *Anabaena*. *Anabaena* spp. was able to tolerate and use higher light intensities in the epilimnion than *Aphanizomenon flos-aquae*, which declined in growth conditions due to reduced light intensity below the deep, cold metalimnion. This is confirmed by laboratory light-dependent growth studies of an *Aphanizomenon flos-aquae* strain isolated from Lake Stechlinsee (Mehnert et al. 2010). Accordingly, *Aphanizomenon flos-aquae* had the lowest growth rates of all Nostocales species investigated.

The low  $I_k$  of *Aphanizomenon flos-aquae*, however, enabled photosynthesis of the overwintering population at low light availability under ice (Mehnert et al. 2010; Üveges et al. 2012). Üveges et al. (2012) demonstrated under laboratory conditions that *Aphanizomenon flos-aquae* was still photosynthetically active at temperatures of 2 °C and irradiances of 7.5  $\mu\text{E m}^{-2} \text{s}^{-1}$ . De Nobel et al. (1998) had already reported that *Aphanizomenon flos-aquae* was better adapted to light-limited conditions than other cyanobacterial species.

Our results indicate that the population of *Aphanizomenon flos-aquae* was able to recruit exclusively from overwintering filaments, and that akinetes do not necessarily play a role in the life cycle of Nostocales in temperate regions.

Akinetes have not been detected in cultures of *Aphanizomenon flos-aquae* isolated from Lake Stechlinsee so far (Ballot A, personal communication).

Remaining in the form of vegetative cells from winter to spring (Head et al. 1999; Simona 2003; Yamamoto and Nakahara 2009) was contrary to most of the other studies. However, Jones (1979) had already reported *Aphanizomenon flos-aquae* overwintering in Kinnego Bay as filaments, and the production of akinetes was not a necessary strategy for perennation of the species.

Hence, questions arise as to what benefit do non-akinetete forming species like *Aphanizomenon flos-aquae* have in deep stratified lakes such as Lake Stechlinsee, and how do they prevent their vanishing from the water-column with a small ecological optimum during harsh conditions in winter? One explanation could be a higher energy efficiency to form colonies in the form of bundles with active buoyancy regulation and remaining in the pelagic zone under the ice cover to be first in spring rather than going a circuitous route.

Another consideration includes the chemical and physical differentiation of their habitats, i.e. the dimictic pelagic zone of deep lakes which are seasonally in dynamic process by circulation and are subject to temporarily abruptly changing environmental conditions, such as light and nutrient limitation. *Aphanizomenon flos-aquae* in the deep nutrient-scarce Lake Stechlinsee built up bundles of up to hundred filaments, which influenced the buoyancy regulation of the species. Buoyant gas vesicles enabled the species under low-light conditions to reach depths of better light availability (Reynolds et al. 1987). The resistance of larger, gas vacuolated bundle-forming *Aphanizomenon flos-aquae* to sinking loss or consumption by grazers provided significant advantages (Reynolds 1987). The buoyancy regulation of *Aphanizomenon flos-aquae* was found to be highest under CO<sub>2</sub>-limiting conditions and these conditions promoted bloom formation (Reynolds and Walsby 1975). As detected, a bloom of those flake-like filaments was capable of stabilizing in thermally stratified periods due to their comparably high surface area. Contrarily, Nostocales species in well-mixed small eutrophic lakes are characterized generally by a low intrinsic settling velocity (Reynolds and Walsby 1975). As our data indicate, *Aphanizomenon* in Lake Stechlinsee presented another ecostrategy than the individually occurring filaments of akinete-producing species *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* observed in Lake Scharmützelsee and Lake Melangsee.

For the deep, dimictic mesotrophic Lake Scharmützelsee, we showed that *Aphanizomenon flos-aquae* in combination with *Aphanizomenon gracile* formed dormant akinetes to overwinter, contrary to this specie's strategy in

Lake Stechlinsee. However, in the shallow, polymictic eutrophic Lake Melangsee, water temperature in March was very low for akinete germination (Figure 4.3A). Thus, overwintering was assumed vegetatively by a very small number of *Aphanizomenon gracile* filaments. This could be related to essential differences in nutrient availability (Table 4.1) and ecological adaptation to prevailing morphological and physical lake conditions.

On the other hand, considering the genus *Anabaena*, filaments decreased at the beginning of winter in the pelagic zone of Lake Scharmützelsee or were absent in Lake Melangsee and Lake Stechlinsee, respectively, during the “*enriched sampling*” programme. This demonstrated the requirement for the dominant part of the *Anabaena* species to produce akinetes for next year’s population growth. The recruited benthic population of cyanobacteria is known to be potentially important as the inoculum of the planktonic population (Karlsson-Elfgren and Brunberg 2004; Rengefors et al. 2004; Takamura et al. 1984). *Anabaena* used this strategy to overwinter and absorb nutrients from lake sediments on an annual basis, and thus, are more independent of nutrient changes in the water-column. However, the akinetes in the sediments of Lake Scharmützelsee were also subject to loss processes (Figure 4.4C), such as infections from viruses, predation by fungi or digestion by invertebrates, as shown in previous studies (Cmiech et al. 1988; Sigeo et al. 2007; Ståhl-Delbanco and Hansson 2002).

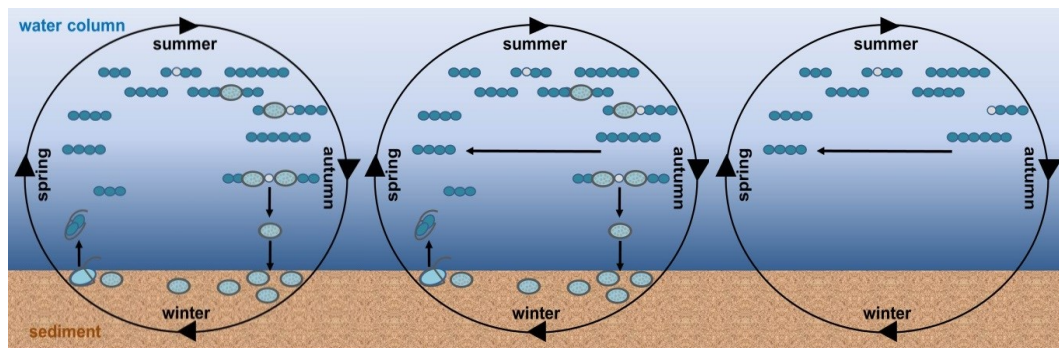
The subsequent germination of intact viable akinetes in the spring after dormant overwintering was probably triggered by the increase in water temperature of  $> 8\text{ }^{\circ}\text{C}$ , which was assumed by Rucker et al. (2012), and was observed  $> 10\text{ }^{\circ}\text{C}$  in many other species (Baker and Bellifemine 2000; Cmiech et al. 1984; Huber 1985). Kim et al. (2005) reported that most active akinete germination of *Anabaena flos-aquae* occurred from the end of March to early April ( $8\text{ to }12\text{ }^{\circ}\text{C}$ ). The optimum temperature for germination corresponded to the optimum growth temperature for cell growth and varied between species of the *Anabaena* genera (Baker and Bellifemine 2000). However, in all lakes, *Anabaena* spp. was observed in “*standard samples*” annually in summer reaching their population maximum in late summer every year.

#### 4.5.2 Summary

Different overwintering strategies of Nostocales were ascertained in lakes of different morphology and trophic states (summary in Figure 4.8). Intraspecific differences occurred in the case of *Aphanizomenon flos-aquae*: with akinete formation in the deep dimictic mesotrophic Lake Scharmützelsee and without akinete formation in the deep dimictic oligo-mesotrophic Lake Stechlinsee.

Interspecific differences were observed between *Anabaena* spp. and *Aphanizomenon* spp. in all lakes. *Anabaena* spp. were exclusively dormant over winter in the form of akinetes in sediments (Figure 4.8, left). Thus, our observations are consistent with those of the Baltic Sea (Suikkanen et al. 2010).

*Aphanizomenon gracile* overwintered both as dormant akinetes and vegetative filaments in the shallow polymictic eutrophic Lake Melangsee (Figure 4.8, middle). Similar results were observed by Suikkanen et al. (2010) for this genus. In the deep stratified oligotrophic lake, *Aphanizomenon flos-aquae* occurred perennially exclusively as vegetative cells without any akinete formation (Figure 4.8, right).



**Figure 4. 8** Schematic representation of overwintering strategies of Nostocales in the studied lakes. Left – dormant overwintering as akinetes in sediments, middle – ‘vegetative and dormant overwintering’, right – vegetative overwintering without akinete formation.

Our investigations showed that filaments of Nostocales species overwinter in lakes and, theoretically, could contribute to building Nostocales populations in the spring. It is likely that a changing climate may shift the meaning of different overwintering strategies and, thus, may influence the interspecific competition between the phytoplankton species in the spring.

## 5. Occurrence of non-cylindrospermopsin-producing *Aphanizomenon ovalisporum* and *Anabaena bergii* strains in Lake Kinneret (Israel)<sup>2</sup>

### 5.1 Abstract

The cylindrospermopsin (CYN)-producing cyanobacterium *Aphanizomenon ovalisporum* is currently known from the Mediterranean area of Europe and the Middle East and from North America and Australia. *Aphanizomenon ovalisporum* was observed for the first time in Lake Kinneret (Sea of Galilee, Israel) in 1994. It appeared with an exceptional bloom and has persisted since then. To gain further insight into biodiversity and chemotype composition of this invasive cyanobacterium, we isolated, cultured and analyzed six putative *Aphanizomenon ovalisporum* strains from Lake Kinneret in 2010. In a polyphasic approach, the strains were investigated for their morphology and phylogeny (based on 16S rRNA gene and PC-IGS) as well as for CYN production (by enzyme-linked immunosorbent assay (ELISA) and LC-MS) and the presence of four fragments of the CYN-encoding gene cluster (*aoaA/cyrA*, *aoaB/cyrB*, *aoaC/cyrC* and *cyrJ*). Two isolated strains were assigned to *Aphanizomenon ovalisporum*, whereas four strains were identified as *Anabaena bergii*. Unlike previous reports of toxic *Aphanizomenon ovalisporum* from Lake Kinneret, all strains isolated in this study tested negative for CYN by ELISA and LC-MS. All strains contained *aoaA/cyrA*, *aoaB/cyrB* and *aoaC/cyrC* fragments of the CYN gene cluster. The *cyrJ* fragment was not detected in any of the isolated strains. This is the first report describing non-CYN-producing *Aphanizomenon ovalisporum* strains and the presence of the cyanobacterium *Anabaena bergii* in Lake Kinneret.

### 5.2 Introduction

The cyanobacterium *Aphanizomenon ovalisporum* (Forti) was recorded for the first time in the phytoplankton community of Lake Kinneret in 1994 (Pollinger et al. 1998). *Aphanizomenon ovalisporum* is currently known from the Mediterranean area of Europe and the Middle East, e.g. in Greece, Italy, Israel and Spain (Bazzichelli and Abdelahad 1994; Gkelis et al. 2005; Pollinger et al. 1998; Quesada et al. 2006), and from North America and Australia (Shaw et al. 1999;

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<sup>2</sup> Ballot, A., Ramm, J., Rundberget, T., Kaplan-Levy, R. N., Hadas, O., Sukenik, A. & Wiedner, C. (2011) Occurrence of non-cylindrospermopsin-producing *Aphanizomenon ovalisporum* and *Anabaena bergii* in Lake Kinneret (Israel). *Journal of Plankton Research* **33**, 1736–1746.



Yilmaz et al. 2008). *Aphanizomenon ovalisporum* is a heterocytous cyanobacterium and is regarded as a cyanobacterial species with the potential to invade temperate areas (Mehnert et al. 2010). It is morphologically close to *Anabaena bergii* Ostefeld, another potentially invasive species (Komárek and Ettl 1958; Stüken et al. 2009). Both species have some morphological characters resembling those of the genera *Aphanizomenon* and *Anabaena*, but both are genetically closer to the genera *Nodularia* and *Anabaenopsis* than to the genera *Aphanizomenon* and *Anabaena* (Stüken et al. 2009). A recent study demonstrated that *Anabaena bergii* and *Aphanizomenon ovalisporum* can be distinguished morphologically and phylogenetically (Stüken et al. 2009).

One cylindrospermopsin (CYN)-producing *Aphanizomenon ovalisporum* strain ILC-164 has been isolated from Lake Kinneret in 1994 (Banker et al. 1997). CYN-production has also been demonstrated in *Aphanizomenon ovalisporum* strains isolated from Australian, North American and Spanish water bodies (Fergusson and Saint 2003; Schembri et al. 2001; Wormer et al. 2008; Yilmaz et al. 2008). It is unknown whether non-CYN-producing *Aphanizomenon ovalisporum* strains exist. Some CYN-producing *Anabaena bergii* strains have also been identified (Fergusson and Saint 2000; Schembri et al. 2001). However, the ability of *Anabaena bergii* to produce CYN is disputed. Fergusson and Saint (Fergusson and Saint 2000) suggested that CYN-producing *Anabaena bergii* strain ANA283A and *Aphanizomenon ovalisporum* strain APH028A are morphological variants of the same cyanobacterium. This was confirmed by Stüken et al. (2009), who demonstrated that the two known Australian CYN-producing *Anabaena bergii* strains ANA283A and ANA366B are most likely misidentified and have to be assigned phylogenetically to *Aphanizomenon ovalisporum*. Other cyanobacterial toxins have not been reported from *Aphanizomenon ovalisporum* and *Anabaena bergii* strains so far.

The distribution of *Anabaena bergii* has been described from tropical to temperate climate zones (Stüken et al. 2006). It was originally described from brackish or seawater habitats (Caspian Sea and Aral Sea) and designated as *Anabaena bergii* and *Anabaena bergii* var. *minor*. *Anabaena bergii* var. *minor* Kiselev and *Anabaena bergii* f. *minor* (Kiselev) Kosinskaja are synonyms for *Anabaena bergii* (Hindák 2000). Populations found in freshwater habitats in France and Switzerland with a similar morphology were designated as *Anabaena bergii* var. *limnetica* (Couté and Preisig 1978; Hindák 2000).

CYN-producing *Aphanizomenon ovalisporum* and non-CYN-producing *Anabaena bergii* strains are found to possess genes (*aoaA/cyrA* homolog, *aoaB/cyrB* homolog, *aoaC/cyrC* homolog) encoding polyketide synthases (PKS) and peptide synthetases (PS) putatively involved in CYN synthesis (Rasmussen et al. 2008; Shalev-Alon et al. 2002; Schembri et al. 2001). A study by Mihali et al. (2008) revealed that *cyrJ*, a sulfotransferase-encoding gene, was present only in CYN-producing strains, supporting the involvement of this gene in the biosynthesis of CYN. The *cyrJ* gene was therefore suggested as a good candidate for a genetic marker to detect CYN-producing cyanobacterial strains (Mihali et al. 2008).

The complete CYN gene clusters of *Cylindrospermopsis raciborskii* strain AWT 205 and *Aphanizomenon* sp. strain 10E6 have been described by Mihali et al. (2008) and Stüken and Jacobsen (2010). The entire putative CYN gene cluster in *Cylindrospermopsis raciborskii* strain AWT 205 spans 43 kb and comprises 15 open reading frames required for the biosynthesis, regulation and export of the toxin (Mihali et al. 2008). Among others, four PKSs, one PKS/non-ribosomal peptide synthetase (NRPS) hybrid and one amidinotransferase are involved in the CYN biosynthesis (Mihali et al. 2008). Comparisons with the partial CYN gene cluster of the Israeli *Aphanizomenon ovalisporum* strain ILC-164 revealed a high gene sequence similarity, but also extensive rearrangements of gene order (Stüken and Jacobsen 2010).

In order to find out more about the phylogeny and toxicity of *Aphanizomenon ovalisporum*, six putative *Aphanizomenon ovalisporum* strains were isolated from Lake Kinneret in April 2010. In a polyphasic approach, they were classified morphologically and phylogenetically, screened for CYN-production with enzyme-linked immunosorbent assay (ELISA) and LC-MS and tested for the presence of putative CYN biosynthesis encoding genes. Additionally, *Aphanizomenon ovalisporum* strain ILC-164 producing CYN- and 7-Epicylindrospermopsin was included in this study (Banker et al. 2000). It was originally isolated from Lake Kinneret in 1994 (Banker et al. 1997). As four of the isolated strains were later identified as *A. bergii*, three non-CYN-producing *Anabaena bergii* strains isolated from Lake Melangsee, Germany (Ballot et al. 2010), were additionally included in this study.

### 5.3 Material and Methods

#### 5.3.1 Isolation of strains and morphological characterization

Six putative *Aphanizomenon ovalisporum* strains were isolated from the warm monomictic freshwater, Lake Kinneret, Israel, located at 32°42'–32°55' N latitude; 35°31'–35°39' E longitude. Phytoplankton sampling was performed in April 2010 by towing a 60 µm plankton net at 1 m depth for 5 min.

Using a microcapillary, single putative *Aphanizomenon ovalisporum*, filaments were isolated. They were washed five times and placed in wells on microtiter plates containing 300 µL Z8 medium (Kotai, 1972). After successful growth, the samples were placed in 50 mL flasks containing 20 mL Z8 medium and maintained at 20 °C and a photon flux density of 80 µE m<sup>-2</sup> s<sup>-1</sup>. Additionally, CYN-producing *Aphanizomenon ovalisporum* strain ILC-164 from Lake Kinneret (Banker et al. 1997; Stüken et al. 2009) and three *Anabaena bergii* strains (AB2008/43, AB2008/44, AB2008/45; Ballot et al. 2010) from Lake Melangsee (Germany) were included in this study. All strains used in this study are maintained at the Norwegian Veterinary Institute, Oslo, Norway.

Morphological studies were conducted using an Olympus BX51 light microscope and color view imaging system (Olympus, Germany). Strains were classified based on morphological traits according to Hindák (2000), Komárek and Komárková (2006) and Stüken et al. (2009). For the morphological identification of *Aphanizomenon ovalisporum* and *Anabaena bergii* strains, the following attributes were considered: (i) size of vegetative cells, heterocytes and akinetes and (ii) nature and shape of filament. Length and width of 50 – 500 vegetative cells and of 50 heterocytes were measured. Akinetes were only found in smaller amounts in one *Aphanizomenon ovalisporum* and two *Anabaena bergii* strains (Table 5.1).

Table 5. 1 Morphological characteristics of *Anabaena bergii* and *Aphanizomenon ovalisporum* strains from Lake Kinneret, Israel, grown under culture conditions.

Strain	shape of trichomes	Shape of end cells	veg. cells		heterocytes		akinetes	
			length ( $\mu\text{m}$ )*	width ( $\mu\text{m}$ )*	length ( $\mu\text{m}$ )*	width ( $\mu\text{m}$ )*	length ( $\mu\text{m}$ )*	width ( $\mu\text{m}$ )*
<i>A. bergii</i>								
AB2010/02	straight	bluntly rounded	3.8 (1.6, 6.8)	5.9 (3.7, 7.2)	7.4 (6.1, 9.6)	7.6 (6.2, 9.0)	n.o.	n.o.
AB2010/03	straight	bluntly rounded	3.4 (1.5, 6.6)	5.4 (3.9, 6.5)	7.1 (5.8, 8.4)	7.1 (6.1, 8.1)	n.o.	n.o.
AB2010/07	straight	bluntly rounded	4.2 (1.9, 7.0)	6.1 (4.4, 7.6)	7.8 (5.6, 9.3)	7.9 (6.1, 9.2)	20.4 (13.7, 27.12)	11.7 (6.8, 16.6)
AB2010/08	straight	bluntly rounded	4.2 (2.1,7.60)	6.4 (4.1,7.4)	8.0 (6.1, 9.9)	8.1 (6.1, 9.6)	n.o.	n.o.
AB2008/43	straight	bluntly rounded	4.0 (1.6, 8.6)	5.1 (2.2, 6.8)	7.2 (5.0, 8.8)	7.1 (5.9, 8.0)	17.9 (17.9, 17.9)	11.3 (11.3, 11.3)
AB2008/44	straight	bluntly rounded	3.8 (2.0, 6.9)	6.4 (2.3, 8.8)	7.9 (5.8, 10.1)	8.3 (6.2, 10.0)	n.o.	n.o.
AB2008/45	straight	bluntly rounded	3.5 (1.8, 6.7)	5.7 (4.2, 6.9)	7.8 (6.3, 9.9)	7.9 (6.7, 9.5)	n.o.	n.o.

Table 5. 1 (continued) Morphological characteristics of *Anabaena bergii* and *Aphanizomenon ovalisporum* strains from Lake Kinneret, Israel, grown under culture conditions.

Strain	shape of trichomes	Shape of end cells	veg. cells		heterocytes		akinetes	
			length ( $\mu\text{m}$ )*	width ( $\mu\text{m}$ )*	length ( $\mu\text{m}$ )*	width ( $\mu\text{m}$ )*	length ( $\mu\text{m}$ )*	width ( $\mu\text{m}$ )*
<i>A. ovalisporum</i>								
ILC-164	straight	bluntly rounded	4.7 (2.6, 8.5)	5.6 (4.1, 7.0)	8.3 (6.0,10.5)	7.8 (6.3,9.0)	12.3 (7.1, 20.0)	9.6 (6.1, 14,5)
AB2010/05	straight	conically shaped	6.3 (3.1,15.7)	5.7 (3.5, 7.1)	7.4 (6.1, 10.0)	7.4 (6.1, 9.0)	n.o.	n.o.
AB2010/06	straight	conically shaped	6.1 (3.4, 9.9)	5.9 (4.2,6.6)	7.8 (5.6, 9.6)	7.6 (6.0, 8.7)	n.o.	n.o.

\*numbers are means (minimum, maximum values); n.o. = not observed

### 5.3.2 Genomic DNA extraction/PCR amplification and sequencing

Fresh culture material of all cyanobacterial strains was frozen and thawed three times and then boiled for 5 min and subsequently centrifuged for 5 min (16,000 g). The supernatants were discarded. Each pellet was resuspended in 100  $\mu$ L distilled water and vortexed for 1 min. Genomic DNA was extracted using the MoleStrips DNA blood kit and the DNA-Cyano protocol on GeneMole (Mole Genetics, Lysaker, Norway) according to the manufacturer's instructions.

All PCRs were performed on a Peltier thermal cycler PTC 200 (MJ Research, Inc., San Francisco, CA, USA) using the Taq PCR core kit (Qiagen GmbH, Hilden, Germany). The reaction mixture contained 0.1  $\mu$ L Taq DNA polymerase (5 U/ $\mu$ L), 0.5  $\mu$ L deoxynucleoside triphosphate mix (10 mM), 2  $\mu$ L Qiagen PCR buffer, 1  $\mu$ L each forward and reverse primer (10 mM) and 1  $\mu$ L genomic DNA, yielding a total volume of 20  $\mu$ L. PCR of the 16S rRNA gene was conducted for six *Anabaena bergii* and *Aphanizomenon ovalisporum* strains from Lake Kinneret and for three *Anabaena bergii* strains from Lake Melangsee using primers pA and B23S (Edwards et al. 1989; Gkelis et al. 2005). The primers PCbf and PCar were used to amplify the intergenic spacer and flanking regions of the *cpcB* and *cpcA* genes of the phycocyanin operon (PC-IGS) (Neilan et al. 1995). PCR was also used to check whether the isolated strains were potential producers of CYN. The PS gene and PKS gene (*aoaA/cyrA*, *aoaB/cyrB*, *aoaC/cyrC* genes) of the CYN gene cluster were analyzed with modified PCR methods after Schembri et al. (2001) using the primer M13 and a newly designed primer Cyli2 (ragcatytcscccaagaaggt) and the primer pair M4/K18. To amplify flanking regions of the PS region, the primer pairs Cyli11f (tgctgagaggatccatcaccgcag) and Cyli11r (gagtcctgttgacaagtcacc) and Cyli333f (tgcaggtcgatcgcccttgga) and Cyli333r (actggtgccagtgggtcgtcca) were newly designed. The primers cover a sequence length of 2450 bp of the *aoaA/cyrA* and *aoaB/cyrB* genes. Amplification of *cyrJ* gene fragment was conducted using primers cynsulF and cynamR (Mihali et al. 2008).

The cycling protocol for the 16S rRNA gene fragment was one cycle for 5 min at 94 °C, then 30 cycles of 30 s at 94 °C, 30 s at 57 °C and 1 min at 70 °C; with a final elongation step of 72 °C for 3 min. To amplify the PC-IGS fragment, one cycle of 5 min at 94 °C and then 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C with a final elongation step of 72 °C for 10 min were used.

The protocol for *aoaA/cyrA*, *aoaB/cyrB* and *aoaC/cyrC* gene fragments was one cycle of 4 min at 94 °C and then 35 cycles of 10 s at 94 °C, 20 s at 55 °C and 1 min at 72 °C. The protocol for *cyrJ* was one cycle at 95 °C for 3 min, followed

by 35 cycles of 45 s at 95 °C, 30 s at 50 °C, 1 min at 72 °C with a final elongation step of 72 °C for 3 min. For all strains studied, a second PCR was conducted with the same protocol using the PCR products and the same primers. The exception was CYN-producing *Aphanizomenon ovalisporum* strain ILC-164, where a good amplification was always obtained during the first PCR. The PCR products were visualized by 1.5 % agarose gel electrophoresis with ethidium bromide staining and UV illumination.

Amplified 16S rRNA gene, PC-IGS and CYN gene products were purified through Qiaquick PCR purification columns (Qiagen, Hilden, Germany), and the DNA was eluted in elution buffer according to the manufacturer's protocol. Sequencing of the purified 16S rRNA gene and PC-IGS products was performed using the same primers as for PCR. For each PCR product, both strands were sequenced on an ABI 3100 Avant genetic analyzer using the BigDye terminator V.3.1 cycle sequencing kit (Applied Biosystems, Applied Biosystems GmbH, Darmstadt, Germany) according to the manufacturer's instructions.

### 5.3.3 Phylogenetic analysis

Sequences of the 16S rRNA gene and PC-IGS locus were analyzed for all *Anabaena* sp. and *Aphanizomenon* sp. strains using Seqassem version 04/2008 (Hepperle 2008). The Align (version 03/2007) MS Windows-based manual sequence alignment editor (Hepperle 2008) was used to obtain DNA sequence alignments, which were then corrected manually. Segments with highly variable and ambiguous regions and gaps making proper alignment impossible were excluded from the analyses. A 16S rRNA gene set containing 1142 positions and a PC-IGS set containing 544 positions were used. *Microcystis aeruginosa* (AB035549) and *Nodularia* sp. (AY78467) were employed as outgroups in the 16S rRNA gene and PC IGS tree, respectively. Thirty-four additional Nostocales 16S rRNA gene sequences and 21 Nostocales PC-IGS sequences derived from GenBank were included in the 16S rRNA and PC-IGS analyses, respectively. Phylogenetic trees for 16S rRNA gene and PC-IGS were constructed using the maximum likelihood (ML) algorithm in PAUP \* v 10b (Swofford 2002). In the ML analyses, evolutionary substitution models were evaluated using the AIC criterion in jModelTest v.0.1.1 (Guindon and Gascuel 2003; Posada 2008). TPM3uf + I + G was found to be the best-fitting evolutionary model for the 16S rRNA gene. The HKY + G evolutionary model was used for PC-IGS. ML analyses of all trees were performed with 1000 bootstrap replicates using PAUP\* v.10b (Swofford 2002).

### 5.3.4 Nucleotide sequence accession numbers

The sequence data were submitted to the EMBL Nucleotide Sequence Database under the accession numbers listed in Table 5.2.

**Table 5.2** *Aphanizomenon ovalisporum* and *Anabaena bergii* strains evaluated by ELISA, LC-MS/MS, and genetic properties

Strain	Detection of CYN		Detection of gene fragments			accession number	
	ELISA	LC-MS/MS	aoaB	aoaA	aoaC	16S	PC-IGS
<i>A. bergii</i>							
AB2010/02	-	-	+	+	+	FR822618	FR822624
AB2010/03	-	-	+	+	+	FR822619	FR822625
AB2010/07	-	-	+	+	+	FR822622	FR822628
AB2010/08	-	-	+	+	+	FR822623	FR822629
AB2008/43	-	-	+	+	+	FR822615	FN552379
AB2008/44	-	-	+	+	+	FR822616	FN552380
AB2008/45	-	-	+	+	+	FR822617	FN552381
<i>A. ovalisporum</i>							
ILC164	+	+	+	+	+	EF529482	EF529470
AB2010/05	-	-	+	+	+	FR822620	FR822626
AB2010/06	-	-	+	+	+	FR822621	FR822627

### 5.3.5 Cyanotoxin analysis

#### Enzyme-linked immunosorbent assay

All *Aphanizomenon* and *Anabaena* strains were tested for CYN by using the Abraxis CYN ELISA kit (Abraxis LLC, Warminster, PA, USA) following the manufacturer's instructions. The test is a direct competitive ELISA designed to detect CYN based on specific antibody recognition. For each culture strain, total CYN (dissolved and cell-bound CYN) was determined. Five milliliters of culture



material were frozen and thawed three times to extract the toxins. The color reaction of the ELISA test was evaluated at 450 nm using a Biotek Synergi 2 microtiter plate reader (Biotek, Bad Friedrichshall, Germany). The detection limit for CYN using the CYN ELISA kit is 0.040 ppb ( $\mu\text{g L}^{-1}$ ).

### **LC-MS/MS analysis**

Fresh culture material of all cyanobacterial strains was frozen and thawed three times, ultrasonicated for 5 min and filtered through Spin-X centrifuge tube filters (Corning Inc., Corning, NY, USA), at 10,000 g. The filtrate was mixed with acetonitrile (1:4).

Liquid chromatography was performed on a SeQuant ZIC-HILIC column (3.5  $\mu\text{m}$ , 150 x 2.1 mm) (Merck, Darmstadt, Germany), using an Accela HPLC module (Thermo Scientific, San Jose, CA, USA). Separation was achieved using step gradient elution at 0.2 mL  $\text{min}^{-1}$  starting with 20 % A (water containing 5 mM ammonium acetate and 0.1 % acetic acid) and 80 % B (95 % CAN containing 5 mM ammoniumacetate and 0.1 % acetic acid) for 8 min, then rising to 60 % A over 15 min, return to 20 % A and hold for 8 min before next injection. The HPLC system was coupled to a TSQ Quantum Access triple quadrupole mass spectrometer operating with an ESI interface (Thermo Scientific). Typical ESI parameters were a spray voltage of 3.5 kV, heated capillary temperature at 250 °C and nebulizer gas at 600 L  $\text{h}^{-1}$  of  $\text{N}_2$ . The mass spectrometer was operated in an MS/MS mode with argon as the collision cell gas at  $1.4 \cdot 10^{-3}$  Torr. Ionization and MS/MS collision energy settings (typically 25–30 eV) were optimized while continuously infusing (syringe pump) 200 ng/mL of CYN, at a flow rate of 5  $\mu\text{L min}^{-1}$ . Screening of CYN was performed with multiple reaction monitoring in a positive ionization mode; CYN 416.1 > 176.0, 416.1 > 194.0. A certified CYN (NRC CRM-CYN) from National Research Council, Halifax, NS, Canada, was used as the standard. The detection limit for CYN was 10 ppb ( $\mu\text{g L}^{-1}$ ).

## **5.4 Results**

### **5.4.1 Morphological and phylogenetical characterization**

All strains grew with straight filaments. The vegetative cells of strains AB2010/05 and AB2010/06 were longer than they were wide and the end cells were bluntly shaped. Strains AB2010/02, AB2010/03, AB2010/07 and AB2010/08 had vegetative cells wider than they were long with conically shaped end cells (Table 5.1). In all strains, spherical intercalary heterocytes were found. Akinetes were observed only in strains AB2010/07, AB2008/43 and ILC-164 (Table 5.1). Based on their morphological features, the isolated strains were identified as

*Aphanizomenon ovalisporum* (AB2010/05 and AB2010/06) and *Anabaena bergii* (AB2010/02, AB2010/03, AB2010/07 and AB2010/08) (Table 5.1, Figure 5.1).

Phylogenetic relationships of the strains investigated are presented in the ML tree of the 16S rRNA gene (Figure 5.2) and the PC-IGS region (Figure 5.3). In both phylogenetic trees, the *Aphanizomenon ovalisporum* strains (cluster II) were clearly separated from the *Anabaena bergii* strains (cluster I) (Figures 5.2 and 5.3). The phylogenetic results clearly support the morphological observations that divided the isolated strains between two species, *Aphanizomenon ovalisporum* and *Anabaena bergii*.

#### **5.4.2 Identification of toxin producers**

As determined by ELISA and LC-MS/MS, only *Aphanizomenon ovalisporum* strain ILC-164 from Lake Kinneret was confirmed to produce CYN (Table 5.2). The other nine *Aphanizomenon ovalisporum* and *Anabaena bergii* strains tested negative for CYN by both ELISA and LC-MS/MS techniques (Table 5.2).

#### **5.4.3 Presence of CYN gene cluster**

All *Aphanizomenon ovalisporum* and *Anabaena bergii* strains were investigated for the presence of fragments of the CYN gene cluster. All strains investigated showed amplification products of the *aoaA/cyrA*, *aoaB/cyrB* and *aoaC/cyrC* genes applying the original or modified PCR protocol and using PS and PKS primers according to Schembri et al. (2001) and newly designed primers Cyli333f/r, Cyli11f/r and Cyli 2.

Sequences were obtained for parts of the *aoaC/cyrC* gene for all investigated strains. Parts of the *aoaA/cyrA* and *aoaB/cyrB* genes were successfully sequenced for *Aphanizomenon ovalisporum* strain ILC-164 and *Anabaena bergii* strains AB2008/43, AB2008/44 and AB2008/45. In all four strains, we were able to sequence 2433 bp of the *cyrB* and *cyrA* segment of the CYN gene cluster. For the remaining strains, sequencing products could not be obtained. In the case of the *cyrJ* gene, a PCR product was obtained for CYN-producing *Aphanizomenon ovalisporum* strain ILC-164. No amplification product was found for the *cyrJ* gene fragment in the other nine *Aphanizomenon ovalisporum* and *Anabaena bergii* strains (Table 5.2).

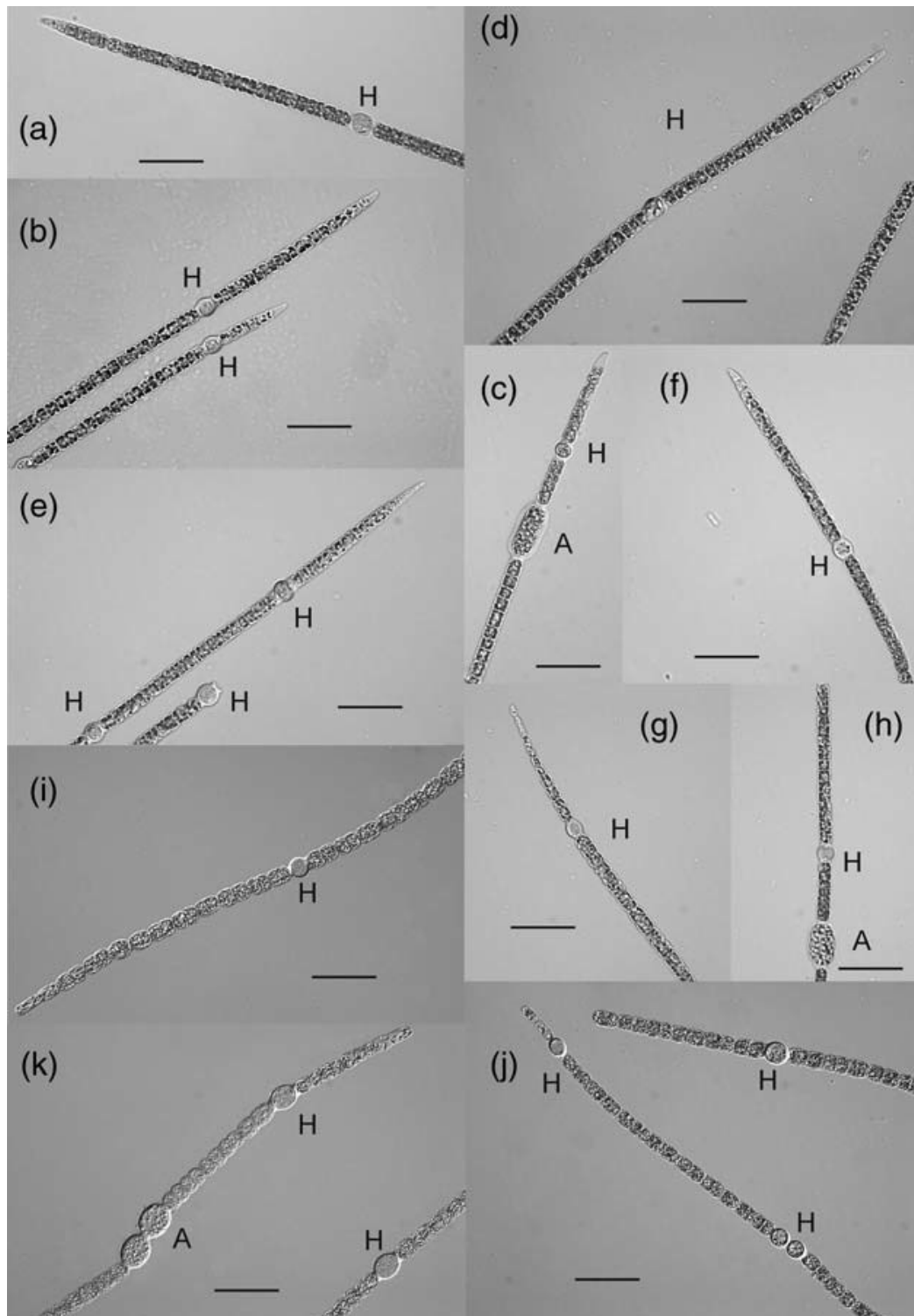


Figure 5.1 Micrographs of *Anabaena bergii* strains (a) AB2010/02, (b) AB2010/03, (c) AB2010/07, (d) AB2010/08 and *Aphanizomenon ovalisporum* strains (i) AB2010/05, (j) AB2010/06, (k) ILC-164 from Lake Kinneret and *Anabaena bergii* strains (e) AB2008/44, (f) AB2008/45, (g and h) AB2008/43 from Lake Melangsee; A, akinete, H, heterocyte; Scale bars indicate 25  $\mu$ m.

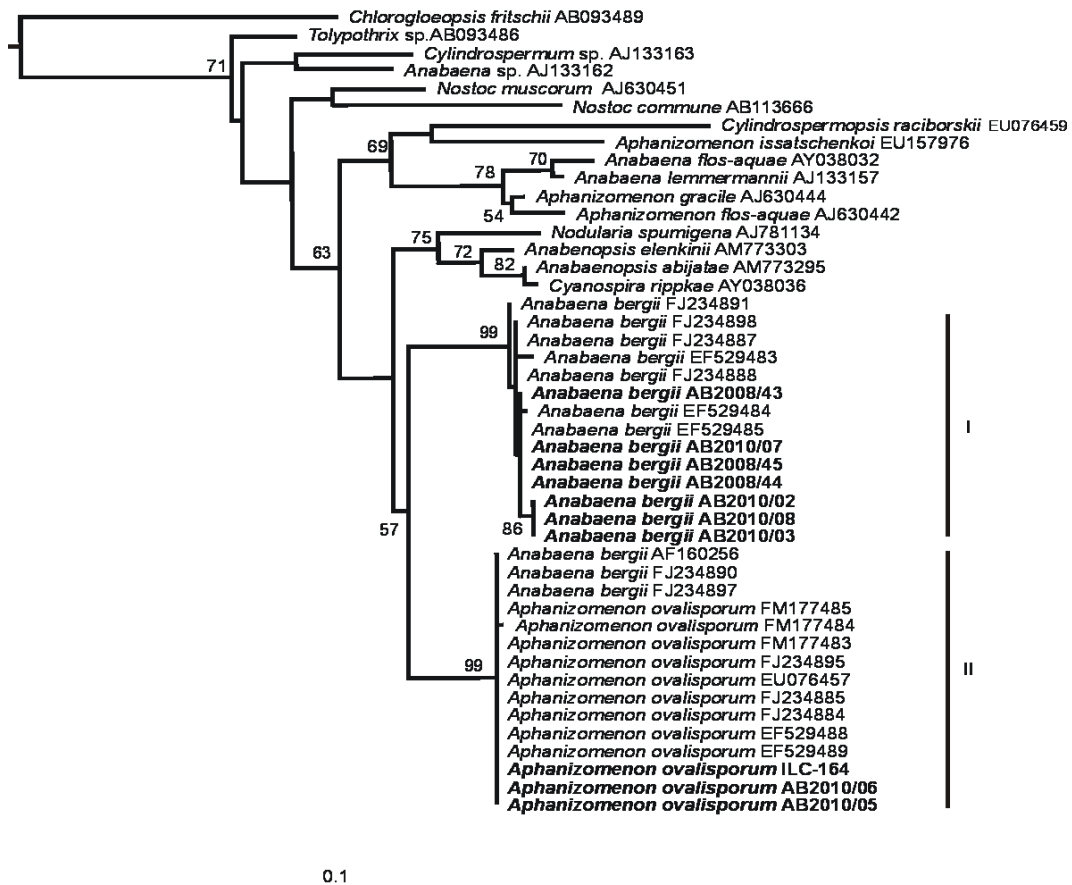


Figure 5. 2 Maximum likelihood tree based on the partial 16S rRNA gene sequences of 52 Nostocales strains. Strains from this study are marked in bold. Outgroup = *Microcystis aeruginosa* (AB035549). Bootstrap values above 50 are included. The scale bar indicates 10 % sequence divergence.

## 5.5 Discussion

Our study is the first to report the presence of non-CYN-producing *Aphanizomenon ovalisporum* and the presence of *Anabaena bergii* in the phytoplankton community of Lake Kinneret.

*Aphanizomenon ovalisporum* was recorded in Lake Kinneret for the first time in 1994 forming a prominent toxic bloom (Banker et al. 1997; Pollingher et al. 1998). Smaller blooms caused by *Aphanizomenon ovalisporum* were observed in the following years (Hadas et al. 2002). A CYN-producing *Aphanizomenon ovalisporum* strain (ILC-164) has been isolated from the 1994 bloom (Pollingher et al. 1998). *Anabaena bergii* has not been mentioned as a member of the phytoplankton community in Lake Kinneret so far.

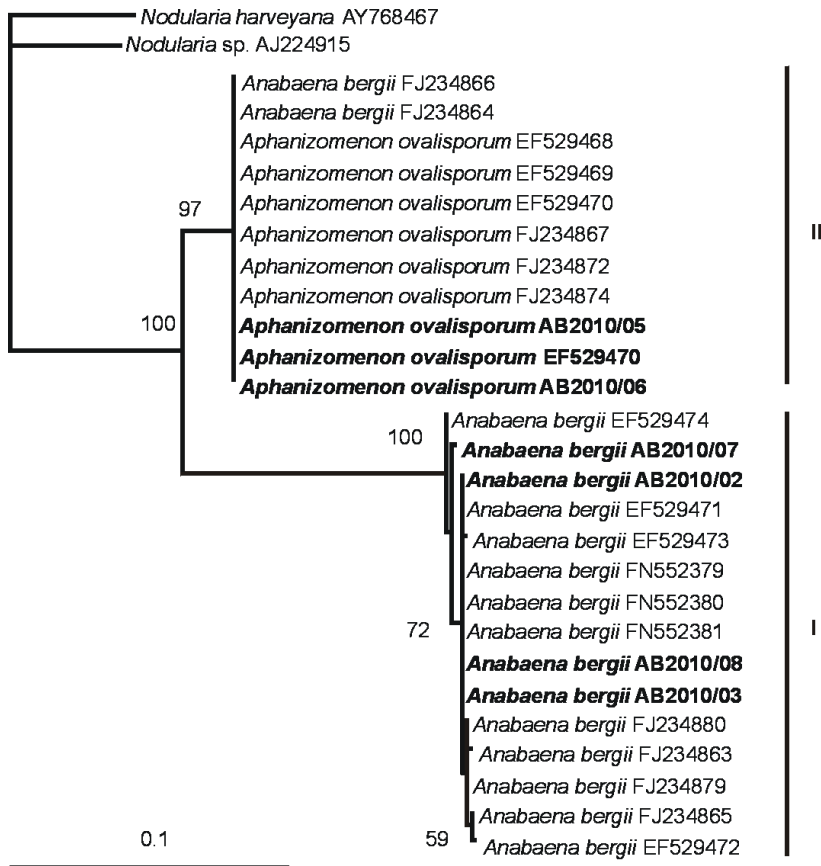


Figure 5. 3 Maximum likelihood tree based on partial PC-IGS gene sequences of 28 Nostocales strains. Strains from this study are marked in bold. Bootstrap values above 50 are included. The scale bar indicates 10 % sequence divergence.

These two species are difficult to distinguish by morphological criteria and can easily be misidentified. Hence, *Anabaena bergii* might have contributed to the phytoplankton community of Lake Kinneret previously.

The assignment of *Aphanizomenon ovalisporum* and *Anabaena bergii* into one single species has been suggested (Komárek and Ettl 1958; Yilmaz et al. 2008). However, Stüken et al. (2009) have clearly demonstrated that both species are closely related but morphologically and genetically distinct. Our study confirms the findings of Stüken et al. (2009). Similarly, we found the vegetative cells of all *Aphanizomenon ovalisporum* strains (AB2010/05 and AB2010/06) to be longer than wide and of all *Anabaena bergii* strains to be wider than long. Interestingly, in *Aphanizomenon ovalisporum* strain ILC-164, the vegetative cells were wider than long. This may be related to the extended period of laboratory cultivation, as the strain was already isolated in 1994 from Lake Kinneret by Hadas, O. Several studies have shown that cyanobacterial cultures tend to change morphologically

with time and with changing culture conditions, and can differ from strains growing under natural conditions (Laamanen et al. 2002; Zapomělová et al. 2008). Another feature distinguishing both species was the form of the apical cells. In the case of *Aphanizomenon ovalisporum* (strain ILC-164 and the newly isolated strains), the end cells were bluntly rounded, which contrasts with the conically elongated apical cells of the *Anabaena bergii* strains (Table 5.1). The same characteristic was also described by Stüken et al. (2009). Accordingly, the putative *Aphanizomenon ovalisporum* strains isolated from Lake Kinneret were identified as *Aphanizomenon ovalisporum* and *Anabaena bergii* (Figure 5.1). The morphological differences are supported by the phylogenetic 16S rRNA gene and PC-IGS trees (Figures 5.2 and 5.3). In the 16S rRNA gene tree and the PC-IGS trees, cluster I comprises *Anabaena bergii* strains and cluster II comprises *Aphanizomenon ovalisporum* strains. Both distinct clusters reflect the obvious morphological differences between the *Aphanizomenon ovalisporum* and *Anabaena bergii* strains observed in this study. However, p-distances between the 16S rRNA gene sequences of cluster I (*Anabaena bergii*) and cluster II (*Aphanizomenon ovalisporum*), which were in the range of 97.3–97.6 %, confirm the close relationship of both species (Table 5.3).

**Table 5.3** Calculated 16S rRNA gene sequence similarities between cluster I and II (Figure 5.2)

Clusters 16S rDNA sequences (1142 bp)	Cluster I	Cluster II
Cluster I	99.5 – 100	97.3–97.6
Cluster II	97.3–97.6	99.7 - 100

Studies by Stackebrandt and Goebel (1994) and Hagström et al. (2002) have shown that a 16S rRNA gene sequence similarity of 97 % is a reasonable level for grouping bacteria into species. However, Oren (2004) has identified different bacterial species sharing identical 16S rRNA genes. Accordingly, closely related species may not always be recognizable using 16S rRNA as a genetic marker and 16S rRNA gene sequence analyses are not in each case a foolproof criterion to support species identity (Fox et al. 1992; Oren 2004).

The six *Aphanizomenon ovalisporum* and *Anabaena bergii* strains isolated from Lake Kinneret were investigated with ELISA and LC-MS/MS for CYN production and tested negative. Our study is the first to describe the presence of

non-CYN-producing *Aphanizomenon ovalisporum* strains in Lake Kinneret. Former studies conducted in Europe, Asia and Australia have always found *Aphanizomenon ovalisporum* strains to produce CYN (Schembri et al. 2001; Wormer et al. 2008; Yilmaz et al. 2008). As depicted in the 16S rRNA and PC-IGS trees, the non-toxic *Aphanizomenon ovalisporum* strains isolated in our study cannot be distinguished phylogenetically from *Aphanizomenon ovalisporum* strain ILC-164, which was found to produce CYN- and 7-epicylindrospermopsin (Banker et al. 1997, 2000).

In other studies, the entire putative CYN gene clusters from the Australian *Cylindrospermopsis raciborskii* strain AWT205 and the German *Aphanizomenon* sp. strain 10E6 have been characterized (Mihali et al. 2008; Stüken and Jacobsen 2010). Parts of the CYN-gene cluster were identified in CYN-producing *Aphanizomenon ovalisporum* strain ILC-164 from Lake Kinneret (Shalev-Alon et al. 2002). Stüken and Jacobsen (2010) recently showed that the CYN gene clusters of *Cylindrospermopsis raciborskii* strain AWT205, *Aphanizomenon* sp. strain 10E6 and *Aphanizomenon ovalisporum* strain ILC-164 had a high gene sequence similarity, but with an extensive rearrangement of gene order.

Regarding *Anabaena bergii*, all isolated strains have either been found to be non-CYN-producing or in the case of CYN production they had to be renamed as *Aphanizomenon ovalisporum* (Ballot et al. 2010; Fergusson and Saint 2000; Stüken et al. 2009). Other toxins have not been described from *Anabaena bergii* so far.

Interestingly, we found parts of the CYN gene cluster (*aoaA/cyrA*, *aoaB/cyrB* and *aoaC/cyrC*) in all our non-CYN-producing *Aphanizomenon ovalisporum* and *Anabaena bergii* strains from Lake Kinneret. However, only the positive control *Aphanizomenon ovalisporum* strain ILC-164 showed amplification products using a PCR protocol according to Schembri et al. (2001). All other *Aphanizomenon ovalisporum* and *Anabaena bergii* strains showed only amplification products of *aoaA/cyrA*, *aoaB/cyrB* and *aoaC/cyrC* when a prolonged PCR protocol with 35 cycles was applied, followed by a second similar PCR using the amplification product of the first PCR as a template and the same primers. This includes also *Anabaena bergii* strains AB2008/43, AB2008/44 and AB2008/45, which were recently tested negative by Ballot et al. (2010) for the PS gene (*aoaB/cyrB*) using the protocol of Schembri et al. (2001). Sequences of *aoaA/cyrA* (185 bp) and *aoaB/cyrB* (2238 bp) were obtained for *Aphanizomenon ovalisporum* ILC-164 and three German *Anabaena bergii* strains but not for the other strains isolated from Lake Kinneret. That implies that further work is required for absolute

confirmation that the six *Aphanizomenon ovalisporum* and *Anabaena bergii* strains isolated from Lake Kinneret possess *aoaA/cyrA* and *aoaB/cyrB* genes. However, a short fragment of the *aoaC/cyrC* gene was sequenced for all 10 *Aphanizomenon ovalisporum* and *Anabaena bergii* strains. All sequences are characterized by 100 % similarity to the corresponding gene fragment of CYN-producing *Aphanizomenon ovalisporum* strain ILC-164. *AoaA/cyrA* spans 1176 bp and encodes an amidinotransferase which is involved in the first step of the formation of the carbon skeleton of CYN (Mihali et al. 2008). The *aoaB/cyrB* gene spans 8.7 kb and catalyzes the next step in the CYN synthesis. It encodes a PKS/NRPS hybrid (Mihali et al. 2008). The *cyrC* gene spans 5.0 kb and encodes a PKS (Mihali et al. 2008).

Other studies have shown the presence of parts of *aoaA/cyrA*, *aoaB/cyrB* and *aoaC/cyrC* genes associated with the biosynthesis of CYN in non-CYN-producing cyanobacterial strains such as *Anabaena bergii* strain ANA360H or *Aphanizomenon issatschenkoi* strain CAWBG02 (Rasmussen et al. 2008; Wood et al. 2007). Mihali et al. (2008) have found that the *cyrJ* gene is only present in the genome of CYN-producing cyanobacteria, suggesting it as a good genetic marker. In their study, it was shown that six CYN-producing *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* strains possessed the *cyrJ* gene while nine non-CYN-producing *Cylindrospermopsis raciborskii* and *A. circinalis* did not possess the *cyrJ* gene. *CyrJ* is supposed to be a sulfotransferase-encoding gene involved in CYN synthesis and it has a size of 780 bp (Mihali et al. 2008). Mazmouz et al. (2010) confirmed the presence of the *cyrJ* gene in four CYN-producing *Oscillatoria* strains and the lack of this gene in one non-CYN-producing *Oscillatoria* strain. In our study, only CYN-producing *Aphanizomenon ovalisporum* strain ILC-164 showed an amplification product for the *cyrJ* gene. All non-CYN-producing strains of this study lacked the *cyrJ* gene, supporting the results of Mihali et al. (2008) and Mazmouz et al. (2010).

The coexistence of toxic and non-toxic cyanobacterial strains was first described for microcystin- and non-microcystin-producing *Microcystis* strains (Meißner et al. 1996). It was later also confirmed for other cyanobacteria, e.g. *Nodularia* (nodularin), *Cylindrospermopsis* (CYN), *Aphanizomenon gracile* (paralytic shellfish poisoning (PSP) toxins) and *Aphanizomenon issatschenkoi* (anatoxin-a) (Ballot et al. 2010; Gugger et al. 2005; Laamanen et al. 2001; Wood et al. 2007). Nonetheless, genes involved in the biosynthesis of different cyanobacterial toxins, e.g. microcystins or PSP toxins, have been found in toxin- and in non-toxin-producing cyanobacteria belonging to various genera (Ballot et al. 2010; Mbedi et al. 2005; Mikalsen et al. 2003; Moustafa et al. 2009). Mechanisms such as



horizontal gene transfer, mutations and insertions/deletions have been proposed as leading to the coexistence of toxin- and non-toxin-producing cyanobacteria (Christiansen et al. 2008; Moustafa et al. 2009; Tooming-Klunderud et al. 2008).

In conclusion, this is the first study which describes the presence of non-CYN-producing *Aphanizomenon ovalisporum* strains and of *Anabaena bergii* strains in Lake Kinneret. Although none of the strains investigated showed production of CYN, all strains contained *aoaA/cyrA*, *aoaB/cyrB* and *aoaC/cyrC* segments of the CYN gene cluster but lacked the *cyrJ* gene of the CYN gene cluster.

## 6. Conclusions and outlook

### 6.1 Conclusions

This thesis confirmed that Nostocales species are distributed and further proliferate in deep lakes of temperate and subtropical regions. The key findings of this study are summarized hereinafter. Only a small percentage (10-20 %) of sedimentary akinete pool was estimated to potentially contribute as inoculum to pelagic population in deep temperate lakes. Different strategies of Nostocales to hibernate were verified the first time in temperate lakes. Overwintering can be conducted exclusively as vegetative and dormant cells or intermediate. A new method for better visual akinete identification and faster detection in sediment samples was successfully developed and applied. *Anabaena bergii* was observed for the first time in Lake Kinneret. To further conclude this thesis, questions raised in the introduction will attempt to be answered.

**Chapter 2 - Do seasonal and spatial variability occur in the akinete abundance in the sediments of deep stratified lakes depending on water depth and lake basin morphometry? Which sediment surface areas can potentially contribute as sedimentary akinete inoculum to pelagic population in spring?**

Akinete formation and sedimentation in autumn and winter, as well as germination in spring and summer causes fluctuations in akinete abundance in sediment surfaces. The akinete abundance at the sediment surface is also positively correlated with depth of above laying water column. Since akinetes do not germinate in the absence of light only those akinetes deposited in littoral sediments are relevant as inoculum for the pelagic population. In the lakes studied about 10 % – 20 % of the viable deposited akinetes are located in available sediment surfaces of assumed light and temperature conditions for germination.

The differences in species composition of the akinete pool between the polymictic and the dimictic basin of Lake Scharmützelsee highlight the occurrence of horizontal transport at the sediment surface, especially due to the fact that the pelagic Nostocales species composition is very similar in both basins.

Viable akinetes are only abundant in the uppermost part of a vertical sediment profile. The total amount of sedimented akinetes seems to be influenced by trophic conditions in lake, because fossil akinetes remaining along a vertical profile are abundant in sediment layers deposited during time of enhanced trophy.

Akinetes in sediments of deeper water depths or deeper sediment regions can contribute either to predict and early detect potentially new or invasive species or to reconstruct Nostocales populations.

**Chapter 3 - Could akinetes be identified and enumerated in deep lake sediments via a developed catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) method? Is this method a useful tool for akinete enumeration and more time advantageous versus the Utermöhl method (1958)?**

A CARD-FISH method was established to identify and quantify akinetes in sediment samples. The method presents a decisive time advantage due to better visibility of akinetes in sediment samples by their fluorescence. Therefore, locating and identifying of akinetes is improved by the combination of fluorescence signal and morphological characteristics. The CARD-FISH and the Utermöhl method, both yield similar counting results for akinetes in sediment samples. Due to minimized expenditure of time for microscopical counting CARD-FISH reduces expense, although sample preparation is complex and materials are expensive. This method can be further modified and used to estimate potential inoculum size of sedimentary akinete pool in sediments or for labelling specific Nostocales species of interest.

**Chapter 4 - Do species- or genus-specific differences exist in the overwintering strategies of Nostocales in the deep and shallow lakes studied? Can the detection limit of filaments in winter plankton samples be reduced by enrichment?**

Different mechanisms for overwintering of Nostocales, as described in marine systems, were verified in the limnic environments studied. The detection of vegetative cells of Nostocales in samples throughout the winter documents the persistence of a pelagic population all year round. *Anabaena* spp. overwinters exclusively as akinetes. While *Aphanizomenon* spp. forms akinetes, their vegetative cells can be found in the pelagic zone during winter even though in low abundances. The population of *Aphanizomenon flos-aquae* growing in Lake Stechlinsee does not form any dormant cells, therefore overwinters as vegetative state. Akinetes are of essential ecological meaning for next year population development in lakes with short water retention time of less than half a year, since vegetative population is washed out during winter. The findings indicate that especially for *Anabaena* spp. akinetes are mandatory in all lake types studied.

To return to our second question, low abundances of Nostocales filaments can be better assessed by enrichment of the plankton during winter in order to lower detection limit.

**Chapter 5 - Do non-CYN-producing strains coexist with toxic strains of *Aphanizomenon ovalisporum* in Lake Kinneret? Can we find any evidence of other CYN-producing Nostocales species in this lake?**

Putative CYN-producing strains of *Aphanizomenon ovalisporum* have been isolated in Lake Kinneret. Non-CYN-producing strains of *Aphanizomenon ovalisporum* and *Anabaena bergii* have been observed for the first time in Lake Kinneret. The new detected species *Anabaena bergii* differs morphologically and phylogenetically from *Aphanizomenon ovalisporum*. In all strains no CYN production can be observed due to lack of *cyrJ* gene, although gene fragments involved in toxin synthesis are present. Coexistence of toxic and non-toxic Nostocales strains in lakes reveals the necessity to isolate various strains of one species in order to conclude about potential toxicity. The ecological mechanisms regulating CYN production in lakes need to be investigated.

## **6.2 Outlook**

The main findings of this thesis can be included in further investigations concerning management of deep stratified water bodies. Nutrient concentrations in subtropical and temperate lakes need to be further reduced to control cyanobacterial dominance. Nevertheless, the population growth of nitrogen fixing cyanobacteria cannot be limited by a deficiency of dissolved inorganic nitrogen, and thus, they are competitively favored. Hence, reducing nitrogen inputs could intensify the relative proportion and dominance of Nostocales on total phytoplankton biomass and enhancing expansion of high adaptable potentially toxic invasive species. External and internal sources of phosphorus ought to be further controlled and reduced and must be one crucial management step.

As drinking water production is restricted by blooms of Nostocales, which cause depletion of oxygen and high concentrations of secondary metabolites like CYN, regulating measures on Nostocales population could be necessary to ensure basic services. The knowledge of the distribution of akinetes in sediments as inoculum is valuable to plan these regulating activities. Early intervention on resuspension could be feasible to influence success of akinete germination in littoral sediments.

The trend of rising temperatures in the course of global climate change in deep lakes can positively affect the proliferation of Nostocales species and cause changes in their overwintering strategies. Warming winters could lead to a

shifting of vegetative population, thus, loss rates will be lower. This suggests a higher phytoplankton biomass of nostocalean cyanobacteria and can change physical factors and processes such as transparency and ratio of stratification. The potential inoculum size of sedimentary akinete pool might be influenced under changed germination conditions, higher turbidity and turbulences in the lakes.

Current mathematical models of Nostocales life cycle (Hellweger et al. 2008; Jöhnk et al. 2011; Wiedner et al. 2007) considering akinetes and loss rates as parameters in shallow lakes or reservoirs. Modelling of a general simple life cycle of Nostocales in deep stratified lakes requires the most difficult part concerning different overwintering strategies and annual variations of population dynamics.

Further investigations ought to be conducted on other lake morphotypes to study variability in Nostocales species composition and to simulate impact of lake basin morphometry on inoculum of sedimentary akinete pool. Future analysis could use a modified CARD-FISH protocol to estimate occasionally occurring filaments in winter plankton samples. Due to differences figured out in akinete appearance of *Aphanizomenon flos-aquae* in the lakes studied, it would be worthwhile to analyze strain-specific genetic variances in akinete formation. Hence, it would be interesting to find out which genes are involved in formation processes and how they are functioning. The question remains open whether Nostocales species without akinete formation can be taxonomically differentiated.

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## Appendix

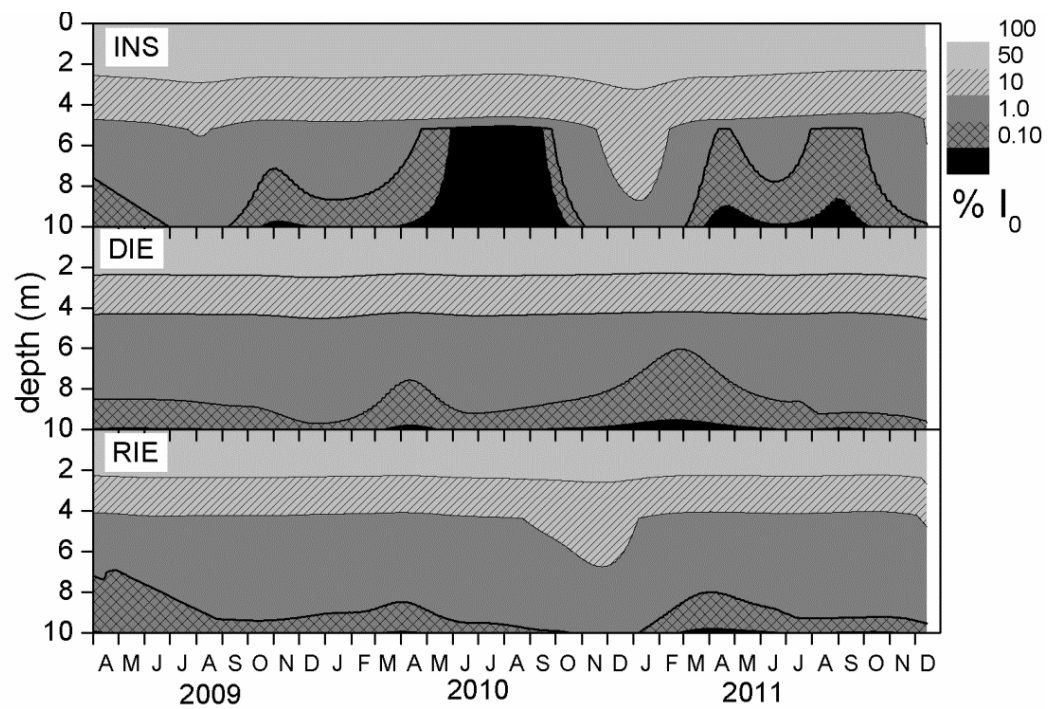


Figure 6.1 Calculated light intensity ( $\% I_0$  – global radiation) in different water depths of the Northern Bay (INS), Middle Basin (DIE) and Southern Basin (RIE) of Lake Scharmützel in the years 2009-2011.

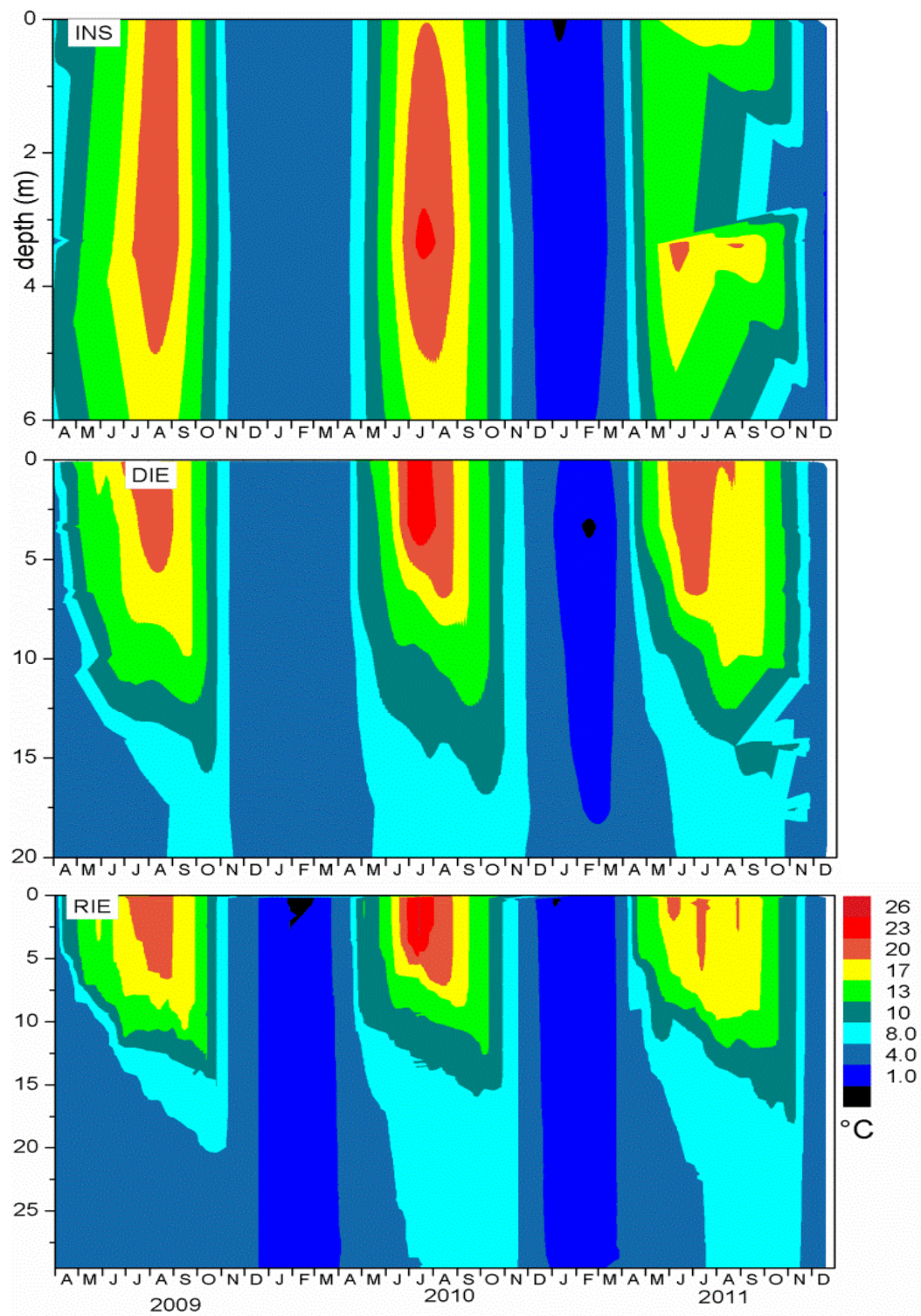


Figure 2. Vertical profiles of temperature in the Northern Bay (INS), Middle Basin (DIE) and Southern Basin (RIE) of Lake Scharmützelsee during the years 2009-2011.



