JOURNAL OF PLANKTON RESEARCH | VOLUME 32 | NUMBER 6 | PAGES 927–936 | 2010

Polyphasic evaluation of *Aphanizomenon issatschenkoi* and *Raphidiopsis mediterranea* in a Mediterranean lake

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Received November 24, 2009; accepted in principle February 3, 2010; accepted for publication February 7, 2010

Corresponding editor: John Dolan

Aphanizomenon issatschenkoi and *Raphidiopsis mediterranea* were studied in the Mediterranean Lake Doirani during a cyanobacterial bloom (August–October 2004). *Aphanizomenon issatschenkoi* was clearly morphologically distinct from *R. mediterranea* on the basis of apical cells. The two species demonstrated an almost inverse temporal distribution, consistent preference for different water depths and negative correlation between their net growth rates. *Raphidiopsis mediterranea* exhibited a higher net growth rate (0.17 $days^{-1}$) and dominated during warmer water periods, with high Z_{max}/Z_{sec} ratio and low but available inorganic nitrogen. *Aphanizomenon issatschenkoi* was more generalist, exhibiting slower net growth (0.10 $days^{-1}$) and a broader distribution in relation to temperature and light, regularly forming heterocytes under low nitrogen conditions. *Aphanizomenon issatschenkoi* was also phylogenetically distinct from *R. mediterranea* on the basis of partial 16S rRNA gene sequence. The *R. mediterranea* typical morphotype corresponded to one phylotype in the well-defined 16S rRNA gene sequence cluster of *Cylindrospermopsis raciborskii*. The formation of two specialized reproductive cells in the same individual of *R. mediterranea* is described for the first time and may indicate unknown alternative reproductive modes and lifestyles in planktonic Cyanobacteria.

KEYWORDS: alternative reproductive modes; *Aphanizomenon issatschenkoi*; cyanobacterial bloom; polyphasic taxonomy; *Raphidiopsis mediterranea*

INTRODUCTION

Cyanobacterial blooms, including toxic species, are a water quality problem of great concern both for lake ecological status and for human health. *Aphanizomenon issatschenkoi* and *Raphidiopsis mediterranea* are among the known toxic cyanobacteria forming blooms, while recent reports describe newly discovered toxic strains of these species (Wood *et al.*, 2007; Li *et al.*, 2008). However, it is known that a large number of cyanobacterial strains have been misidentified (Komárek and Anagnostidis, 1989). This may result in confusion regarding the identity of the toxic strains. Wood *et al.*

doi:10.1093/plankt/fbq019, available online at www.plankt.oxfordjournals.org. Advance Access publication March 2, 2010

(Wood *et al.*, 2007) highlighted the problems associated with morphological misindentifications and demonstrated the added value of a polyphasic approach. However, polyphasic evaluation of these cyanobacteria was focused on culture strains (Wood *et al.*, 2007; Li *et al.*, 2008), largely ignoring their natural populations (Moustaka-Gouni *et al.*, 2009). Nevertheless, we still do not understand what a bacterial species is. For several practical applications, it is necessary to define species in a coherent fashion by combining phenotypic/genetic diversity and distinct ecology (Fraser *et al.*, 2009).

In the Botanical Classification System (Komárek and Anagnostidis, 1989), the genera *Raphidiopsis* and *Aphanizomenon* belong to the order Nostocales. Under the Bacteriological Code, *Aphanizomenon* belongs to the subsection IV of the cyanobacteria (Rippka *et al.*, 2001), whereas *Raphidiopsis* has not yet been given valid published names. Recently, molecular analysis has shown that *Aphanizomenon* is a heterogeneous genus, and consequently, *A. issatschenkoi* has been revised as *Cuspidothrix issatschenkoi* (Rajaniemi *et al.*, 2005). In contrast, morphological and phylogenetic analyses suggest that *Raphidiopsis* and *Cylindrospermopsis* represent the same genus in Lake Kastoria (Moustaka-Gouni *et al.*, 2009).

Raphidiopsis mediterranea usually accompanies blooms of *Cylindrospermopsis raciborskii* (Skuja, 1937; Hill, 1970; Moustaka-Gouni *et al.*, 2009). Also, *A. issatschenkoi* has been observed to grow together with these species in tropical, Mediterranean and northern temperate freshwaters (Hindák and Moustaka, 1988; Fabbro and Duivenvoorden, 2000; Mischke, 2003). *Aphanizomenon issatschenkoi* is a member of the functional group H1, a diazotroph tolerant of low nitrogen and sensitive to mixing and low light conditions (Reynolds *et al.*, 2002). Modification of *R. mediterranea* from functional group S2 of Oscillatoriales to S_N of *C. raciborskii* (with mixed attributes of Nostocales and Oscillatoriales) has recently been suggested (Padisák *et al.*, 2009).

Until recently, biogeographic studies were mostly focused on macroorganisms, largely ignoring microorganisms (Hughes Martiny *et al.*, 2006). To better understand biogeography and the successful spread of cyanobacterial species to temperate freshwaters during the twentieth century (Padisák, 1997; Neilan *et al.*, 2003; Haande *et al.*, 2008), there is a clear need to provide a coherent taxonomy for natural populations, particularly for those of the closely related species *A. issatschenkoi*, *R. mediterranea* and *C. raciborskii*. In nearby Lake Kastoria, type locality of *R. mediterranea*, morphological and phylogenetic evidence suggest that this species represents non-heterocytous life-cycle stages of *C. raciborskii* (Moustaka-Gouni *et al.*, 2009). In another locality (Lake Hakanoa, New Zealand), phylogenetic analysis showed that non-heterocytous *A. issatschenkoi* was morphologically misidentified as *R. mediterranea* (Wood *et al.*, 2007).

Aphanizomenon issatschenkoi and *R. mediterranea* dominated in the Mediterranean Lake Doirani, providing us the opportunity to examine their relations in natural populations. This is the first paper that combines morphological, phylogenetic and ecological features of these closely related populations and evaluates their position with a polyphasic approach, including reproductive features.

METHOD

Lake Doirani (41°11′ N, 22°45′ E, altitude of 140 m) is traversed by the border between Greece and FYROM (Former Yugoslav Republic of Macedonia). It has a surface area of 28 km^2, maximum depth of ~5.0 m and a long water retention time (>2 years). It is a karstic lake, and is a remnant of a much larger Pleistocene lake, the Peonie (Stanković, 1931). Since 1986, there has been a drastic drop of 3 m in the water level of the lake. It is a polymictic or a warm monomictic lake in very warm and dry years. It is located in a semi-arid Mediterranean area. In 2004, the summer was dry (July–August rainfall: 17 mm), whereas September was rainy (77 mm) and warm (air temperature at noon in the range of 22.2–32.4°C, mean 27.2°C). Weather during this period was characterized by calm days and weak winds (except for the first week of August when wind speed reached 10 m s^{-1}) and few windy days late in August (max. 7 m s^{-1}) and early September (max. 10 m s^{-1}) (Hellenic National Meteorological Service data). Phytoplankton studies in Lake Doirani are scarce (Temponeras *et al.*, 2000a, b), although floristic data were published in the early twentieth century (Schröder, 1921; Stanković, 1931). Recent studies on toxic cyanobacterial blooms and cyanotoxins in Greek freshwaters have shown that toxic cyanobacteria are members of the lake's phytoplankton (Vardaka *et al.*, 2005; Gkelis, 2006).

Sampling was conducted at a fixed site in the deep part of the lake (max. depth, z_{max} 4.7 m) and carried out weekly from August 5 to October 10 of 2004. Water temperature, pH and transparency (Secchi depth, z_{sec}) were measured *in situ* using a portable WTW type pH-meter and a Secchi disk. The z_{max}/z_{sec} ratio characterizes the optical properties of the lake and represents the proportion of the euphotic zone depth. Water samples were collected at three discrete depths (0.0–1.2, 1.5–2.7 and 3.0–4.2 m) in the water column using a 1.2 m long water sampler. A set of three samples was collected from each depth and mixed in a plastic container. From each mixed sample, four subsamples were taken. Sub-samples were examined within

3 h under a light microscope (fresh samples). Sub-samples were also preserved in Lugol's and formaldehyde solution (preserved samples). Sub-samples for 16S rRNA were stored in sterile polyethylene bottles and kept under cool and dark conditions until return to the laboratory (<1 h).

Soluble reactive phosphorous (SRP), nitrate (NO_3–N), nitrite (NO_2–N) (APHA, 1985) and ammonium nitrogen (NH_4–N) (Grasshoff, 1976) concentrations were measured in Whatman GF/C filtered water samples. Dissolved inorganic nitrogen (DIN) expresses the total nitrate, nitrite and ammonium nitrogen.

Fresh and preserved samples were examined using an inverted microscope (Nikon SE 2000) with phase-contrast, and photographs were taken using a digital camera (Nikon DS-L1).

Species were identified using the taxonomic papers of Skuja (Skuja, 1937) and Hindák and Moustaka (Hindák and Moustaka, 1988) and the classification system of Komárek and Anagnostidis (Komárek and Anagnostidis, 1989). Phytoplankton counts (trichomes) were performed using 2 mL sedimentation chambers with the inverted microscope method. At least 400 individuals (trichomes) of each *A. issatschenkoi* and *R. mediterranea* were counted in each sub-sample to calculate population density, which yields a precision of ±10% within 95% confidence limits (Lund *et al.*, 1958). Mean trichome biovolume was estimated using geometric formulae after measuring the dimensions of 30 individuals. Biomass was calculated by multiplying the population density of each species by the mean biovolume of its trichomes by assuming a specific density of cyanobacterial cells of 1 g cm^{-3}. Trichomes of the typical *R. mediterranea* morphotype (>98% of the total) and very few trichomes which were indistinguishable from non-heterocytous *C. raciborskii, Raphidiopsis*-like trichomes, were counted together and assigned to morphospecies *R. mediterranea*.

Population net growth rate (r) was determined from changes in the population biomass between two consecutive samples (B_i, B_{i-1}) collected at the time t_i and t_{i-1} (in days) according to the equation:

$$r = \frac{\ln B_i - \ln B_{i-1}}{t_i - t_{i-1}} \ (\text{days}^{-1})$$

The following parameters were selected to describe the morphology and morphometry of the species studied: the trichome shape, length and width, the presence or absence of terminal heterocytes, the number and shape of trichome apical cells, vegetative cells, heterocytes and akinetes. At least 300 measurements of the trichome length and 100 measurements of the trichome width were made for each of the species *A. issatschenkoi* and *R. mediterranea*.

Upon return to the laboratory, 100 mL of lake water was filtered on a Whatman GF/C filter and the filter was stored at −20°C. DNA was extracted using the UltraClean Soil DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's protocol after slicing the filters with a sterile scalpel.

For the cyanobacterial 16S rRNA PCR amplification, we used 0.5 μL of the DNA template and the cyano-specific primers CYA106f (5′-CGGACGGGTG AGTAACGCGTGA-3′) and an equimolar mixture of CYA781r (a) GACTACTGGGGTATCTAATCCCATT) and CYA781r (b) (5′-GACTACAGGGGTATCTAAT CCCTTT-3′) (Nübel *et al.*, 1997). All amplification reactions (25 μL) contained 10–20 ng genomic DNA, determined with the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington), as template, each primer at a concentration of 0.5 μM and PCR buffer supplied with the GoTaq polymerase (Promega, USA) enzyme (2.5 U), dNTPs (200 μM) and $MgCl_2$ (1.5 μM). PCR conditions were similar to those described by Berger *et al.* (Berger *et al.*, 2006) for cyanobacteria. We applied 24 PCR cycles for each sample, after we performed cycle optimization (i.e. to use the lowest number of PCR cycles giving a visible PCR product instead of the commonly used 30 cycles) in order to eliminate PCR innate limitations (Spiegelman *et al.*, 2005) and to achieve less differential representation of 16S rDNA genes with low (i.e. underrepresented phylotypes) and high (i.e. abundant phylotypes) copy numbers in the clone libraries. Amplified DNA was separated by 1.2% w/v agarose gel electrophoresis run at 70 V for 45 min in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 7.8) and visualized by UV transillumination after staining with ethidium bromide (0.5 μg mL^{-1}).

The PCR products were purified using the Montage purification kit (Millipore, USA) and the purified PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen, USA) using chemically competent cells according to the manufacturer's specifications. Approximately 70 clones were randomly selected and checked to make sure that they had the correct insert size (ca. 680 bp). All positive clones were grown in liquid LB medium with 50 μg mL^{-1} kanamycin and their plasmids were purified using the Nucleospin Plasmid QuickPure kit (Macherey-Nagel, Germany). DNA sequencing was done by Macrogen (Korea) using capillary electrophoresis by the BigDye Terminator kit (Applied Biosystems Inc., USA) with the primer M13F (5′-GTAAAACGACGGCC AG-3′). Each read was ~850 bp.

The retrieved sequences were compared with those in the GenBank databases by BLAST (Altschul *et al.*, 1990). Sequences with ≥98% similarity were grouped as identical operational taxonomic units (OTU).

Detection of chimeric DNAs was performed by the CHECK-CHIMERA program of the Ribosomal Database Project (Maidak *et al.*, 2001), and all chimeras found were excluded from further analysis. In this study, we report only the sequences which were phylogenetically affiliated to the *A. issatschenkoi* and *Raphidiopsis/Cylindrospermopsis* group of species. The 16S rRNA of these sequences has GenBank accession numbers FJ204842 and FJ204843.

Sequence data were compiled using the MEGA4 software (Tamura *et al.*, 2007) and aligned with sequences obtained from the GenBank (www.ncbi.nlm.nih.gov) databases, using the ClustalX aligning utility. Phylogenetic analyses were performed using minimum evolution and parsimony methods implemented in MEGA4 (Tamura *et al.*, 2007). Heuristic searches under minimum evolution criteria used 1000 random-addition replicates per data set, each followed by tree bisection-reconnection topological rearrangements. The topology of the tree was based on neighbour-joining according to Jukes-Cantor. Bootstrapping under parsimony criteria was performed with 1000 replicates.

Spearman's correlation coefficient was used to examine correlation between physical–chemical parameters (water temperature, Z_{max}/Z_{sec}, DIN, ammonium nitrogen, N:P atomic ratio) and biological parameters (*R. mediterranea* and *A. issatschenkoi* biomass) or between biological parameters (Sokal and Rohlf, 1981). Vertical differences between mean values of chemical parameters were assessed using analysis of variance (ANOVA), followed by a multiple comparison test (LSD) (Sokal and Rohlf, 1981). Canonical correspondence analysis (CCA) was performed in order to investigate the vertical distribution of *R. mediterranea*, *A. issatschenkoi* and physical–chemical parameters (water temperature, pH, DIN, SRP) (Legendre and Legendre, 1998). For this, ordination was produced using mean water depth values of each parameter over the period of study. The relationship between the net growth rate of *R. mediterranea* and *A. issatschenkoi* at each water depth was analysed using linear correlation analysis (Sokal and Rohlf, 1981). Significant relationships were defined as $P < 0.05$.

RESULTS

Micrographs of the trichomes corresponding to the morphospecies *A. issatschenkoi* and *R. mediterranea* are shown in Fig. 1. *Aphanizomenon issatschenkoi* trichomes were solitary, straight or slightly curved, generally constricted at the cross walls (Fig. 1A_{1-8}), short to moderate length (range 61.0–280.3 μm, mean 171.3 μm) and narrow (range 1.6–2.9 μm, mean 2.1 μm), apical cells 1–3 in number,

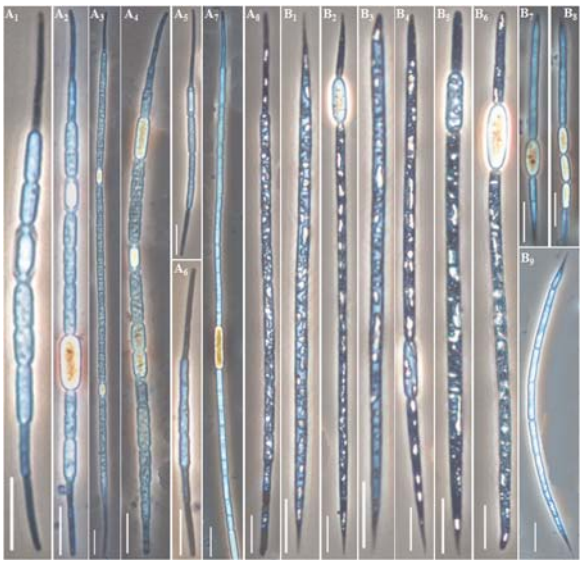

Fig. 1. *Aphanizomenon issatschenkoi* and *R. mediterranea* morphotypes. (A_1–B_9) Light micrographs (phase contrast) of trichomes. (A_{1-8}) *Aphanizomenon issatschenkoi* trichomes; (B_{1-9}) *R. mediterranea* trichomes. Scale bar is 10 μm. A colour version of this figure is available online.

gradually narrowed, pointed at the apices, hair-like, without or with 1–2 heterocytes, occasionally with akinetes (up to three) distant from heterocytes, and vegetative cells with or without gas vacuoles. Trichomes assigned to *R. mediterranea* were solitary, straight or rarely curved, rarely constricted at the cross walls (Fig. 1B_{1-9}), from very short (11.5 μm) to moderate length (maximum 206.1 μm, mean 111.5 μm) and narrow (range 1.4–2.8 μm, mean 2.0 μm) with apical cells conical, sharply pointed to needle-like, rarely bluntly pointed, often with akinetes (1–3) and the vegetative cells with or without gas vacuoles. In Fig. 1B_{5-6}, trichomes with overlapping characters of *R. mediterranea* and non-heterocytous *C. raciborskii* are shown.

Reproduction by trichome fragmentation was common in the morphospecies studied. Central division by diameter reduction was observed in long trichomes of *Aphanizomenon* (Fig. 2A). In *Raphidiopsis*, in addition to (i) central fragmentation (Fig. 2C), (ii) multiple constrictions in trichome positions of fragmentation (Fig. 2D) as in *Aphanizomenon* (Fig. 2B) and (iii) trichome disintegration into several fragments (Fig. 2E), (iv) a new mode of trichome reproduction described by Moustaka-Gouni *et al.* (Moustaka-Gouni *et al.*, 2009) was observed (Fig. 2F–N). This mode of trichome reproduction by an apical structure was common in Lake Doirani throughout the study period and resulted in young trichomes of typical *Raphidiopsis* (Fig. 2O). In few trichomes of *Raphidiopsis*, both types of specialized reproductive cells, namely the akinete and the apical reproductive structure, were observed (Fig. 2M).

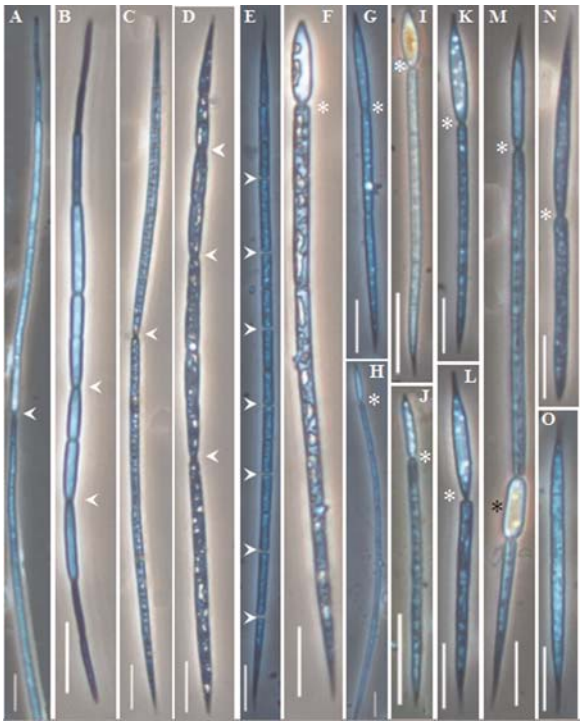

Fig. 2. Trichome reproduction in *A. issatschenkoi* and *R. mediterranea*. (A–O) Light micrographs (phase contrast) of reproduction stages. (A) *Aphanizomenon issatschenkoi* central division by narrow constriction (arrow head); (B) *A. issatschenkoi* trichome fragmentation in several parts (arrow heads); (C) *R. mediterranea* trichome fragmentation by central division (arrow head); (D) *R. mediterranea* trichome fragmentation in several parts (arrowheads); (E) *R. mediterranea* trichome disintegration (arrow heads); (F–N) *R. mediterranea* trichome reproduction by development of a structurally distinct apical cell (white asterisk); (M) *R. mediterranea* trichome with two different reproductive cells, an apical structure (white asterisk) and an akinete (black asterisk); (O) young *R. mediterranea* trichome produced by an apical reproductive structure. Scale bar is 10 μm. A colour version of this figure is available online.

Of all the clones analysed, phylotypes ND2-4-32 and ND2-1-3 were related to the *Raphidiopsis/Cylindrospermopsis* group and *Aphanizomenon issatschenkoi*, respectively (Fig. 3). In particular, ND2-4-32 was 99.8% similar to *R. mediterranea* strain HB2, 99.7% to *Cylindrospermopsis/Raphidiopsis* morphotypes NK2-532 and >99% with other *Cylindrospermopsis* strains. ND2-1-3 was 99.2% similar to *A. issatschenkoi* strains CAWBG02 and 0tu37s7.

During the study period (August–October 2004), mean water temperature ranged from 25.3 ± 0.5°C in August to 19.6 ± 0.7°C in October (Fig. 4A). Vertical temperature gradients of <1°C m^{-1} occurred. Transparency was low ranging from 0.30 to 0.85 m. The Z_{max}/Z_{sec} ratio was always >4.5 (max. 15.7) (Fig. 4B). pH ranged from 8.9 to 9.1 (Fig. 4C). Mean SRP concentrations varied between 12.2 ± 2.7 and 29.6 ± 4.2 μg L^{-1} (Fig. 4D). Nitrate nitrogen (Fig. 4E) dropped to low levels (2.6 ± 2.1 μg L^{-1}) and ammonium nitrogen (Fig. 4F) reached highest concentrations (16.96 ± 23.5 μg L^{-1}) in early September, whereas it was undetectable in October (Fig. 4C). Mean DIN was relatively low throughout the study period (12.5–51.1 μg L^{-1}) with ammonium nitrogen constituting 40.7%. Inorganic N:P atomic ratio was <15 throughout the study period (range 1.5–7.4).

A cyanobacterial bloom consisting of *R. mediterranea* and *A. issatschenkoi* developed during August–September (max. 7795 and 4031 trichomes mL^{-1}, respectively) (Fig. 5). *Raphidiopsis mediterranea* initial increase was associated with windy days (wind speed reached 10 m s^{-1}) and a high content of suspended particles in the surface layer resulting in the highest Z_{max}/Z_{sec} ratio and available nitrogen (Fig. 4). Comparing the population dynamics of the co-dominating species at each water depth (Fig. 5), an almost inverse relationship can be seen over time. A vertical separation between the two populations with the *A. issatschenkoi* population exhibiting its highest density in the middle water layer (1.5–2.7 m), whereas *R. mediterranea* in the deep water layer (3.0–4.2 m) was shown with CCA (Fig. 6).

Correlating biomass net growth rate of the co-dominating *A. issatschenkoi* and *R. mediterranea* at each water depth, a negative linear correlation was clear (Fig. 7). Nevertheless, this negative correlation was statistically significant (Pearson's $r = -0.859$, $P < 0.05$) only for the deep water layer, where the highest *R. mediterranea* population density was observed. Mean net growth rate was higher for *R. mediterranea* (0.17 $days^{-1}$) than for *A. issatschenkoi* (0.10 $days^{-1}$).

On August 20, the bloom rapidly diminished whereas the species recovered within 1–3 weeks. Microscopic analysis of the samples of August 12th revealed bacterial attack (Fig. 8). In 600 trichomes examined, more than 80% of the *Raphidiopsis* trichomes were attacked and >10% of them were dead (Fig. 8 inset) whereas fewer trichomes of *A. issatschenkoi* were attacked (<20%). Other filamentous cyanobacteria present (*Aphanizomenon flos-aquae*, *Planktolyngbya limnetica* and *Planktothrix* cf. *agardhii*) were not affected (Supplementary Fig. S1).

Raphidiopsis mediterranea biomass was positively correlated with temperature (Spearman's $r = 0.623$, $P < 0.05$) following the changes in Z_{max}/Z_{sec} with a lag time of a week (positive but insignificant correlation, Spearman's $r = 0.537$, $P > 0.05$). No significant correlation was found between its biomass and DIN, ammonium nitrogen or N:P ratio. *Aphanizomenon issatschenkoi* biomass was not correlated with any of the physical (water temperature, Z_{max}/Z_{sec}) or chemical (DIN, ammonium nitrogen and N:P ratio) parameters. *Aphanizomenon issatschenkoi* regularly formed heterocytes under low nitrogen conditions (DIN <50 μg L^{-1}).

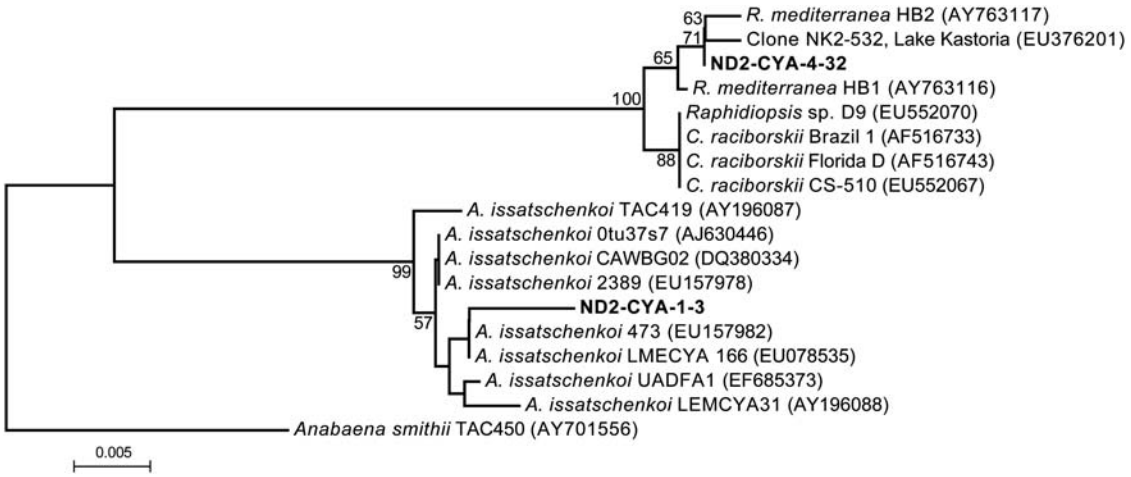

Fig. 3. Neighbour-joining phylogenetic tree showing the relationship of the Lake Doirani cyanobacterial phylotypes ND2-4-32 and ND2-1-3 with strains of *A. issatschenkoi* and *R. mediterranea*. Numbers at nodes represent the bootstrap percentages from 1000 replicates. Values below 50% are not shown. Bar indicates the number of substitutions per site. *Anabaena smithii* (Nostocales) was used as an outgroup.

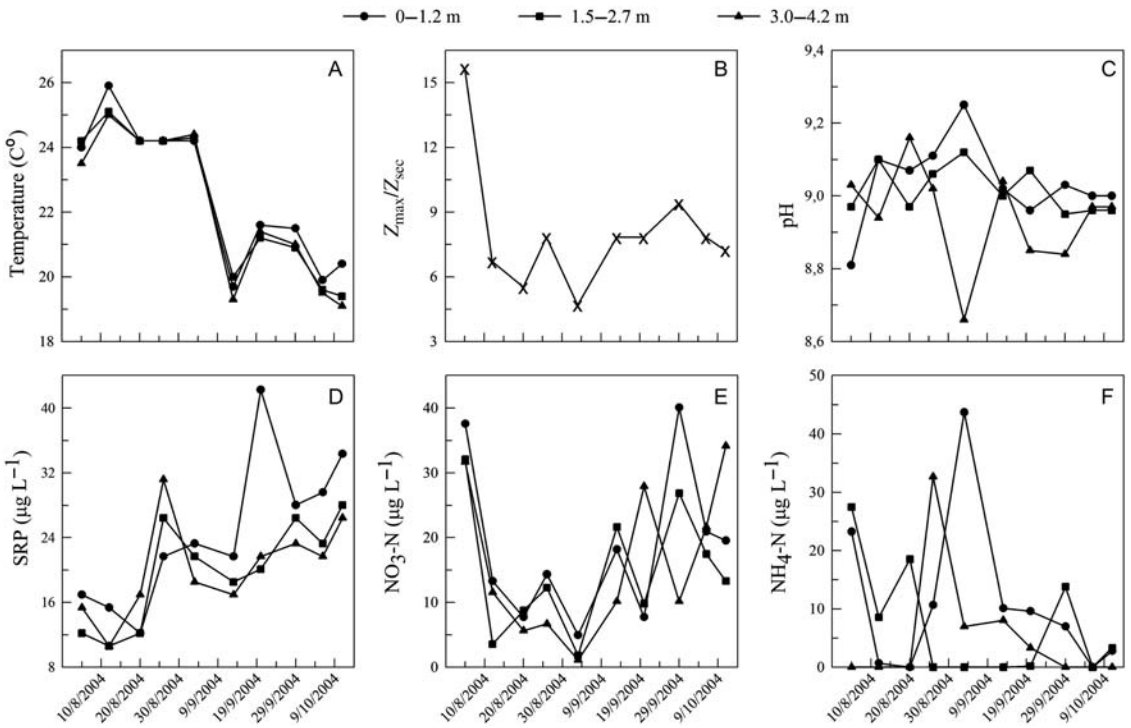

Fig. 4. Physical and chemical parameters in Lake Doirani from August 5 to October 10: (**A**) water temperature, (**B**) ratio of Z_{max}/Z_{sec}, (**C**) pH, (**D**) SRP, (**E**) NO_3–N and (**F**) NH_4–N.

DISCUSSION

We investigated morphological, phylogenetic and ecological features of *A. issatschenkoi* and *R. mediterranea* in the Mediterranean Lake Doirani, during a cyanobacterial bloom. It was not difficult to distinguish (i) the heterocytous trichomes and (ii) the non-heterocytous trichomes of *A. issatschenkoi* from the trichomes assigned to *R. mediterranea*. Non-heterocytous *A. issatschenkoi* was distinguished from *R. mediterranea* on the basis of trichome apical cells. They were 1–3 in number, gradually narrowed, and pointed at the apices, hair-like, whereas in *R. mediterranea*, the apical cells were of longitudinally conical shape, bluntly or sharply pointed to needle-like. A variety very similar to, in this aspect, *A. issatschenkoi*,

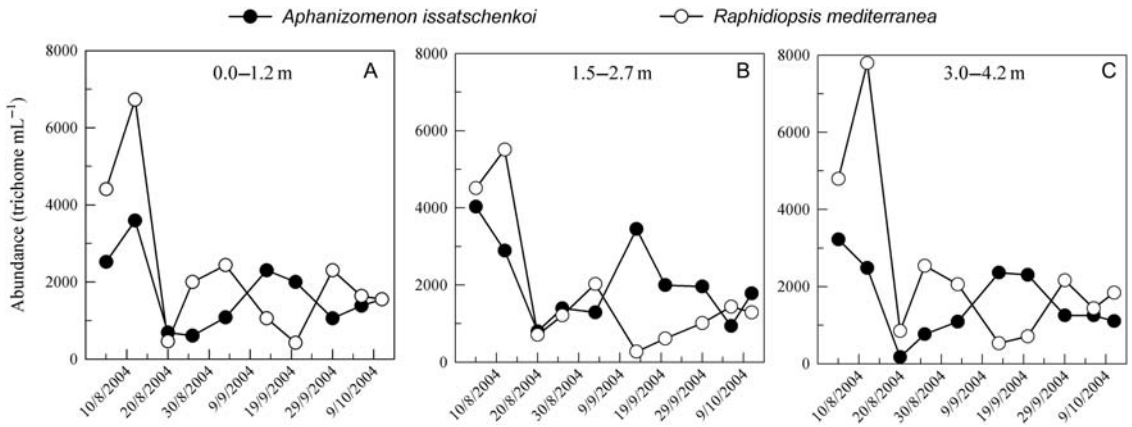

Fig. 5. *Aphanizomenon issatschenkoi* and *R. mediterranea* abundance in Lake Doirani from August 5 to October 10: (**A**) in the surface water layer (0.0−1.2 m), (**B**) in the middle water layer (1.5−2.7 m) and (**C**) in the deep water layer (3.0−4.2 m).

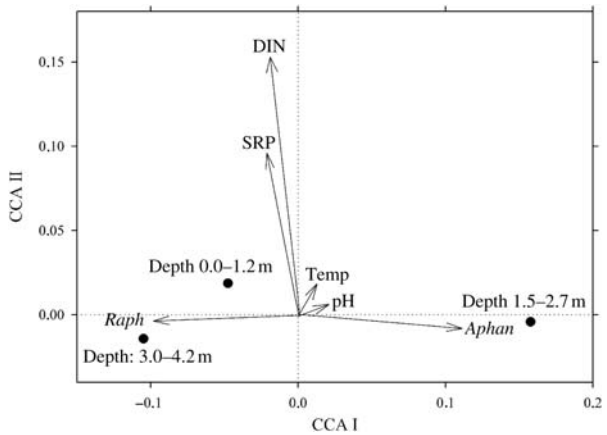

Fig. 6. CCA ordination biplot representing the water depths (black circles) and *R. mediterranea* (*Raph*), *Aphanizomenon issatschenkoi* (*Aphan*), water temperature (Temp), pH, DIN and SRP (arrows). Axes I and II explain 98.5% and 1.5% of the variation, respectively.

the *R. mediterranea* Skuja, var. *grandis* Hill was described in 1970 (Hill, 1970). This variety seems to be a non-heterocytous life-stage of *A. issatschenkoi* with akinetes. Subsequently, *R. mediterranea* reported from the Swedish Lake Trummen (Cronberg, 1973) and a toxic strain of *R. mediterranea* isolated from Lake Biwa (Watanabe *et al.*, 2003) corresponding to the description of Hill (Hill, 1970) shows high similarity with non-heterocytous *A. issatschenkoi*. *Aphanizomenon issatschenkoi* from Lake Hakanoa was also confused with *R. mediterranea* (Wood *et al.*, 2007). As a consequence, misidentifications of non-heterocytous *A. issatschenkoi* as *R. mediterranea* are possibly associated with the description and establishment in the phycological research of *R. mediterranea* Skuja, var. *grandis* Hill.

Apart from the distinguishing feature of apical cells, *A. issatschenkoi* and *R. mediterranea* shared some similar features (morphology, length and width of trichomes) and reproduction modes (trichome fragmentation and akinete formation). However, an unusual mode of reproduction of *Raphidiopsis*, reported from Lake Kastoria (Moustaka-Gouni *et al.*, 2009) which has been commonly observed in *Raphidiopsis*, in this study, has not been documented in *Aphanizomenon*.

In the type locality of *R. mediterranea* (Lake Kastoria), the novel mode of reproduction was observed in few trichomes, and *Raphidiopsis* and *Cylindrospermopsis* morphotypes were found in a ratio of 1:2 (Moustaka-Gouni *et al.*, 2009). In contrast, in this study (Lake Doirani), the unusual mode of reproduction was common over time and the typical *R. mediterranea* morphotype was dominant (>98% of the trichomes throughout the study). In this lake, life under specific conditions of poor underwater light, high content of suspended particles and low but available nitrogen may have strong feedback on the life-cycle described by Moustaka-Gouni *et al.* (Moustaka-Gouni *et al.*, 2009), triggering low morphotype diversity towards typical *R. mediterranea*.

Development of two different types of reproductive cells in the same individual was observed for the first time in planktonic Cyanobacteria in this study; the akinete, a typical dormant offspring of Nostocales (Komárek and Anagnostidis, 1989) and the novel apical reproductive structure (Moustaka-Gouni *et al.*, 2009), an active offspring forming young trichomes of typical *R. mediterranea*. These alternatives to binary fission that result in different offspring of the same individual may provide opportunities for flexibility to accommodate the different lifestyles of this cyanobacterium (Angert, 2005).

The morphotypes we examined using microscopy could be assigned to two phylotypes, very closely affiliated (i) to *A. issatschenkoi*, >99% similar to

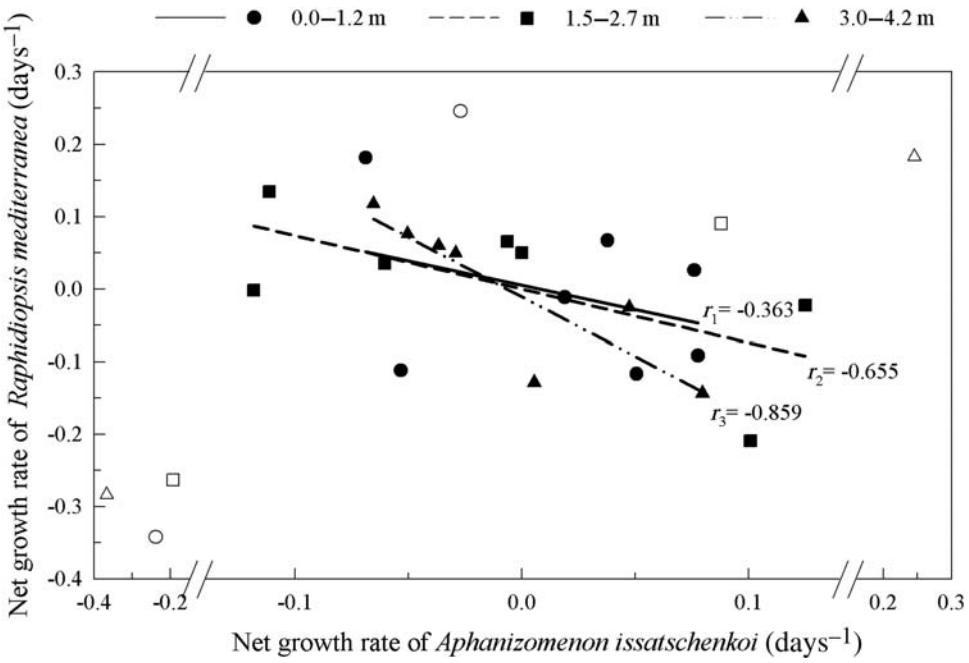

Fig. 7. The linear relationship between the net growth rate of biomass of *R. mediterranea* and that of *A. issatschenkoi* in Lake Doirani from August 5 to October 10. In the samples indicated by white circles, triangles and squares, population dynamics is regulated by bacterial parasites. For this reason, they were excluded from linear correlation analysis.

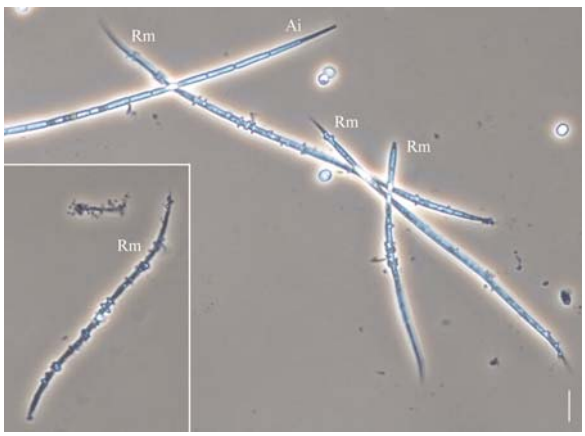

Fig. 8. Bacterial attack on *R. mediterranea* (Rm) and *A. issatschenkoi* (Ai) trichomes in Lake Doirani. Inset: *R. mediterranea* (Rm) dead trichome. Scale bar is 10 μm. A colour version of this figure is available online.

A. issatschenkoi strains examined by Wood *et al.* (Wood *et al.*, 2007) and Rajaniemi *et al.* (Rajaniemi *et al.*, 2005) revised as *Cuspidothrix issatschenkoi* and (ii) to *Raphidiopsis/Cylindrospermopsis* strains with >99% similarity to strains and natural populations examined by Li *et al.* (Li *et al.*, 2008) and Moustaka-Gouni *et al.* (Moustaka-Gouni *et al.*, 2009), respectively. The slight variation of 16S rRNA gene sequence in populations of the neighbouring lakes Kastoria and Doirani (<1% divergence) contrasted with the obvious difference in morphotype diversity, implying that the two populations might have multiple strategies of adaptation and diversification, as has been suggested for tropical oceanic N_2-fixing cyanobacteria with low genomic diversity (Zehr *et al.*, 2007).

By investigating the ecological patterns, we observed both similarities and important differences between the dominant *A. issatschenkoi* and *R. mediterranea*. In general, both showed a rapid decline during a severe attack by bacteria. However, *R. mediterranea* was preferred by bacteria and showed the highest amount of trichome attack (>80% of the trichomes, followed by <20% of *A. issatschenkoi*). To the best of our knowledge, bloom termination of *R. mediterranea* by bacterial attack has not been reported. Nevertheless, evidence for bloom termination by cyanobacteria-lysing bacteria has been published since the 1970s (Daft *et al.*, 1975). Nowadays, these bacteria are considered as agents of potential biological control of cyanobacterial blooms, although their importance in regulating cyanobacterial population dynamics has been largely overlooked (Rashidan and Bird, 2001; Gumbo *et al.*, 2008).

Apart from the period of bacterial effect on the bloom, the population dynamics of *A. issatschenkoi* and *R. mediterranea* at different depths of the shallow water column demonstrated an almost inverse relationship over time. Moreover, the two species consistently

preferred different water depths, and showed negative correlation between their net growth rates, with *R. mediterranea* dominating the deep water layer. These findings indicate a vertical niche separation. The species are motile (gas-vacuolated cells), a particular strategy of vertical niche separation (Lampert and Sommer, 1997). As nutrient concentrations exhibited insignificant vertical differences (ANOVA $P > 0.05$), low light conditions (Z_{max}/Z_{sec} ratio > 4.5 throughout the study) could be a major environmental factor for vertical separation of species. Low light conditions and high water temperature positively affected the temporal distribution of *R. mediterranea*, but not that of *A. issatschenkoi*. The results of this study support the recent modification and assignment of *R. mediterranea* in the functional group S_N (Padisák *et al.*, 2009) together with the phylogenetically indistinguishable *C. raciborskii*. *Aphanizomenon issatschenkoi* has been assigned to the functional group H1, indicating similar tolerances with *Raphidiopsis* for low nitrogen (same requirements) but different abilities and life-styles to satisfy their requirements (heterocyte formation and N-fixation). Phytoplankton species can co-dominate either if they are ecologically dissimilar or if they are ecologically similar and show vertical separation in the water column (Lampert and Sommer, 1997). *Raphidiopsis mediterranea* exhibited a higher net growth rate and dominance in each period of water temperature increase above 20°C. Net growth rate of *A. issatschenkoi* was comparable to that published for a natural population in Langer See (Mischke, 2003). *Raphidiopsis mediterranea* net growth rate was higher than the published data for *C. raciborskii*, both in Langer See and in Melangsee (Mischke, 2003).

In conclusion, the results of this study demonstrate that *A. issatschenkoi* is clearly distinct morphologically, phylogenetically and ecologically from *R. mediterranea*. Morphological misidentifications are possibly associated with the description of *R. mediterranea* Skuja, var. *grandis* Hill and can be solved by careful examination of the morphology of 2–3 apical cells. *Raphidiopsis mediterranea* exhibited specialized dominance in periods with high water temperature, high Z_{max}/Z_{sec} ratio and low but available inorganic nitrogen. *Aphanizomenon issatschenkoi* was more generalist showing a different strategy to satisfy nitrogen requirement. The two species consistently preferred different water depths. Phylogenetically, *R. mediterranea* morphospecies fell into the well-defined 16S rRNA gene sequence cluster of *C. raciborskii*. In this study, for the first time in planktonic cyanobacteria, *R. mediterranea* individuals developed two different specialized reproductive cells, exhibiting alternative reproduction modes that may indicate flexibility for different lifestyles in Nostocales.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

ACKNOWLEDGEMENTS

We thank the two unknown reviewers and the corresponding editor John Dolan for valuable comments that improved the manuscript. We thank the 5th Office of the Hellenic National Meteorological Service for providing meteorological data. We also thank Suzanne Thomas for suggesting linguistic improvements for the manuscript.

FUNDING

This work was partially funded by the Greek Ministry of Foreign Affairs (Hellenic Aid) and the Greek Biotope Wetland Center, Goulandris Museum (ANAP-46-2003).

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Figure S1. Bacterial parasites on *Raphidiopsis mediterranea* (Rm) along with co-occurring unaffected filaments of *Aphanizomenon flos-aquae* (Afa), *Aphanizomenon issatschenkoi* (Ai), *Planktolyngbya limnetica* (Pl) and *Planktothrix* cf. *agardhii* (Pa) in Lake Doirani. Scale bar is 10 μm.