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ORIGINAL ARTICLE

Evaluation of different DNA sampling techniques for the application of the real-time PCR method for the quantification of cyanobacteria in water

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early warning, genotype composition, *Microcystis*, *Planktothrix*, quantitative PCR, *Taq* nuclease assay, toxicity, water monitoring.

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Abstract

Aims: To evaluate different types of sample storage and DNA extraction techniques for the real-time PCR quantification of cyanobacteria in water.
Methods and Results: Two different filter types for the cell harvest of *Microcystis* sp. and *Planktothrix* spp. that were either freeze-dried or stored frozen, and two different methods for DNA extraction were compared. DNA extraction was achieved by standard phenol-chloroform extraction or by a faster commercially available purification kit (DNeasy®, QIAGEN). In general there was good agreement between the cell number equivalents of phycocyanin (PC) genotypes that were estimated using the *Taq* nuclease assay (TNA) between both filter types and the storing of samples. The standard DNA extraction procedure gave higher numbers of PC genotypes when compared with the DNeasy procedure. TNA results obtained from *Planktothrix* from natural samples extracted with the standard procedure revealed a significant correlation with the cell numbers estimated via the microscope.
Conclusions: Freeze-drying of samples gives quantifiable data. The standard DNA extraction is considered to be the most reliable and accurate, although the DNeasy procedure is useful for early warning monitoring.
Significance and Impact of the Study: Application of quantitative genotype analysis in cyanobacteria from freeze-dried samples collected during recent and past sampling programmes.

Introduction

Cyanobacteria of the genera *Microcystis* sp. and *Planktothrix* spp. are frequently found in fresh water. Cyanobacteria are considered as important source of organic toxic compounds deteriorating drinking water quality worldwide (WHO 2004). Typically, nontoxic strains and strains containing different toxins co-occur. Parallel to the elucidation of the genes that are involved in toxin synthesis (see Dittmann and Börner 2005 for a recent review) techniques have been developed in order to detect and quantify toxic genotypes directly in water. The real-time PCR technique, i.e. the *Taq* nuclease assay (TNA) has been introduced as tool for the quantification of toxic genotypes in water (Kurmayer and Kutzenberger 2003). The intensity of a genotype-specific signal induced by a fluorescent TaqMan probe [i.e. the microcystin synthetase gene (*mcy*) of *Microcystis* sp.] is related to the signal specific of the total population [i.e. the phycocyanin (PC) operon of *Microcystis* sp.]. Due to the binding of the primers and the probe, the TNA has been found to be highly specific for the target DNA and to produce reliable results even in the presence of complex background such as in field samples (Kurmayer and Kutzenberger 2003).

In the course of the EU-project PEPCY (Toxic and other bioactive peptides in cyanobacteria, QLRT-2001-02634, http://www.pepcy.de, 23 April 2005) a number of water bodies located within European countries were selected for quantifying toxin-producing genotypes. Two problems arising from the sampling schedule were

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investigated: (i) how the field samples should be stored and posted and (ii) how DNA from cyanobacteria in field samples can be quantitatively extracted. A standard phenol-chloroform extraction procedure has been derived from established protocols (Franche and Damerval 1988) in an earlier study by Kurmayer *et al.* (2003) and has been shown to produce quantitative results (Kurmayer and Kutzenberger 2003). However, it is also considered to be time intensive regarding a large number of field samples and particularly for early warning monitoring a more time efficient and easy-to-use technique for DNA extraction would be required. Such a new technique needs to be applicable to all morphologies of cyanobacteria, for example *Microcystis* is growing as single cells embedded in mucilage while *Planktothrix* is forming rigid filaments consisting of cells tightly attached to each other.

In this study, we tested the TNA results obtained for (i) using two types of filters for cell harvest which were either freeze-dried or frozen and (ii) different amounts of cells from strains of *Microcystis* sp. and *Planktothrix* spp. extracted using a commercially available DNA extraction kit (DNeasy; QIAGEN) as opposed to the standard phenol-chloroform extraction procedure. TNA was specifically designated for the intergenic spacer region of the PC operon (PC-IGS).

Materials and methods

Cultivation and cell harvesting

For establishing the calibration curve *Planktothrix rubescens* strain PCC7821 (L. Gjersjøen, NO) was grown in BG_{11} medium (Rippka 1988) at 20°C and 30 μE (continuous light, Philips TLD, 36W/965; Phillips, Vienna, Austria) and harvested under logarithmic growth phase conditions using low vacuum filtration onto glass fibre filters (BMC, Ederol, Vienna, Austria). As for all experiments aliquots were fixed with formaldehyde (2% final concentration) for cell enumeration in parallel to cell harvesting. Because all cyanobacteria contain the PC-IGS, DNA from *Microcystis* sp. strains HUB53 and HUB524 (Pehlitzsee, D) and *Synechococcus* strains MW15#2SUB (Mondsee, AT) was extracted and used as background DNA in order to test the specificity of the new TNA designed for PC-IGS of *Planktothrix agardhii* and *P. rubescens*.

Planktothrix strain PCC7821 and *Microcystis* strain HUB524 were grown and harvested as described above for comparing the TNA results between DNA extracts obtained (i) from glass fibre filters (Ederol, type BMC, Ø 47 mm, which is equivalent to the GF/C filter type, Whatman, Kent, UK) *vs* membrane filters (ME, Schleicher and Schüll, Dassel, Germany; RC55, Ø 50 mm) and (ii) from freeze-dried samples *vs* frozen samples. Filters were freeze-dried using a speed vac (vacuum centrifuge concentrator 5301; Eppendorf, Hamburg, Germany) or were directly stored wet frozen at −20°C. There were three parallels per treatment. To compare DNA extraction methods, the following strains were used: *P. rubescens*: No. 6 (Mondsee, AT), No. 108 (Irrsee, AT), No. 61 (Schwarzensee, AT), No. 67 (Wörthersee, AT), No. 34 (Ammersee, D), No. 75 (Zürichsee, CH); *P. agardhii*: No. 31/1 (Wannsee, D), PCC7805 (Veluwermeer, NL), CCAP1459/17 (Blelham Tarn, UK), CYA126/8 (L. Langsjön, FI); *Microcystis aeruginosa*: *M. aeruginosa* Hofbauer and *Microcystis flos-aquae* (Neusiedlersee, AT), HUB524, HUB53, P461 (Pehlitzsee), W368, W334, W75, W61 (Wannsee, D), PCC7806 (Braakman Reservoir, NL). These strains were grown as described above and harvested during the stationary phase.

Enumeration of cells

Formaldehyde-fixed samples were stained with DAPI according to standard protocols and filtered onto 0·2-μm pore-size polycarbonate filters (GTBP, Ø 25 mm, Millipore). At least 400 cells per transect were counted at 1000× (Axioplan; Zeiss, Göttingen, Germany) and cells per ml were calculated by way of extrapolation. Field samples integrated from the surface down to 8 m of depth from Lake Wannsee (Berlin, Germany) from June 1999 to October 2000 were analysed for cell numbers using the inverted microscope as described (Kurmayer *et al.* 2003).

DNA extraction

Filters were freeze-dried and stored at −20°C until DNA extraction. The standard phenol-chloroform DNA extraction procedure was performed as described in Kurmayer *et al.* (2003) and used for all analyses. For comparing extraction methods isolation of DNA was also achieved using the DNeasy Plant system (QIAGEN, VWR). Cells were not incubated in liquid nitrogen before digestion by incubating the filter using a lysis buffer (containing RNase A) for 10 min at 65°C. Subsequently polysaccharides and proteins were precipitated and incubated for 5 min on ice. The undigested cellular debris was removed using the QIAshredder spin column. A binding buffer (containing ethanol) was added to the cleared lysate and the DNA was purified using a DNeasy mini spin column (QIAGEN 2003). In order to exclude the possibility of saturation of the extraction columns by excess amounts of DNA strain HUB524 (15×10^6 cells ml^{-1}) was harvested from stationary phase on GF/C filters ($5{\cdot}4 \times 10^7$ cells per filter) and extracted in 1, 1/2, 1/4, 1/10, 1/100, 1/200, 1/400, 1/800 amounts. If saturation of the extraction

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column occurred it was expected that yields on a per cell basis should improve when cell numbers per extraction column were decreased.

Taq nuclease assay

Primers and the TaqMan probe that were specifically bound to PC-IGS of *Microcystis* sp. were designed by Kurmayer and Kutzenberger (2003). Primers and probes that were specifically bound to PC-IGS of *Planktothrix rubescens* and *P. agardhii* (*sensu* Suda *et al.* 2002) were designed during this study. The following PC-IGS sequences from cyanobacteria were aligned using Clustal W 1·8: *Synechococcus* sp. (AY151231, AY151230, AY151224, AY151222, AF223463, AF223465, AF223464), *Synechocystis* sp. (AJ003180), *Chroococcus* sp. (AJ003188), *Microcystis* sp. (strain PCC7806, AF195177), *Planktothrix* spp. (AJ558135, AJ132279, AJ131820, AJ558138, AJ558140, AJ558160, AJ401186, AJ401185), *Aphanizomenon* sp. (AJ243968), *Lyngbia* sp. (AJ401187), *Nostoc* sp. (NC 003272). In addition, PCR for the PC-IGS region of the *Microcystis* strains HUB524 and HUB53 was performed using the primers designed by Neilan *et al.* (1995) and the PCR products were sequenced and submitted to the DDBJ/EMBL/GenBank (AJ965489, AJ965490). *Planktothrix*-specific primers were designed from gene regions that are homogeneous within *P. rubescens* and *P. agardhii* but are variable enough when compared with other genera: PlPC fwd: 5′-GAGCAGC ACTGAAATCCAAG-3′, PlPC rev: 5′-GCTTTGGCTGC TTCTAAACC-3′). The TaqMan probe (PlPC 5′-6FAM-TTTGGCTTGACGGAAACGACCAA XT-PH-3′) had a fluorescent reporter dye (6-carboxyfluorescein) covalently attached to the 5′-end (6-FAM) and a TAMRA(X) fluorescent quencher dye (6-carboxytetramethylrodamine). The size of the amplification product was 72 bp. TNAs were performed using a GeneAmp 5700 sequence detection system (ABI, Vienna, Austria) as described (Kurmayer and Kutzenberger 2003). The primer and probe concentrations were optimized following the manufacturer's instructions. The 25 μl reaction mix for PC-IGS of *Planktothrix* consisted of 12·5 μl (2×) TaqMan Universal PCR Master Mix (ABI), 50 nmol l^{-1} forward primer, 300 nmol l^{-1} reverse primer, 100 nmol l^{-1} TaqMan probe, 5 μl of template and 6·5 μl Millipore water. Each measurement was done in triplicate. Standard curves were established by relating the cell numbers to the measured threshold cycle (the cycle number where fluorescence exceeds the threshold set at 0·1). PC-IGS of *Planktothrix* PCC7821 was analysed by comparing the calibration curve in the absence and presence of background DNA (*Microcystis*, *Synechococcus*) which was added in two concentrations, $1{\cdot}7 \times 10^4$ and $1{\cdot}7 \times 10^5$ cells per template consisting of 13% HUB53, 11% HUB524 and 76% MW15#2SUB.

Results

Sensitivity and amplification efficiency of TNA

The regression equation for the detection of PC-IGS using the TNA showed a significant linear curve between the amount of DNA in the template (expressed as cell number equivalents) and the C_t-value obtained: $y = 38{\cdot}20 - 3{\cdot}61x$ ($R^2 = 0{\cdot}99$, $n = 6$, $P < 0{\cdot}0001$), where y is the C_t-value and x is the amount of DNA per template calculated in $\log_{10}$ cell number equivalents (data not shown). The dilution with the lowest detectable signal corresponded to one cell per template. The variation in C_t-values in the presence of both dilutions of the DNA background was small ($\Delta C_t < 1$) within the central region of the standard curve (40–40 000 cells per template), but more pronounced towards higher (400 000 cells, $\Delta C_t = 1{\cdot}2$) or lower cell numbers (<1 cell, $\Delta C_t = 3{\cdot}4$). A DNA concentration equivalent to 400 cells per template was found with the lowest deviation, and in further analysis all of the DNA extracts were diluted to this concentration.

Influence of filter types and sample storage on TNA results

Independent of the storage method and the filter type, the total variability in C_t-values obtained from standard DNA extracts was low and corresponded to the cell number determination in the microscope. At the day of cell harvest *Planktothrix* PCC7821 had $5{\cdot}3 \times 10^4$ cells ml^{-1} and *Microcystis* HUB524 had $1{\cdot}7 \times 10^6$ cells ml^{-1}. For strain PCC7821 C_t-values for ME filters wet frozen and freeze-dried were 23·5–24·6 (min–max) and 23·8–25·4, respectively, and for GF/C filters wet frozen and freeze-dried 25·4–29·5 and 22·6–26·5, respectively. For strain HUB524 C_t-values for ME filters wet frozen and freeze-dried were 29·2–31·0 and 29·4–30·1, respectively, and for GF/C filters wet frozen and freeze-dried 29·0–30·7 and 29·4–32·3, respectively. There were no significant differences between treatments, and for *mcyB* identical results were observed (Table 1).

Variation in TNA results using two different DNA extraction techniques

All strains showed comparable cell numbers (1×10^6 cells ml^{-1}–7×10^7 cells ml^{-1}) during harvesting. With both extraction techniques DNA >12 kb in size was visible after ethidium bromide staining and electrophoresis on 1% agarose gels in 0·5x Tris-borate-EDTA buffer.

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Table 1 C_t-values and cell numbers extract^{-1} (mean ± 1 SE) estimated using TNA (PC, *mcy*B) from DNA extracts obtained from two different types of filters (GF/C, glass fiber; ME, membrane) and two different types of storage (wet frozen, freeze-dried). P, significance level calculated from Kruskal–Wallis one-way analysis of variance on ranks (three parallels per treatment)

	ME frozen	ME freeze-dried	GF/C frozen	GF/C freeze-dried	*P*
C_t-values					
HUB524 (PC)	30.1 ± 0.4	29.8 ± 0.2	29.9 ± 0.5	31.0 ± 0.8	0.52
HUB524 (*mcy*B)	36.9 ± 0.3	36.5 ± 0.6	37.5 ± 0.7	37.8 ± 0.3	0.35
PCC7821 (PC)	24.1 ± 0.24	24.6 ± 0.5	27.1 ± 1	24.4 ± 0.9	0.12
PCC7821 (*mcy*B)	25.0 ± 0.3	25.0 ± 0.2	27.8 ± 1.3	25.4 ± 0.5	0.12
Cells extract^{-1}					
HUB524 (PC)	$2.6 \times 10^8 \pm 5.9 \times 10^7$	$2.9 \times 10^8 \pm 3.3 \times 10^7$	$3.0 \times 10^8 \pm 9.6 \times 10^7$	$1.7 \times 10^8 \pm 8.7 \times 10^7$	0.52
HUB524 (*mcy*B)	$3.9 \times 10^8 \pm 8.9 \times 10^7$	$4.8 \times 10^8 \pm 1.5 \times 10^8$	$3.0 \times 10^8 \pm 9.7 \times 10^7$	$2.2 \times 10^8 \pm 3.7 \times 10^7$	0.31
PCC7821 (PC)	$1.9 \times 10^8 \pm 2.7 \times 10^7$	$1.5 \times 10^8 \pm 3.9 \times 10^7$	$4 \times 10^7 \pm 2.0 \times 10^7$	$2.1 \times 10^8 \pm 1.1 \times 10^8$	0.09
PCC7821 (*mcy*B)	$1.6 \times 10^8 \pm 2.8 \times 10^7$	$1.6 \times 10^8 \pm 2.4 \times 10^7$	$4.7 \times 10^7 \pm 3.2 \times 10^7$	$1.3 \times 10^8 \pm 9.8 \times 10^5$	0.18

For *Microcystis* sp. the standard DNA extraction procedure had C_t-values that were significantly lower (23·6–30·9) when compared with C_t-values obtained from the DNeasy Plant system extraction (26·3–32·5) (*t*-test, $P < 0{\cdot}001$, $n = 10$, Fig. 1a). For *Planktothrix* sp. the difference between the standard DNA extraction (23·6–29·2) and the DNeasy Plant system (25·2–29·2) was less pronounced albeit significant (t-test, $P = 0{\cdot}006$, $n = 10$, Fig. 1b). For both cyanobacteria the calculated cell numbers were found to be significantly higher in DNA extracts obtained using the standard phenol-chloroform procedure when compared with the DNeasy Plant system extractions (1·6- to 18·8-fold in *Microcystis*, 0·21- to 6·2-fold in *Planktothrix*). The DNeasy Plant system did not reveal improved yields on a per cell basis when cell numbers per extraction column were decreased linearly down to 800-fold [total filter, $C_t = 33{\cdot}2 \pm 0{\cdot}32$ (1SE); 1/2 filter, $C_t = 32{\cdot}0 \pm 0{\cdot}33$; 1/4, $C_t = 33{\cdot}5 \pm 0{\cdot}20$; 1/10, $C_t = 32{\cdot}6 \pm 0{\cdot}2$; 1/100, $C_t = 33{\cdot}9 \pm 0{\cdot}3$; 1/200, $C_t = 33{\cdot}1 \pm 0{\cdot}1$; 1/400, $C_t = 32{\cdot}4 \pm 0{\cdot}3$; 1/800, $C_t = 33{\cdot}0 \pm 0{\cdot}2$] and yields compared with the yield extracted using the standard procedure ($C_t = 27{\cdot}2 \pm 0{\cdot}06$).

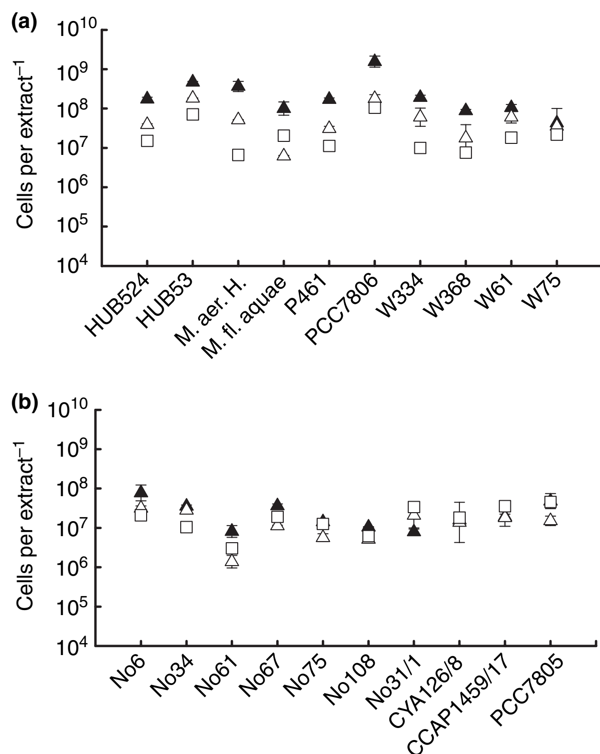

Figure 1 Cell numbers (mean ± 1SE) of 10 *Microcystis* sp. (a) and 10 *Planktothrix* spp. strains (b) as determined by *Taq* nuclease assay from DNA extracts obtained either through DNA extraction according to the standard phenol-chloroform procedure (▲) or the DNeasy Plant system (△) and quantified by epifluorescence microscopy (□).

In order to find out whether impurities in the DNA that are inhibitory to the PCR may cause the lower yields obtained by the DNeasy Plant system the following tests with *M. flos-aquae* extracts (showing 18·8-fold lower cell numbers) were performed: (i) DNA obtained by the DNeasy Plant system was spiked with DNA obtained by the standard procedure to balance for the 18·8-fold lower cell number estimate and (ii) the DNA extract obtained by the DNeasy Plant system was 18·8-fold less diluted. Compared with the standard procedure extract ($C_t = 29{\cdot}1 \pm 0{\cdot}02$) the spiking treatment ($C_t = 29{\cdot}5 \pm 0{\cdot}51$) and the dilution treatment ($C_t = 28{\cdot}6 \pm 0{\cdot}12$) showed DNA yields that did not differ significantly.

In order to test the influence of DNA extraction on sensitivity of TNA, DNA extracts obtained through both extraction techniques from filters containing varying cell numbers (10^2–10^8 cells per filter) then amplified by TNA were compared with cell numbers estimated by microscopy. No significant difference was detectable when comparing microscopic cell counts to cell numbers estimated by TNA from DNA extracts obtained from *Microcystis* strain HUB53 (Mann–Whitney test, $P = 0{\cdot}86$, $n = 6$) and *Planktothrix* strain No. 75 (Mann–Whitney

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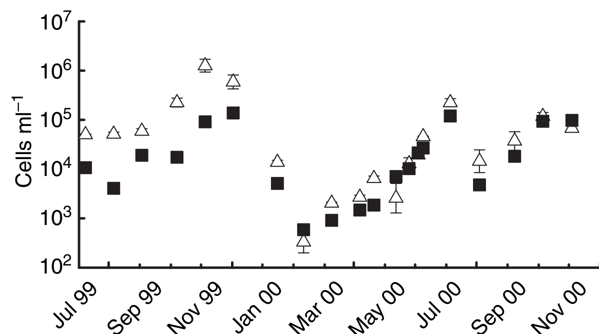

Figure 2 Number of *Planktothrix* cells in Lake Wannsee from June 1999 to October 2000, determined by counting under the inverted microscope (■) or by Taq nuclease assay quantification (△) of the phycocyanin operon (mean ± 1SE).

test, $P = 0{\cdot}62$, $n = 6$). Irrespective of the DNA extraction method, cells of both strains were equivalently detected down to a concentration of 10^5 cells per extract. Below 10^5 cells per extract the results had greater standard deviation. The lowest detectable signal corresponded to 10 cells per template extracted from 10^2 cells on a filter.

Application of TNA

In Lake Wannsee *Planktothrix* cell numbers estimated in the microscope varied from $5{\cdot}9 \times 10^2$ to $1{\cdot}4 \times 10^5$ ml^{-1} (Fig. 2). The variation measured by TNA ranged from 4×10^2 to $1{\cdot}4 \times 10^6$ ml^{-1}. TNA results on cell number obtained from DNA samples corresponded significantly with cell numbers counted in the microscope. The regression equation was $y = -0{\cdot}08 + 1{\cdot}07x$ ($R^2 = 0{\cdot}76$, $n = 20$, $P < 0{\cdot}0001$), where y is the $\log_{10}$ cell number determined by TNA and x is the $\log_{10}$ cell number counted in the microscope.

Discussion

Filter types and sample storage

The quantitative analyses of water samples require the appropriate storage of samples as well as efficient and reliable DNA extraction. In contrast to the more rigid nature of ME filters the softer GF/C filters do form a distinct pellet after centrifugation subsequent to DNA extraction using phenol-chloroform-isoamyl alcohol. This allows for a distinct separation of the water phase from the phenol-chloroform phase. In contrast the phase separation is disturbed by the rigid pieces of ME filters. Further the results are relevant with regard to the storage and postage of samples that have been collected through the course of recent and past sampling programmes. We conclude that freeze-drying of samples gives quantifiable data and that DNA extraction and subsequent TNA of historic samples would be possible. Such a TNA analysis would provide information on toxin genotype composition in populations over longer time periods.

DNA extraction techniques

In this study, we found that cell numbers estimated via TNA were highest in extracts obtained by the standard phenol-chloroform procedure. Although cells were harvested during the stationary phase we do not believe that DNA dissolving from dead cells influenced the results obtained. As aliquots of cell harvest were treated identical before the experiment on DNA extraction an influence due to dissolved DNA in the medium during the growth phase seems unlikely. The DNeasy procedure has been developed for DNA extraction from leaves/needles of higher plants containing complex polymers such as cellulose (QIAGEN 2003). Because the reduction of cell numbers for DNA extraction down to 1/800 did not reveal higher DNA yields per cell the possibility of over-saturation of the DNA extraction columns can be excluded. In addition, no impurities that might inhibit the PCR were detected in consequence to spiking the DNeasy kit extracts with DNA obtained by the standard procedure. It is concluded that the lysis and/or extraction efficiency of the DNeasy kit is generally lower. Owing to the variation in polysaccharide composition among cyanobacteria (De Phillipis and Vincenzini 1998) and *Microcystis* sp. (Doers and Parker 1988) and the variation between strains observed in this study we cannot exclude strain-specific differences in DNeasy extraction efficiency in field samples.

When testing the sensitivity between both DNA extraction procedures no difference was detectable. The TNA results were predictable down to 10^5 cells ml^{-1} implying that if 1 l of water is filtered effective cell numbers could be as low as 100 cells ml^{-1} in order to obtain reliable results. This concentration is below the concentration of 20 000 cells ml^{-1} that has been considered of relatively mild or low probability of adverse health effects (Falconer *et al.* 1999). Consequently the DNeasy procedure might be considered an alternative to standard phenol-chloroform DNA extraction for early warning monitoring tasks, particularly to achieve a high throughput in DNA analyses for the health safety analysis of environmental samples. The DNeasy procedure needs only 2 h as opposed to 8 h required for the standard extraction procedure and also replaces cumbersome phenol and chloroform extraction.

The results of this study obtained from field samples from Lake Wannsee demonstrate the application of DNA extraction and subsequent TNA. The overestimation of

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cell numbers by the TNA during the first year cannot be explained, however, may also be due to errors in sedimentation and/or counting under the microscope. The TNA developed in this study is also expected to provide reliable results applied to samples from other populations of *P. agardhii* and *P. rubescens.*

Acknowledgements

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