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First report of cyanobacterial paralytic shellfish toxin biosynthesis genes and paralytic shellfish toxin production in Polish freshwater lakes

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ABSTRACT

In central and southern Europe, *Aphanizomenon* spp., *A. gracile* Lemmermann in particular, have been associated with paralytic shellfish toxin (PST) production. In western Poland, *A. gracile* is very common, and *Cylindrospermopsis raciborskii* (Woloszyńska) Seenayya & Subba Raju, another potentially PST-producing species, is often found as well. To date it is, however, unknown if the cyanobacterial populations in this area harbour the genetic capability to produce PSTs, and to what extent toxin biosynthesis occurs. The objective of this study was to survey the prevalence of potentially PST-producing cyanobacteria by measuring paralytic shellfish toxin biosynthesis gene *sxtB* copy numbers, *sxtA*, *sxtG* and *sxtS* gene presence, and PST concentrations in Polish freshwater lakes. In total, 34 lakes in western Poland were sampled twice during summer 2010. The presence of PST biosynthesis genes *sxtA*, *sxtG* and *sxtS* was determined using conventional qualitative PCR. Quantitative PCR (qPCR) was used to measure *sxtB* copy numbers, and the samples were analysed for PSTs using ion-pair high performance liquid chromatography with post-column oxidation and fluorescence detection (HPLC-FLD). Cyanobacteria carrying the *sxtB* gene were present in 23.5% of all samples (n=16) and in 14 lakes of the studied 34. Gene copy numbers ranged from 8.2×10^4 to 5.1×10^7 *sxtB* copies L^{-1} (mean 3.8×10^6). The median was 4.5×10^5 *sxtB* gene copies L^{-1} and the majority of results clustered at the lower end of the *sxtB* qPCR linear range. In 12 out of the 16 samples positive for *sxtB* the gene co-occurred with the other three targeted PST biosynthesis genes *sxtA*, *sxtG* and *sxtS*. However, five additional samples lacked one or two of the targeted four genes. Thirteen samples contained PSTs, of which 12 samples at levels <0.072 µg L^{-1}, *i.e.*, close to or below the quantitative detection limit of the HPLC-FLD method (0.01 µg L^{-1}). One sample contained 0.57 µg L^{-1} saxitoxin, co-occurring with all four *sxt* genes studied. No correlation between PST and *sxt* gene occurrence or copy numbers was observed. *A. gracile* and *C. raciborskii* occurred in 92% and 50% of samples, respectively, containing PSTs, *sxt* genes or both. In conclusion, the results confirm that potential PST producers constitute an established subpopulation of cyanobacteria in Polish freshwater lakes. However, none of the *sxt* genes targeted in this study could serve as a reliable marker for active PST biosynthesis.

Key words: Saxitoxin; paralytic shellfish toxins; cyanobacteria; lakes; quantitative PCR; PCR.

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INTRODUCTION

Saxitoxins or paralytic shellfish toxins (PSTs) are a group of potent alkaloid neurotoxins produced by a number of freshwater cyanobacterial species, as well as by certain marine dinoflagellates. Paralytic shellfish toxins accumulate in the food chain, and consumption of toxin-contaminated seafood can lead to potentially severe poisoning. Health risks are also associated with the use of water sources harbouring PST-producing cyanobacteria for *e.g.* drinking water production or recreation. In Europe, PSTs have been detected widely in freshwater lakes, rivers and reservoirs; in Czech Republic (Jančula *et al.*, 2014) and Spain PST production has been associated with blooms of *Aphanizomenon* spp. (Wörmer *et al.*, 2011). In Finland (Rapala *et al.*, 2005; Savela *et al.*, 2015) and Denmark (Kaas and Henriksen, 2000) *Dolichospermum lemmermannii* (Richter) P. Wacklin, L. Hoffmann & J. Komárek (formerly *Anabaena lemmermannii*, Wacklin *et al.*, 2009), a species closely related to *Aphanizomenon gracile* Lemmermann (Cirés and Ballot, 2016), has been implicated as the most likely PST-producing species. Moreover, biosynthesis of PSTs has been confirmed in numerous strains of *A. gracile* from Germany, Portugal and France (Pereira *et al.*, 2004; Ballot *et al.*, 2010; Ledreux *et al.*, 2010; Casero *et al.*, 2014; Cires *et al.*, 2014) and *Cuspidothrix issatschenkoi* (Usachev) P. Rajaniemi, Komárek, R. Willame, P. Hrouzek, K. Kastovská, L. Hoffmann & K. Sivonen (formerly *Aphanizomenon issatschenkoi*, Rajaniemi *et al.*, 2005) from Portugal

(Pereira *et al.*, 2000; Li *et al.*, 2003). Other PST-producing cyanobacteria encountered elsewhere in the world include *Cylindrospermopsis raciborskii* (Woloszyńska) Seenayya & Subba Raju (Lagos *et al.*, 1999), *Dolichospermum circinale* (Rabenhorst ex Bornet & Flahault) P. Wacklin, L. Hoffmann & J. Komárek (formerly *Anabaena circinalis*, Wacklin *et al.*, 2009) and *Plectonema wollei* Farlow ex Gomont (Carmichael *et al.*, 1997), *Raphidiopsis brookii* Hill (Yunes *et al.*, 2009), *Scytonema* sp. (Smith *et al.*, 2011), *Geitlerinema* spp., *Phormidium uncinatum* Gomont ex Gomont, and *Cylindrospermum stagnale* Bornet & Flahault (Borges *et al.*, 2015). Of the above mentioned species, *C. raciborskii* is commonly found also in European lakes, including Poland (Kokociński and Soininen, 2012), but has not been associated with PST production in Europe to date.

PSTs affect vertebrate nerve function by reversibly blocking voltage-gated ion channels, primarily Na^+ (Llewellyn, 2006), but also K^+ and Ca^{2+} channels are affected (Kao, 1986, Wang *et al.*, 2003, Su *et al.*, 2004). Ion channel blockage leads to inhibition of neuronal signal transduction, the extent of which is directly dependent on PST concentration. Disruption of neuronal signals leads to symptoms ranging from slight numbness and tingling of extremities to full respiratory arrest and death in extreme cases (Llewellyn, 2006). The effectivity of ion channel blockage depends on the structural characteristics of each given PST variant, 57 of which have been identified (Wiese *et al.*, 2010). The biosynthesis of all PSTs is directed by a suite of biosynthetic genes (*sxt*), first described in *C. raciborskii* strain T3 (Kellmann *et al.*, 2008). Since then, toxin-specific molecular genetic analysis has been employed in the study of PST-producing cyanobacteria in culture and in the field. Several studies on European cyanobacteria have been carried out on strains isolated from freshwater lakes and reservoirs, and many have targeted the PST biosynthesis gene *sxtA* showing that while all PST-producing strains do carry the gene, it can also be found in *Anabaena*, *Anabaenopsis* and *Aphanizomenon* strains which appear to lack the ability to produce the toxins (Ballot *et al.*, 2010; Ledreux *et al.*, 2010; Cires *et al.*, 2014). Other *sxt* genes, including *sxtG*, *sxtB*, *sxtI* and *sxtX,* have been studied to a lesser extent (Casero *et al.*, 2014, Gkelis and Zaoutsos, 2014, Savela *et al.*, 2015). Only a few studies have investigated the co-occurrence of *sxt* genes and PSTs in environmental samples in Europe (Gkelis and Zaoutsos, 2014; Savela *et al.*, 2015) or elsewhere in the world (Al-Tebrineh *et al.*, 2012; Bowling *et al.*, 2013).

Cyanobacterial toxins are common in western Polish lakes; cylindrospermopsin, produced primarily by *A. gracile*, is found at concentrations exceeding WHO recommendations (Kokociński *et al.*, 2013). Hepatotoxic microcystins are another common cyanotoxin group in Polish lakes (Mankiewicz-Boczek *et al.*, 2006). However, neither the prevalence of potentially PST-producing cyanobacteria, indicated by the presence of biosynthesis genes (*sxt*), nor PSTs in Polish lakes has been previously studied (Kobos *et al.*, 2013). In this study, qualitative PCR methods targeting the *sxtA* and *sxtG* genes and a quantitative method for the detection of *sxtB*, which have been previously employed in the analysis of lakes and brackish coastal waters in Åland Islands, Finland (Savela *et al.*, 2015) were applied to investigate freshwater lakes in western Poland. Targeting previously studied genes allowed comparisons to existing data. To assess the potential benefit of adding a new gene target to the analysis, primers for the detection of the PST biosynthesis gene *sxtS* were designed and used to complement existing methods. This study reports the first analysis of Polish freshwater lakes for the prevalence of selected cyanobacterial paralytic shellfish toxin biosynthesis genes as well as PST prevalence and concentrations.

METHODS

Study site and sampling

Sampling was carried out in randomly selected freshwater lakes (n=34) in Western Poland twice during summer 2010; first between June 6th and July 23rd (Tab. 1) and again between August 20th and October 1st (Tab. 2). The limnological characteristics and geographical locations of the lakes as well as the sampling procedure have been described in detail by Kokociński *et al.* (2013). Briefly, 3 to 4 subsamples were taken at 1 m vertical intervals using a 0.5 m Limnos sampler starting at the depth of 1 m. One litre of the pooled subsamples from each location was transported to the laboratory and used for phytoplankton identification and biomass calculations (as described in Kokociński *et al.*, 2013) and molecular and toxin analyses.

Sample preparation for molecular analyses

Aliquots (20 mL) of the 1 L subsample were filtered onto 25 mm diameter Whatman GF/C filters and stored frozen (-20°C). Addition of exogenous amplification control and heat treatment (80°C, 10 min) in sterile deionized water (V=1.5 mL) water to lyse the cyanobacteria were carried out as described previously (Savela *et al.*, 2014). The resulting lysate was stored frozen (-20°C) until further analysis. Spectrophotometric analysis of DNA quantity and quality was not applicable to this sample type.

Qualitative analysis of *sxtA*, *sxtG* and *sxtS*

Sample lysates were analysed for the presence of PST biosynthesis genes *sxtA*, *sxtG* and *sxtS* using primers listed in Tab. 3. Successful amplification resulted in 648 bp, 519 bp and 382 bp PCR products, respectively. The specificity

of *sxtA* and *sxtG* primers has been validated previously (Savela *et al.*, 2015). For this study, the *in silico* specificity of *sxtS* primers was confirmed by running a query against the GenBank nr database (as available in February 2011) using blastn 2.2.29+ for highly similar sequences (Altschul *et al.*, 1997) and optimized for short input sequences. The *sxtS* target is located at bases 130-511 of the *D. circinale* AWQC131C (DQ787201) and the *Aphanizomenon* sp. NH-5 (EU603710) *sxtS* gene and bases 216-597 of the *C. raciborskii* T3 (DQ787200) *sxtS* gene. The *in vitro* specificity was confirmed by analysing PST-producing and non-producing cyanobacterial strains following the same procedures as for *sxtA* and *sxtG* (Savela *et al.*, 2015). For environmental sample analysis, *sxtA*, *sxtG* and *sxtS*-specific PCR reactions (total volume 20 µL) contained 1X Phire Reaction buffer and 0.4 µL Phire II Hot-Start DNA polymerase (Thermo Scientific), 0.2 mM dNTPs (Bioline), 0.5 µM appropriate forward and reverse primers and 4 µL sample lysate. One ng of *D. circinale* CS-537/13 genomic DNA was used as the positive control. Thermal cycling was performed using a Bio-Rad C1000 Touch cycler: initial denaturation at 98°C for 30 s, then 35 cycles of 98°C 5 s, 62°C 5 s and 72°C 10 s, final extension at 72°C for 1 min (Savela *et al.*, 2015). Reaction outcome was determined on 1% w/v agarose gels stained with 1X (final conc.) SYBR®Safe dye (Thermo Fisher Scientific). UV illumination and gel imaging was carried out using a Bio-Rad GelDoc XR system.

Tab. 1. The prevalence of *sxtA*, *sxtG*, *sxtS*, potentially PST-producing cyanobacterial species, the quantity of *sxtB* gene copies and the concentration and identity of PSTs in western Polish lakes in June-July of 2010.

Lake	*sxtB* (copies L^{-1})	*sxtA* (+/-)	*sxtG* (+/-)	*sxtS* (+/-)	PSTs (µg L^{-1})	*A. gracile* (+/-)	*C. issatschenkoi* (+/-)	*A. flos-aquae* (+/-)	*C. raciborskii* (+/-)
Biezdruchowo	-	-	-	-	-	+	-	-	-
Biskupieckie	-	-	-	-	-	-	-	-	-
Biskupińskie	-	-	-	-	-	+	-	-	+
Bnińskie	-	-	-	-	-	+	-	-	+
Boczowskie	-	-	-	-	0.043 (STX)	+	-	-	+
Busko	-	-	-	-	-	+	-	+	-
Buszewskie	4.98×10^5	+	+	+	-	+	-	-	+
Bytyńskie	-	-	-	-	-	+	-	-	+
Długie	-	-	-	-	-	-	-	-	-
Głębokie	-	-	-	-	-	-	-	-	-
Grylewskie	-	-	-	-	-	+	-	-	+
Ilno	-	-	-	-	-	+	-	+	-
Jelonek	-	-	-	-	-	+	+	-	+
Kierskie	-	-	-	-	trace (STX)	+	-	-	-
Kierskie Małe	2.53×10^6	+	+	+	trace (STX)	+	+	-	-
Kowalskie	-	-	-	-	-	+	+	-	-
Kruchowskie	-	-	-	-	-	-	-	-	-
Kursko	-	-	-	-	-	+	+	+	+
Lednica	-	-	-	-	-	-	-	-	-
Lubosińskie	-	+	+	-	-	+	-	-	-
Lusowskie	-	-	-	-	-	-	-	-	-
Mogileńskie	8.20×10^4	-	+	-	trace (dcSTX)	+	+	-	-
Niepruszewskie	-	-	-	-	-	-	-	-	-
Niesłysz	-	-	-	-	-	-	-	-	-
Pniewskie	-	-	-	-	-	+	-	-	-
Sarbsko	4.68×10^5	+	+	+	0.57 (STX)	+	+	-	-
Strykowskie	-	-	-	-	-	+	-	-	-
Strzyżewskie	1.29×10^5	+	+	+	-	+	+	-	-
Świętokrzyskie	-	-	-	-	-	+	-	-	+
Szydłowskie	-	-	-	-	-	+	+	-	-
Tomickie	-	-	-	-	-	+	-	-	+
Witobelskie	-	-	-	-	-	+	-	-	-
Żabiniec	-	-	-	-	-	-	-	-	-
Zbąszyńskie	-	-	-	-	-	+	+	+	-

Quantitative analysis of *sxtB* copy numbers

The quantitative PCR (qPCR) method used to detect and measure *sxtB* copy numbers in cyanobacteria, and the method used to assess possible inhibition in environmental sample lysates have been described previously in detail by Savela *et al.* (2014, 2015).

Primer and probe sequences are listed in Tab. 3. Briefly, the labelling of the *sxtB* detection probe with an intrinsically fluorescent terbium(III) chelate ({2,2,′2′′, 2′′′-{{6,6′-{4′′-[2-(4-Isothiocyanatophenyl)ethyl]-pyratzole-1′′,3′′-diyl}bis(pyridine)-2,2′-diyl}bis(methylenenitrilo)}-tetrakis(acetato)}Tb(III)), University of Turku, Turku, Finland) was carried out as described by Nurmi *et al.* (2002). The 20 µL qPCR reactions contained 0.2 U DyNAzyme II HotStart polymerase, 1X DyNAzyme II HS buffer, 0.2 mM dNTPs (Finnzymes, Espoo, Finland), 0.4 mM both primers, 24 nM sxtB_Q quencher probe, 3 nM labelled sxtB_P detection probe and 4 µL template; standard or sample lysate. Quantification standards were produced from *D. circinale* CS-537/13 genomic DNA, quantified, purified and diluted as described previously (Savela *et al.*, 2015). Thermal cycling [MJ Research (St. Bruno, Canada) PTC-200 Thermal Cycler or Bio-Rad (Hercules, CA, USA) C1000 Touch Thermal Cycler] was performed as follows: initial denaturation, 95°C 5 min, then 40 cycles of 95°C 30 s, 62°C 1 min. Every second cycle, from cycle 8 to cycle 40, after annealing and ex-

Tab. 2. The prevalence of *sxtA*, *sxtG*, *sxtS*, potentially PST-producing cyanobacterial species, the quantity of *sxtB* gene copies and the concentration and identity of PSTs in western Polish lakes in August-October of 2010.

Lake	*sxtB* (copies L^{-1})	*sxtA* (+/-)	*sxtG* (+/-)	*sxtS* (+/-)	PSTs (µg L^{-1})	*A. gracile* (+/-)	*C. issatschenkoi* (+/-)	*C. ovalisporum* (+/-)	*A. flos-aquae* (+/-)	*C. raciborskii* (+/-)
Biezdruchowo	-	-	-	-	-	+	-	-	-	-
Biskupieckie	-	-	-	-	-	-	-	-	-	-
Biskupińskie	-	-	-	-	trace (STX)	+	-	-	-	+
Bnińskie	-	+	+	+	-	+	-	-	-	+
Boczowskie	-	-	-	-	-	+	-	-	-	+
Busko	4.31×10^5	+	+	+	trace (STX)	-	-	-	-	-
Buszewskie	-	-	-	-	trace (dcSTX)	+	-	-	-	+
Bytyńskie	-	-	-	-	-	+	-	-	-	+
Długie	-	-	-	-	trace (STX)	-	-	-	-	-
Głębokie	-	-	-	-	-	-	-	-	-	-
Grylewskie	-	-	-	-	-	+	-	-	-	+
Ilno	5.10×10^7	+	+	+	0.072 (STX)	+	+	-	+	-
Jelonek	-	-	-	-	-	+	-	-	+	+
Kierskie	-	-	-	-	-	+	+	-	+	-
Kierskie Małe	-	-	-	-	-	+	+	-	-	+
Kowalskie	1.28×10^5	+	+	+	-	+	+	-	-	-
Kruchowskie	8.37×10^5	+	+	+	trace (STX)	+	-	-	-	+
Kursko	1.32×10^5	+	+	+	-	+	+	-	+	+
Lednica	-	-	-	-	-	-	-	-	-	-
Lubosińskie	3.97×10^5	+	+	-	-	+	-	-	-	+
Lusowskie	-	-	-	-	-	-	-	-	-	-
Mogileńskie	2.50×10^5	+	+	+	-	+	+	-	-	+
Niepruszewskie	-	-	-	-	-	-	-	-	-	-
Niesłysz	-	-	-	-	-	+	-	-	+	-
Pniewskie	-	-	-	-	0.069 (dcSTX)	+	-	-	-	+
Sarbsko	9.85×10^5	+	+	+	-	+	+	+	-	-
Strykowskie	-	-	-	-	-	+	-	-	-	+
Strzyżewskie	-	-	-	-	-	+	+	-	+	-
Świętokrzyskie	-	-	-	-	-	-	-	-	+	+
Szydłowskie	3.96×10^5	+	+	+	-	+	+	-	-	-
Tomickie	5.49×10^5	+	+	+	-	+	-	+	+	+
Witobelskie	-	-	-	-	-	+	-	-	-	+
Żabiniec	-	-	-	-	-	+	+	-	-	+
Zbąszyńskie	1.14×10^5	+	+	-	-	+	-	-	+	+

tension the temperature was lowered to 35°C for 15 s, during which Tb fluorescence was measured using the standard TRF-Tb protocol on a Victor X4 2030 Multilabel Reader (PerkinElmer, Waltham, MA, USA).

Extraction and analysis of PSTs

Extraction of PSTs from water sample aliquots preserved on filters and the analysis of the resulting extracts using ion-pair high-performance liquid chromatography with post-column oxidation and fluorescence detection (HPLC-FLD) was carried out as described previously (Savela *et al.*, 2015). Certified reference materials included C-toxins (C1-2), gonyautoxins (GTX1–4, GTX2–3, GTX5), decarbamoyl gonyautoxins (dcGTX2-3), saxitoxin (STX), decarbamoyl saxitoxin (dcSTX) and neosaxitoxin (NeoSTX) (NRC-IMB, Halifax, Canada).

Data analysis

Raw fluorescence measurement data from the qPCR experiments was analysed in Microsoft Excel 2010. Amplification curves were generated by plotting well-specific signal-to-background ratios against their respective PCR cycles. Background was determined for each individual reaction separately; the average signal of the first two measurements was used. C_q-values were determined visually, and values deviating from the other replicates by more than one cycle were not considered in the final analysis. Statistical analysis was carried out using R Statistical package version 3.2.0 (R Core Team, 2015).

RESULTS

sxt genes

Specificities of the *sxtA* and *sxtG* PCR methods as well as the properties of the *sxtB* qPCR assay have been described previously by Savela *et al.* (2015). For this study, an additional primer pair targeted at the paralytic shellfish toxin biosynthetic gene *sxtS* was applied to analysis of environmental samples. *In silico* analysis revealed no significant non-target hits, and no amplification was observed in cyanobacterial strains incapable of PST production (Tab. 4, Supplementary Figure S1). The *sxtB* gene was amplified in 14 (41.1 %) out of the 34 lakes investigated in this study. In total, 23.5% of all samples contained the *sxtB* gene, detection frequency increasing towards the end of summer, from 18% in June-July (Tab. 1) to 29% in August-October (Tab. 2). Significant inhibition in the amplification of the exogenous amplification control was not observed. For two of these lakes, Lake Mogileńskie and Lake Sarbsko, the *sxtB* gene was found in samples taken both in June–July and August-–October. Overall, *sxtB* copy numbers were low; in June-July the average was 7.4×10^5 copies L^{-1} (median 4.7×10^5). In August-October the average was 5.1×10^6 copies L^{-1}, but the median only 4.1×10^5. Only one relatively high *sxtB* copy number was measured in a sample collected at Lake Ilno in August 2010, 5.1×10^7 copies L^{-1}. The presence of *sxtA*, *sxtG* and *sxtS* genes was confirmed in 75% (n=12) of the *sxtB*-positive samples. One to two target genes were absent from five samples. In four samples, either the gene *sxtB* or *sxtS* was absent, once both genes *sxtA* and *sxtS* were not detected (Tabs. 1 and 2).

Paralytic shellfish toxins

PSTs were detected in a total of 13 samples. One sample, collected at Lake Sarbsko in July 2010 contained 0.57 µg L^{-1} saxitoxin. In three of the PST-positive samples concentrations between 0.043-0.072 µg L^{-1} were measured, and the majority (n=9) contained trace amounts of PSTs, the concentrations not exceeding the quantitative detection limit of the HPLC-FLD method (0.01 µg L^{-1}, Savela *et al.*, 2015). Only saxitoxin and decarbamoylsaxitoxin were present in the samples, other variants were not detected. PSTs or *sxt* genes were observed in 18 samples of

Tab. 3. Primers and probes for conventional qualitative PCR and qPCR used in this study.

Gene	Name	Sequence (5'–3')
sxtA	sxtA855_F*	GACTCGGCTTGTTGCTTCCCC
	sxtA1480_R*	GCCAAACTCGCAACAGGAGAAGG
sxtG	sxtG432_F*	AATGGCAGATCGCAACCGCTAT
	sxtG928_R*	ACATTCAACCCTGCCCATTCACT
sxtS	sxtS205_F°	GGAGTATTGGCGGGTGACTATGA
	sxtS566_R°	GGTGGCTACTTGGTATAACTCGCA
sxtB	sxtB_F2*	TGTTGTGCTTGCTGCTCTATCAG
	sxtB_R2*	CAGCGTTTTCAGCGTAYCGAC
	sxtB_P detection probe*	aminoC6-CAATCAAAGTTATGCTCCCTATACGA-Phos
	sxtB_Q quencher probe*	GGGAGCATAACTTTGATTG-BHQ®1

*Savela *et al.*, 2015; °this study.

the total 68. Only 6 of the 68 samples contained both PSTs and *sxt* genes. No quantitative correlation between *sxtB* copy numbers and PST concentrations was observed (Spearmans's ρ=0.19, P=0.11).

Identification of potentially PST-producing cyanobacteria

Cyanobacterial populations in the studied lakes at the times of sampling were analysed for potentially PST-producing species (Tabs. 1 and 2). Particularly *A. gracile* was abundant, present in 75% of all samples, being as common in June-July as in August-October. The species was present in 83% and 94% of PST- and *sxt*-gene containing samples, respectively. *C. raciborskii* was also commonly observed, the overall prevalence increasing from 29% to 56% towards the end of summer. It was present in 42% and 44% of PST- and *sxt*-gene containing samples, respectively. Of other potentially PST-producing species, *C. issatschenkoi* was observed in 26% and 29% and *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault in 12% and 26% of all samples in June-July and August-October, respectively. Of these two, *C. issatschenkoi* was present in 50% and 56% of the PST- and *sxt*-gene containing samples. The corresponding percentages for *A. flos-aquae* were 8% and 22%. *C. ovalisporum* was present in two samples (6%) in August-October, one of which contained both PSTs and *sxt* genes.

DISCUSSION

In this study, potentially PST-producing cyanobacteria were for the first time indicated in Polish lakes on the basis of *sxt* gene detection. Additionally, active but low-level biosynthesis of PSTs was demonstrated by the presence of saxitoxin or decarbamoylsaxitoxin in 19% of the 68 samples tested. The results are in line with other studies investigating central European freshwaters (Ballot *et al.*, 2010; Ledreux *et al.*, 2010, Jančula *et al.*, 2014).

On average, the *sxt* gene cluster includes 30 genes, the exact number as well as the composition and arrangement of genes within the cluster being genus-dependent (Mihali *et al.*, 2009). The gene cluster includes so-called "core", or essential genes, without which PST biosynthesis cannot function (D'Agostino *et al.*, 2014), and the majority of these are conserved among PST-producing cyanobacterial genera (Murray *et al.*, 2011). Conserved core *sxt* genes include *sxtA*, *sxtB*, *sxtG* and *sxtS* (Murray *et al*., 2011) which were targeted in this study. These four genes are responsible for the initiation of PST biosynthesis and formation of the three central heterocycles and the second

Tab. 4. Amplification of *sxtS* in in toxin-producing and non-toxic cyanobacteria.

Species	Strain*	*sxtS*°	Toxins produced#
Dolichospermum circinale	CS-337/01	+	PST
Dolichospermum circinale	CS-537/13	+	PST
Dolichospermum circinale	CS-530/05	-	nd
Dolichospermum circinale	CS-533/12	-	nd
Anabaena cylindrica	PCC 73105	-	nd
Anabaena cylindrica	PCC 7938	-	nd
Dolichospermum lemmermannii var. *minor*	NIVA-CYA 83/1	-	MCYST§
Dolichospermum lemmermannii var. *minor*	NIVA-CYA 266/1	-	MCYST§
Cylindrospermopsis raciborskii	CS-505	-	CYN^
Cylindrospermopsis raciborskii	CS-506	-	CYN$
Cylindrospermopsis raciborskii	CS-510	-	nd
Nodularia sphaerocarpa	PCC 7804	-	L-HAr^2-NOD**
Nostoc sp.	PCC 6310	-	nd
Nostoc sp.	PCC 7422	-	nd
Microcystis aeruginosa	PCC 7806	-	MCYST§
Microcystis aeruginosa	NIVA-CYA 140	-	MCYST§
Microcystis aeruginosa	PCC 7005	-	nd
Planktothrix agardhii	NIVA-CYA 15	-	MCYST§
Planktothrix agardhii	NIVA-CYA 299	-	MCYST§
Planktothrix agardhii	NIVA-CYA 12	-	nd

*Strains were obtained from the Australian National Algae Culture Collection (ANACC, CS), Pasteur Culture Collection (PCC) and the Norwegian Institute for Water Research (NIVA). Culture conditions and DNA extraction have been previously described (Savela *et al.*, 2015); °this study; #data from Savela *et al*. 2015, unless otherwise indicated; §Hautala *et al*., 2013; ^Stucken *et al*., 2009 and references therein; $Kokociński *et al*., 2013; **Beattie *et al.*, 2000; nd, not detected; MCYST, microcystins; CYN, cylindrospermopsin; L-HAr^2-NOD, L-homoarginine^2-nodularin.

guanidino group present in all PST variants known to date, and are thus necessary for PST production (Kellmann *et al.*, 2008, Mihali *et al.*, 2009, Wiese *et al.*, 2010). In the majority (75%) of *sxt*-positive samples (n=18) all four genes were present. The presence of all four genes suggests a functional *sxt* gene cluster, although other genes are still needed to complete the biosynthesis route. Interestingly, in some samples (n=5) an apparent lack of one to two of the targeted *sxt* genes was observed. There are two possible explanations for this phenomenon. First, non-amplification might be caused by sequence dissimilarities between target and primers which could exist in natural cyanobacterial populations while being absent from the *sxt* gene sequences available at sequence databases. Both the *sxtA* and *sxtG* primer sequences are identical with their respective binding sites in *Aphanizomenon* sp. and *C. raciborskii* sequences, but nevertheless *sxtA* failed to amplify in one sample despite other *sxt* genes being present, indicating critical dissimilarities between the primers and the target. Both *sxtB* and *sxtS* forward primers share at most one known internal nucleotide mismatch with the corresponding sequences in the abovementioned species. An internal mismatch can affect reaction efficiency, but is not sufficient to abolish amplification, as shown by the robust amplification of *sxtS* in *D. circinale*, which shares an identical primer binding site, including the one mismatch, with *Aphanizomenon* sp. NH-5. Second, mutations in a larger scale, including deletions and insertions within the *sxt* gene cluster would result in a negative outcome in PCR analysis. Such mutations would also result in an inactive gene cluster and effectively a non-toxic cyanobacterial strain. Other cyanotoxin genes, most notably the microcystin biosynthesis genes are known to occasionally contain mutations rendering the biosynthesis pathway inactive (Christiansen *et al.*, 2006; Noguchi *et al.*, 2009; Ostermaier and Kurmayer, 2009). This suggests that similar mutations might be found also in PST-producing cyanobacteria. Indeed, previous studies have shown that the presence of *sxt* genes *e.g. sxtA,* does not always indicate detectable PST production in cyanobacteria; *sxtA* has been detected in non-PST-producing *Aphanizomenon*, *Anabaena* and *Anabaenopsis* strains (Ballot *et al.*, 2010; Casero *et al.*, 2014). Similarly, in this study none of the analysed samples in which a partial set of *sxt* genes was observed contained PSTs. However, the general lack of correlation between *sxt* gene presence and quantity and PST concentrations means that definitive conclusions on the presence of mutated *sxt* gene clusters cannot be drawn, and to confirm the hypothesis a suspected mutant strain would have to be isolated and the *sxt* genes characterized in detail.

An overall lack of correlation between *sxt* gene presence and copy number quantity and PST concentrations in the investigated lakes was apparent. Only a few surveys of environmental samples without prior strain isolation have been carried out, however, findings similar to this study have been obtained in Greece, where amplification of the *sxtI* gene was twice as common as PST detection (Gkelis and Zaoutsos, 2014) and during a three-year survey of lakes in Åland Islands in Finland using the same methods as in this study (Savela *et al.*, 2015) with the exception of the new *sxtS* primers. The observed lack of correlation is not limited to Europe; in Australia, neither the presence of *sxtA* nor the gene's increasing copy numbers did indicate a detectable presence or growing concentrations of PSTs in the environment (Al-Tebrineh *et al.*, 2012; Bowling *et al.*, 2013). Comparisons of qPCR and HPLC-FLD data are complicated by the fact that qPCR can be extremely sensitive (Wittwer *et al.*, 1997), whereas PSTs can be difficult to analyse in complex environmental sample extracts. For this reason, molecular analysis of PST biosynthesis genes have been proposed as an easier alternative for screening environmental samples for potential PST production. In this study, in addition to using HPLC-FLD, samples were originally analysed for PSTs using a hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) method (Diener *et al.*, 2007; Savela *et al*., 2015). Due to the very low PST concentrations, and similarly to previous work reported by Savela *et al*. (2015), only the HPLC-FLD method could be used to detect the toxins; its sensitivity was far superior compared to that of the HILIC-MS/MS method, which employed an ion-trap MS instrument. Notwithstanding methodological limitations, the findings from this and previous studies suggest regulation of PST production at the transcriptional and/or translational level, rather than toxin biosynthesis being a direct consequence of the biosynthetic capability encoded in the genome. Environmental factors including osmotic pressure, ionic stress and availability of organic nitrogen have been shown to affect *sxt* gene expression, PST production and export in *Aphanizomenon* spp., *D. circinale* and *C. raciborskii* (Dias *et al.*, 2002; Pomati *et al.*, 2004; Casero *et al.*, 2014; Ongley *et al*., 2016), suggesting complex regulation mechanisms of toxin biosynthesis tied to the processes of primary metabolism. Therefore the simple approach of measuring *sxt* gene presence or copy number would not serve as a reliable indicator of PST production; measuring gene expression instead might predict active PST biosynthesis better.

Of the potentially PST-producing cyanobacteria, *A. gracile* was present in 83% and 94% of *sxt* gene- and/or PST-containing samples, respectively. The role of *A. gracile* in PST production is supported by its confirmed status as a PST-producing species in *e.g.* north-eastern Germany (Ballot *et al.*, 2010). Previously, a positive correlation between *A. gracile* biomass and cylindrospermopsin, another cyanobacterial toxin, has already been

shown (Kokociński *et al.*, 2013). These observations suggest that *A. gracile* populations in western Polish lakes may contain both cylindrospermopsin- and PST-producing strains. However, the involvement of other *Aphanizomenon* species, which often co-occurred with *A. gracile* in the lakes included in this study, in PST production cannot be ruled out. Further study into the *Aphanizomenon* spp. described here would be required to definitely confirm the identity and possible role in PST production in Polish lakes. The second most common species to co-occur with *sxt* genes and/or PSTs was *C. raciborskii*. To date, the species has not been associated with PST production in European freshwaters, and conclusions of its possible role as a PST producer in western Polish lakes cannot be made without further investigation.

CONCLUSIONS

In this study, both the genetic capability for PST biosynthesis and active PST production in selected freshwater lakes in western Poland was demonstrated for the first time. The most likely potentially PST-producing species is *A. gracile*, however, further studies on isolated strains need to be conducted to confirm this hypothesis. The low *sxtB* gene copy numbers and PST concentrations indicate that PST producers constituted a relatively minor subpopulation in the studied waterbodies. The non-amplification of certain *sxt* genes in the presence of others suggested the possible presence of inactive *sxt* gene clusters in the studied cyanobacterial populations. Since no significant correlation between genes *sxtA*, *sxtB*, *sxtG* and *sxtS*, and measured PSTs was observed, it was concluded that analysis of *sxt* genes indicates the presence of potentially PST-producing cyanobacteria, but is not suitable for predicting active PST biosynthesis in the studied Polish lakes.

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