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Paralytic shellfish toxin producing *Aphanizomenon gracile* strains isolated from Lake Iznik, Turkey

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ABSTRACT

Aphanizomenon gracile is one of the most widespread Paralytic Shellfish Toxin (PST) producing cyanobacteria in freshwater bodies in the Northern Hemisphere. It has been shown to produce various PST congeners, including saxitoxin (STX), neosaxitoxin (NEO), decarbamoylsaxitoxin (dcSTX) and gonyautoxin 5 (GTX5) in Europe, North America and Asia. Three cyanobacteria strains were isolated in Lake Iznik in northwestern Turkey. Morphological characterization of these strains suggested all three strains conformed to classical taxonomic identification of *A. gracile* with some differences such as clumping of filaments, partially hyaline cells in some filaments and longer than usual vegetative cells. Sequences of 16S rRNA gene of these strains were placed within an *A. gracile* cluster including the majority of PST producing strains, confirming the identification of these strains as *A. gracile*. These new strains possessed saxitoxin biosynthesis genes *sxtA*, *sxtG* and their sequences clustered with those of other *A. gracile*. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis demonstrated the presence of NEO, STX, dcSTX and decarbamoylneosaxitoxin (dcNEO) in all strains. This is the first report of a PST producer in any water body in Turkey and first observation of dcNEO in an *A. gracile* culture.

1. Introduction

Saxitoxin and its analogues are a group of guanidinium containing alkaloids produced either by marine dinoflagellates or freshwater cyanobacteria (Wiese et al., 2010). These toxins exert their effect by blocking voltage gated sodium channels, calcium channels and by modifying the gating behavior of potassium channels in mammals (Cusick and Sayler, 2013 and references therein). They are usually transferred to humans and animals via vector organisms such as shellfish (Wiese et al., 2010). In cases of severe mammalian intoxications they cause the paralysis of respiratory muscles and therefore they are collectively called Paralytic Shellfish Toxins (PSTs) (Cusick and Sayler, 2013; Wiese et al., 2010). While in marine environments the main route of intoxication is through the food web (Wiese et al., 2010), in fresh water environments exposure may result both from the food web and drinking water (Batoréu et al., 2005).

PSTs are produced by strains of various cyanobacterial species in freshwater environments. These include *Dolichospermum circinale* in Australia (Humpage et al., 1994), *Cylindrospermopsis raciborskii* (Lagos et al., 1999), *Raphidiopsis brookii* (Yunes et al., 2009), *Geitlerinema* spp., *Phormidium uncinatum* and *Cylindrospermum stagnale* (Borges et al., 2015) in South America, *Microseira wollei* (Foss et al., 2012; Onodera et al., 1997) in North America, *Cuspidothrix issatschenkoi* (Pereira et al., 2000) in Europe, *Scytonema* sp. in New Zealand (Smith et al., 2011) and *Aphanizomenon gracile* in North America, Europe and Asia (Ballot et al., 2010; Cirés et al., 2014; Jung et al., 2003; Ledreux et al., 2010; Liu et al., 2006a; Mahmood and Carmichael, 1986; Pereira et al., 2004; Perez et al., 2008).

Aphanizomenon gracile, one of the most widespread PST producing cyanobacteria, is a species of the temperate regions (Komarek and Komarkova, 2006). The first report of PST production

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in *A. gracile* was from a strain isolated in Durham, NH, USA (strain NH-5) (Mahmood and Carmichael, 1986). Although initially identified as an atypical non-fasciculated *A. flos-aquae*, subsequent molecular work placed this strain within an *A. gracile* cluster (Casero et al., 2014; Li et al., 2003). Isolation of PST producing *A. gracile* in Europe was much later, first in Portugal (Pereira et al., 2004), followed by Germany (Ballot et al., 2010), France (Ledreux et al., 2010), Spain (Cirés et al., 2014) and Norway (Ballot et al., 2016). On the other hand, in Asia, two *A. gracile* strains are known producers of PSTs. *Aphanizomenon gracile* NIES-81 (*A. flos-aquae* f. *gracile*), isolated in Japan in 1978, was shown to produce saxitoxin in 2003 (Jung et al., 2003) and strain DC-1 from China produced various PSTs (Liu et al., 2006a, 2006b).

Aphanizomenon gracile strains isolated to date proved to produce certain PST analogues including saxitoxin (STX), neosaxitoxin (NEO), decarbamoylsaxitoxin (dcSTX) and gonyautoxin-5 (GTX5). Strain NH-5 from USA (Mahmood and Carmichael, 1986), LMECYA-40 from Portugal (Pereira et al., 2004), PMC 627.10 and 638.10 from France (Ledreux et al., 2010) produced only STX and NEO. While strain UAM 529 from Spain produced only STX and dcSTX, the other Spanish strain UAM 531 produced STX, NEO and dcSTX (Cirés et al., 2014). While Japanese strain NIES-81 was shown to produce STX (Jung et al., 2003), German strains from lakes Scharmützel and Melang produced more diverse PSTs that included STX, NEO, dcSTX and GTX5 (Ballot et al., 2010). Strain NIVA-CYA 851 from Norway was more similar to German strains producing STX, NEO and GTX5 (Ballot et al., 2016). Chinese strain DC-1 was different from the above and was reported to produce STX, NEO, GTX5 (Liu et al., 2006a) or dcSTX, dcGTX2, dcGTX3, and GTX4 (Liu et al., 2006b) or NEO, GTX1 and GTX5 (Zhang et al., 2015).

Previous phylogenetic analyses based on 16S rRNA gene demonstrated close genetic relatedness between *A. gracile* and *Aphanizomenon flos-aquae* (Cirés et al., 2014; Gugger et al., 2002; Rajaniemi et al., 2005a; Wu et al., 2010). While some studies suggested that *A. gracile* and *A. flos-aquae* could be separated into different clusters in phylogenetic trees based on 16S rRNA, *rpoB*, *rbcLX*, *cpcBA-IGS* and concatenated genes (Casero et al., 2014; Cirés et al., 2014; De Figueiredo et al., 2010; Rajaniemi et al., 2005a; Wu et al., 2010), other studies argued against it (Gugger et al., 2002; Stüken et al., 2009).

Three cyanobacteria strains, tentatively identified as *A. gracile*, were isolated from Lake Iznik in Turkey in 2016. To our knowledge, this species was not previously reported in Lake Iznik. Therefore, the goals of this study were to perform polyphasic identification of these isolates and the investigation of their PST production.

2. Materials and methods

2.1. Sampling site, cyanobacteria isolation and culture

Lake Iznik is a large freshwater lake located in northwestern Turkey. The lake covers an area of 310 km^2 with depths reaching 80 m. It is a warm monomictic lake with mean annual temperature around 14 °C (Ülgen et al., 2012). The lake is mesotrophic with reported occasional increases of cyanobacteria; including species such as *Sphaerospermopsis aphanizomenoides*, *Planktothrix rubescens*, *Nodularia spumigena* (Akcaalan et al., 2009), *Dolichospermum mendotae* and *Chrysosporum ovalisporum* (Akcaalan et al., 2014).

Aphanizomenon gracile strains were identified and isolated from a plankton tow sample collected on 14 July 2016 in Lake Iznik, Turkey (40°26′13.4″N, 29°42′58.0″E). Single filaments of *A. gracile* were isolated under an inverted microscope (Olympus, CKX41-PH, Tokyo, Japan) at 100x magnification with a 10 µl micropipette. Picked filaments were washed in a drop of 1/20 diluted sterile culture medium twice and finally transferred into 1/20 diluted 200 µl medium in a microwell plate. During isolation and culture, either BG-11 (Stanier et al., 1971) or MLA medium (Bolch and Blackburn, 1996), without a nitrogen source, was used. Filaments in microwell plates were incubated in a growth chamber (Panasonic Healthcare, Tokyo, Japan) at 23 °C and 50 µmol photons.m^2.s^{-1} light intensity with a 12 h:12 h light −dark photoperiod. Filaments were weekly checked under the inverted microscope and mono-cyanobacterial (algae and other cyanobacteria absent, but bacteria present) strains with observable growth were finally transferred into 5 mL of full strength BG-11 (-N) or MLA (-N) medium in screw cap cultures tubes. Tubes were kept under the same conditions as described for microwell plates. Three strains of *A. gracile* (mono-cyanobacterial, non-axenic) designated as AQUAMEB-35, -37 and −38 were deposited in the Culture Collection of Algae and Cyanobacteria at Bursa Technical University.

Cell materials for genomic DNA isolations were obtained from tube cultures. For toxin analyses, cultures were sequentially transferred into larger volumes. Approximately 1 month old 1 L cultures were harvested by centrifugation, pellets were frozen and freeze-dried.

2.2. Morphometric measurements of isolated cyanobacteria

Aphanizomenon gracile filaments were photographed under an inverted microscope (Olympus, CKX41-PH, Tokyo, Japan) at 400X magnification (brightfield and phase contrast). Morphometric measurements were obtained from these pictures using Image J (https://imagej.nih.gov/ij/docs/index.html). Widths and lengths of 5 random vegetative cells from 30 filaments (n = 150), 30 heterocytes and 30 terminal cells were measured for each *A. gracile* strain. Akinetes were observed only for AQUAMEB-37 (n = 4) and −38 (n = 2) and their widths and lengths were measured.

2.3. Genomic DNA isolation, polymerase chain reaction (PCR) and sequencing

A 2 mL culture was pelleted by centrifugation in a Microfuge 16 microcentrifuge, (Beckman Coulter, IN, USA) at 16,000 × *g* for 10 min. Genomic DNA from the pellet was isolated using the Promega Wizard Genomic DNA purification kit (Promega, WI, USA) according to the manufacturer's instructions. Amplification of 16S rRNA gene was performed with 27F1 and 1516R primers as described in Yilmaz et al. (2008). Presence of saxitoxin biosynthesis genes *sxtA* and *sxtG* were also checked with PCR. Approximately 660 bp *sxtA* fragment was amplified with primers sxtAF and sxtAR as described in Yilmaz and Phlips (2011). A 700 bp fragment of *sxtG* was amplified with primer pairs sxtGF and sxtGR as described in Foss et al. (2012). PCR products were purified from agarose gels via the GeneJET gel extraction kit (Thermo Scientific, Wilmington, DE, USA) according to the manufacturer's instructions. Purified PCR products were sequenced on both strands using the PCR primers on an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For 16S rRNA gene sequencings, internal primers reported in Yilmaz et al. (2008) were also used. Sequences obtained in this work were deposited in GenBank with the following accession numbers: MH107237 to MH107239 for 16S rRNA gene sequences; MH113424 to MH113426 for *sxtG* gene sequences, MH113427 to MH113429 for *sxtA* gene sequences of *A. gracile* strains AQUAMEB-35, -37 and-38, respectively.

2.4. Phylogenetic analysis

Raw sequences obtained for 16S rRNA, *sxtA* and *sxtG* genes were manually corrected using Mega, version 6 (Tamura et al., 2013). Corrected 16S rRNA, *sxtA* and *sxtG* gene sequences were