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Short communication

Extraction method for total microcystins in cyanobacteria-laden sludge

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ABSTRACT

Cyanobacteria in water treatment sludge pose a health risk as they continue to be viable, multiply, and produce potentially harmful secondary metabolites. To date, little research has focused on accurately determining cell bound microcystin (MC) concentrations of cyanobacterial cells in water treatment sludge. Three extraction methods (freeze–thaw, lyophilisation, direct methanolic extraction) with three different pre-treatments (homogenisation, (ultra)sonication, combination of both, and controls) were investigated for their MC extraction recovery. It was found that lyophilisation with prior sonication achieved the highest toxin recovery across the two MC analogues (MC-LR, MC-LA) tested. The method was able to extract 69 and 56% of MC-LR and MC-LA, respectively with good reproducibility. Comparable results were also obtained with direct methanolic extraction, with poor reproducibility. The least efficient method was freeze-thawing which achieved poor recoveries and was less reproducible. This study highlights a rapid, efficient, low-cost extraction method for determining total microcystins in cyanobacterial-laden sludge.

1. Introduction

Cyanobacteria represent a serious issue to water authorities and water treatment plant operators due to their persistence in water supplies. As cyanobacteria can produce harmful secondary metabolites such as toxins, they challenge the water treatment process, particularly during bloom episodes (sudden exponential increase of cell numbers in the water column). One of the most common toxins causing a problem in drinking water sources is the hepatotoxin microcystin (MC), for which more than ninety variants have been identified [1]. Therefore, the presence of cyanobacteria in source waters can cause increased financial burden (additional demand of treatment chemicals). Furthermore, it has been shown that high numbers of cyanobacterial cells can accumulate in sludges from water treatment plants during the coagulation/flocculation/sedimentation process. Recent findings by Ho et al. [2] have demonstrated that cyanobacterial cells which undergo conventional water treatment processes (coagulation, flocculation, sedimentation) retain cell integrity, remain viable, reproduce, and continue to produce secondary metabolites (cylindrospermopsin, anatoxin, and geosmin) for up to three days in water treatment sludge. As Sun et al. [3] and Ho et al. [2] have shown, eventually cell integrity becomes compromised and cell bound secondary metabolites are released into the sludge and sludge supernatant. This is particularly problematic if the toxin-laden sludge supernatant is then recycled to the head of the plant as conventional water treatment processes are not efficient at removing dissolved MCs [4]. Therefore, monitoring sludge-bound cyanobacterial material and accurately determining intra-cellular metabolite concentrations is crucial for the operation of water treatment plants with source water that has a persistent cyanobacterial presence.

Several well described methods for the analysis of total MC concentration exist [5–7]. A common method is to apply a series of freeze–thaw cycles (usually three) to open breaches in the cell wall and release the cell-bound toxin. Another option is to lyophilise the samples with the same end-result. Thirdly, cell material can be directly extracted in pure or aqueous methanol. All of these procedures have been used in the past and are accepted methods worldwide for the extraction and analysis of MCs from aqueous matrices [8]. When attempting the analysis of the total MC concentration in cyanobacteria-laden water treatment sludge, standard extraction methods may fail to extract sufficient amounts of toxin, due to the cells being physically enclosed by the sludge particles. This can be especially challenging when the sample volume is low. Therefore, a different approach is needed to allow the

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reliable determination of the total MC concentration in sludge-bound cyanobacteria. Due to the nature of the sludge it may be necessary to break up the floc in order to achieve the extraction of toxin from cells within the flocs. There are several mechanical means of achieving this. A common microbiological method is using mechanical homogenisation in a Potter-Elvehjem homogeniser, which applies repeated shear forces on a sample aliquot [9]. This method is usually employed to break up cell conglomerates in environmental samples to facilitate enumeration in microscopy. Another means of breaking the floc is ultrasonication. This method is also commonly applied in microbiology and molecular biology to release intra-cellular content. The force applied during sonication should have two effects, to break up the treatment flocs and potentially initiate cell lysis. Neither of these pre-treatments requires special chemicals.

To the best of the author's knowledge there has been no systematic and controlled attempt made to determine the extraction efficiencies and reproducibility of the aforementioned extraction procedures on cyanobacterial cells trapped in water treatment sludge. It is of critical importance for water utilities to be able to accurately and reliably determine cyanobacterial metabolite concentrations in sludge storage systems, in order to avoid contamination of the source water with cyanobacterial metabolites at the head of the plant. Therefore, in this study, three commonly applied cell extraction methods were investigated in regards to their MC extraction effectiveness from a water treatment sludge matrix: Freeze-thawing, lyophilisation, and direct methanolic extraction. In addition, three different mechanical pre-treatments were investigated: homogenisation, (ultra)sonication, and a combination of the two procedures. Controls were also investigated in absence of any pre-treatment.

2. Materials and methods

2.1. Materials, cultures, and water source

All solvents were obtained from Merck (Germany) and were of analytical grade. *Microcystis aeruginosa* (AWQC-MIC338) Kutz. emend Elenkin from the Australian Water Quality Centre Culture Collection was cultured in ASM-1 [10] at 20 °C under a 12 h/12 h light/dark cycle at an intensity of 70 μmol photons $m^{-2}s^{-1}$. This *M. aeruginosa* strain is known to produce two MC analogues (MC-LR and MC-LA). The water tested/used was raw water sourced from Happy Valley Reservoir (Adelaide, South Australia, Australia). The ranges of water quality parameters were: temperature 22.1 °C, pH 8.3, turbidity 5 NTU, true $colour_{456nm}$ 59 HU, UV_{254} 0.353, DOC 11.4 mg L^{-1}, SUVA 3.1 L mg^{-1} m^{-1}. There were no cyanobacteria present in the raw water.

2.2. Controls

In order to compare the extraction efficiency of the various methods, a control was created by spiking raw water from Happy Valley Reservoir with a *M. aeruginosa* cell suspension (5×10^5 cells mL^{-1} final concentration) and then vacuum filtered (Whatman GF/C, UK). The filter disks were stored at −20 °C over night and a methanolic extraction was performed (see protocol [5]). The filtrate was concentrated by solid phase extraction (SPE) through C_{18} cartridges (Waters, UK) as published previously [11]. Both methanolic suspensions were reduced to dryness by centrifugal evaporation in a MiVac DuoConcentrator attached to a MiVac DuoPump (both GeneVac, UK), resuspended in methanol (50%), sonicated for 10 min, syringe filtered (0.2 μm), and analysed by high performance liquid chromatography (HPLC) coupled with a photodiode array (PDA) detector. A 2 mL aliquot was collected from each replicate and stored with Lugol's iodine (30 μL) for cell enumeration.

2.3. Production of sludge

Two litres of a cell suspension of *M. aeruginosa* was prepared in Happy Valley Reservoir water with a final concentration of 5×10^5 cells mL^{-1}. The suspension was mixed with 80 mg L^{-1} aluminium sulphate (as $Al_2(SO_4)_3 \cdot 18H_2O$) solution, flash mixed (200 rpm) for 3 min, slow mixed (40 rpm) for 17 min, and allowed to settle overnight. Most of the supernatant (~70%) was then decanted and sludge aliquots (2 mL) were collected with a pipette to evaluate different extraction methods and cell enumeration. Samples for cell enumeration were stored in Lugol's iodine (30 μL).

2.4. Pre-treatments

In order to investigate the different sample pre-treatments in triplicate, nine sludge aliquots (each 2 mL) were homogenised using a homogeniser, nine aliquots were sonicated (50 Hz, Unisonics, Australia) for 10 min, and nine sludge aliquots were first homogenised with a homogeniser and then sonicated for 10 min. Finally, nine sludge aliquots did not undergo any pretreatment and served as controls. Triplicate samples were then subjected to each extraction procedure.

2.5. Freeze–thaw

After the respective pre-treatments, triplicate sludge aliquots (2 mL) were subjected to three cycles of freezing at −20 °C for 2 h (which was considered sufficient time for the formation of ice crystals to facilitate the rupturing of the cyanobacterial cell walls in the low sample volume used [12]) followed by thawing under running water. The samples were then rotary evaporated to dryness, resuspended in methanol (50%), sonicated for 10 min, syringe filtered (0.2 μm) and analysed by HPLC-PDA.

2.6. Lyophilisation

Following the respective pretreatments, triplicate sludge aliquots were stored at −20 °C for 2 h and placed in a Dynavac (FD12) freeze–drier at -33 °C. After samples were lyophilised to dryness they were resuspended in methanol (50%), sonicated for 10 min, syringe filtered (0.2 μm), and analysed by HPLC-PDA.

2.7. Direct methanolic extraction

Having undergone the respective pretreatment steps, triplicate sludge aliquots were placed in methanol (8 mL) and allowed to extract for 1 h with occasional agitation. Samples were then rotary evaporated to dryness, resuspended, sonicated for 10 min, syringe filtered (0.2 μm), and analysed by HPLC-PDA.

2.8. Cell enumeration, toxin and statistical analysis

Cell enumeration was performed with a light microscope (Nikon 50i, Japan) at 200 times magnification using a Sedgewick-Rafter counting chamber.

All concentrated samples were analysed on an Agilent Technologies 1100 series HPLC system consisting of a quaternary pump (G1311A), degasser (G1379A), auto sampler (G1313A), column compartment (G1316A) and photodiode array detector (G1315B) using a method adapted from Ho et al. [13] for MCs.

The statistical relevance of the differences between the treatment methods was tested using Student's *t*-test.