ANALYSIS OF TOTAL MICROCYSTINS USING TIME-INTEGRATED PASSIVE SAMPLING

By

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ANALYSIS OF TOTAL MICROCYSTINS USING

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Abstract:

Microcystins are the most common freshwater cyanotoxin and present a risk to wildlife and humans who may interact with contaminated water bodies. Detecting microcystins in the environment presents a challenge as microcystins can have heavily fluctuating concentrations, and a sensitive analysis is required to detect concentrations below toxic levels. Passive sampling offers a way to integrate potentially fluctuating or low microcystin concentrations with time, which may allow for a more toxicologically relevant analysis of microcystins in the environment. However, current analytical methods associated with passive sampling of microcystins are not well suited for detecting all microcystin variants (of which there are over 200). Therefore, the purpose of this study was to explore the feasibility of coupling passive sampling with the analysis of total microcystins (all variants). To do so, microcystins were harvested directly from cyanobacteria cultures and used to calibrate passive samplers. Both Gas Chromatography—Mass Spectroscopy (GC-MS) analysis and Enzyme-Linked Immunosorbent Assay (ELISA) were assessed as methods for analysis of total microcystins from passive samplers. While both methods had exceptional detection limits for total microcystins, ELISA was chosen as the simpler and more efficient analysis. Three passive sampler designs were tested using polyethersulfone, nylon, or steel membranes to enclose the sampler sorbent to determine the effects of pore size and membrane material on sampling rate of total microcystins. The uptake of dissolved and cell-bound microcystins were then further investigated in the polyethersulfone and nylon samplers. All 3 sampler designs were successfully calibrated for analysis of total microcystins, with nylon and polyethersulfone samplers having exceptional linear uptakes of microcystins over time. Furthermore, it was determined that coupling ELISA analysis with passive sampling using steel or nylon samplers could theoretically reach much lower detection limits than with grab sampling. The high uptake rate of steel and nylon samplers may make them better suited for short-term studies, while the lower uptake of polysulfonate samplers may be better for longer studies. Although it remains unclear how well cell-bound microcystins may bind to the samplers, lower detection limits and the continuous collection of data from these samplers may prove ideal for monitoring of microcystins in the environment.

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CHAPTER I

INTRODUCTION

Microcystins

Cyanobacteria are a phylum of bacteria that can come to dominate harmful algal blooms (Whitton 2012). Some species of cyanobacteria have the ability to produce toxins (cyanotoxins), under certain conditions (Lawton and Codd 1991). Among these, microcystins (Figure 1) are the most common cyanotoxin produced in freshwater systems (Lopez *et al*. 2008). If ingested, microcystins can enter the cell through organic anion transporter peptides where they can bind to and inhibit the phosphatase enzymes PP1, PP2A, and PP5, causing damage to the cytoskeleton. As a results, microcystins most typically cause hepatotoxicity, as organic anion transporter peptides are especially abundant in the liver (MacKintosh *et al*. 1990; Chorus and Welker 2021; Kaya 1996). In the environment, microcystins contamination can potentially have a large area of effect, as their water solubility allows them to easily dissolve and disperse throughout the water column of a contaminated water body. Furthermore, the frequency of microcystin occurrence in the environment is increasing globally, as increased nutrient inputs and climate change lead to a higher incidence of HABs in general (Pham & Utsumi 2018). Due to their increasing occurrence and toxicity, microcystins present a threat to aquatic and terrestrial wildlife, and humans that come into contact with microcystin contaminated water bodies. For this reason, the USEPA has established a safe maximum concentration

of 1.6 ng/mL microcystins in drinking water and 8 ng/mL microcystins in ambient waters (USEPA 2015; USEPA 2019)

Detecting Microcystins

Detecting microcystins at or below the EPA recommended safe concentrations requires sensitive analytical methods. Liquid chromatography coupled with either ultra-violate (UV) or mass spectrometry (MS) detection are common and relatively sensitive methods for detecting microcystins (Aguete *et al.* 2003; Mayumi *et al.* 2006). However, over 200 identified variants of microcystins have been described, each with different levels of toxicity (Spoof and Catharine 2016; Gupta *et al.* 2003; Pichardo *et al.* 2007). Direct analysis of microcystin molecules could not detect total microcystins (all variants) without calibration of for each variant, which would be too costly for practical application. However, analysis of the ADDA moiety (2S, 3S, 8S,9S-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) present in all microcystin variants offers a way to detect total microcystins with a single calibration. ADDA enzyme linked immunosorbent assays (ELISA) is one possible way to detect total microcystins (Fischer *et al.* 2001), and offers exceptionally low limits of quantification at 0.10 ng/mL. Barring the upfront cost of the analytical equipment, gas chromatograph-mass spectrometry (GC-MS) analysis of the ADDA moiety potentially offers a more cost-effective method than ADDA-ELISA. Oxidation of microcystins causes the cleavage of the ADDA moiety to form MMPB (2-methyl-3-methoxy-4-phenylbutanoic acid) (Figure 1), which can then be derivatized and analyzed via GC-MS (Wu *et al*. 2009). Xu *et al.* (2013) achieved this oxidation with potassium permanganate and sodium periodate, and were able to reach a detection limit of 0.56 ng/mL with GC-MS.

Passive Sampling

Most analyses of microcystins in potentially impaired water bodies rely on the use of grab sampling. Grab sampling is inherently insensitive to fluctuations in analyte concentration between samplings, which is especially problematic for microcystins which can have heavily fluctuating concentrations depending on environmental conditions (Kanoshina *et al*. 2003; Morrison *et al.* 2016). Passive sampling, on the other hand, offers a way to both integrate concentration fluctuations with time, and potentially achieve lower detection limits than grab sampling (Górecki & Namieśnik 2002). By placing a sorbent receptive to the analyte of interest in a water body and allowing the sorbent to collect the analyte over a period of time, the time-weighted average concentration of the analyte can be determined. Generally, for aquatic passive samplers, a porous membrane holds the sorbent, which allows a consistent volume of water to reach the sorbent over time (Figure 2; Alverez *et al.* 2004). Assuming the uptake rate of the analyte to the passive sampler is linear (determined via laboratory calibration), fluctuations in the concentration of the analyte can be integrated into the time-weighted average (Alverez *et al.* 2004; Belden *et al.* 2015; Morrison *et al.* 2016). Furthermore, low concentrations of the analyte can be detected with passive samplers due to the continuous collection (and thus concentration) of the analyte onto the sorbent (Figure 3; Górecki & Namieśnik 2002).

Passive Sampler Kinetics and Designs

One important aspect of a sampler design is the uptake rate of the sampler. The sampling rate of the sampler determines the volume of water "sampled" per day while deployed, which governs the amount of analyte reaching the sorbent (Vrana *et al.* 2005).

With regards to sampler design, sampling rate is mainly controlled by the movement of the analyte through the membrane used to enclose the sorbent. Different materials and different pore sizes will allow the analyte to pass through to the sorbent at different rates. In theory, materials with larger pores will have higher sampling rates by allowing more water and analyte to pass through the sampler while deployed (Morrison and Belden 2016). However, the affinity of the membrane material to the analyte also affects sampling rate, and potentially prevents the analyte from reaching the sorbent (Booij *et al.* 2007). Furthermore, higher sampling rates are not necessarily ideal. While deployed, the sorbent in the sampler captures the analyte until it reaches a kinetic equilibrium with or is removed from the water column (Vrana *et al.* 2005). To integrate analyte concentrations with time, the samplers must be removed while the analyte uptake is still linear and before the equilibrium phase is reached (Figure 3). Therefore, the sampling rate of a sampler must be high enough to accumulate a quantifiable amount of the analyte, but not high enough to reach the equilibrium phase in the time frame that the sampler is deployed.

Solid phase absorption toxin tracking (SPATT) samplers have been proposed for use in sampling cyanotoxins, including microcystins (Kudela 2011). SPATT samplers use mesh bags (typically nylon) to enclose the sorbent, which allows for a high accumulation of cyanotoxins over time. However, SPATT bags were originally designed as early warning measures rather than quantitative, time-integrated analyses (MacKenzie *et al.* 2004). Polar organic compound integrative samplers (POCIS), on the other hand, are a common sampler design used for time-integrated analysis, which have also been proposed for sampling microcystins (Kohoutek *et al.* 2010). POCIS style samplers encase

the sorbent in a polyethersulfone membrane, which is sealed between two steal rings (Figure 2; Alverez *et al.* 2004). The small pore size (typically 0.1 µm) creates a low sampling rate, which allows the samplers to be deployed for a long period of time without the sorbent reaching a kinetic equilibrium with the water (Kohoutek *et al.* 2010). Using these conventional POCIS designs, samplers used for microcystins are often deployed for multiple weeks (Brophy *et al.* 2019; Kim *et al.* 2021). However, for studies concerned with shorter sampling periods, higher uptake rates may be preferred. As mentioned, microcystins can have heavily fluctuating concentrations along with a high acute toxicity (Chorus and Welker 2021). As a result, it may be more impactful to deploy samplers at a higher frequency and integrate concentrations over shorter periods of time (days rather than weeks. Morrison and Belden (2016) described a nylon POCIS which combines the steal ring structure of a POCIS with the nylon mesh (35 µm pore size) which might typically be found in SPATT bags. Larger pore sizes, higher uptake rates, and thus shorter sampling periods, may therefore prove ideal for time integrated sampling of microcystins.

Before passive samplers can be deployed in the field for time-integrated analyses, calibration studies are required to determine the uptake rate of a sampler design for a specific analyte (Vrana *et al.* 2005). Although POCIS using 0.1 µm polyethersulfone membranes have been calibrated for analysis of some microcystin variants (Kohoutek *et al.* 2010; Jaša *et al.* 2019), they have not been calibrated for microcystins using membranes of larger pore sizes, like those in SPATT bags or the nylon POCIS of Morrison and Belden (2016). Furthermore, passive sampling studies thus far for have only focused on individual variants of microcystins either purchased as standards or

isolated from cyanobacterial production. To the best of our knowledge, no studies have yet calibrated passive samplers for total microcystins using water harvested directly from cyanobacterial cultures, nor attempted to assess how cell-bound microcystins interact with passive samplers. Although it has been demonstrated that biofouling does not largely affect the uptake kinetics of passive samplers (Rosen *et al*. 2018), microcystins bound to cells will likely bind to the sorbent at a different rate than those dissolved in water, or potentially fail to pass through the membrane at all. In the environment, microcystins are likely majority cell bound as blooms occur, but are then released as blooms decline and the cyanobacteria begin to undergo apoptosis. Once dissolved in the water, microcystins can persist for weeks to months after a bloom has occurred (Peng *et al*. 2020). Therefore, it is important to assess the uptake kinetics of passive samplers for both cell-bound and dissolved microcystins.

Objectives

The goal of this study was to calibrate three POCIS sampler designs for analysis of total microcystins, and to assess their application to dissolved and cell-bound microcystins. These three designs varied in the membrane used to enclose the sorbent and included samplers with the conventional polyethersulfone (0.1 µm pores), and those with nylon (35 µm pores) and steel mesh (150 µm pores) membranes. Use of passive samplers designs that potentially allow for higher uptake rates than conventional POCIS will facilitate more sensitive analyses with shorter study timeframes. In addition to the different sampler designs, two methods of analysis of total microcystins were tested for their compatibility with passive sampling using ADDA-ELISA and GC-MS analyses.

Analysis of total microcystins from passive samplers potentially allows for a more sensitive and time-integrated monitoring of microcystins in contaminated water bodies.

CHAPTER II

METHODS

Chemicals and Reagents

Calibration studies were conducted in activated charcoal filtered tap water from Oklahoma State University, Stillwater Oklahoma (sourced from Lake Carl Blackwell, Stillwater, OK, USA). Hardness was measured at 170 mg/L calcium carbonate, alkalinity at 155 mg/L as calcium carbonate, pH 7.5, and conductivity 511 µs/cm. ELISA analysis of total microcystins was conducted using Abraxis Microcystins/Nodularins ADDA kits (PN 520011OH, Euforins Abraxis, Warminster, PA, USA). A microcystin-LR standard (MilliporeSigma, Burlington, MA, USA) was used to measure the efficiency of oxidative cleavage of ADDA to form MMPB. An MMPB standard was purchased from Tokyo Chemical Industry (Tokyo, Japan) and 4-phenylbutyric acid (4-PB) was used as an internal standard (MilliporeSigma, Burlington, MA, USA). Other reagents included potassium permanganate, sodium periodate, potassium bicarbonate, sodium bisulfite, and *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA-TMS) and were purchased from MilliporeSigma (Burlington, MA, USA). Solvents (methanol and acetonitrile) were purchased from Fisher Scientific (Hampton, NH, USA).

Production of Microcystins

Microcystis aeruginosa (purchased from UTEX, University of Texas at Austin, Austin, TX, USA) was grown for the purpose of harvesting microcystins for calibration of the samplers. A preliminary study was conducted to measure the production of microcystin over time and correlate it to various growth parameters to determine if these parameters could be used to estimate microcystin concentrations in each batch. *M. aeruginosa* was cultured in COMBO growth media (Kilham *et al.* 1998), under 12 hour light/dark cycles provided by fluorescent lights. At 0, 4, 6, and 8 days after inoculation, cell count and phycocyanin content were measured using a hemocytometer and fluorometer respectively, and water samples were frozen for subsequent analysis of microcystins using ADDA-ELISA. For use on passive samplers, batches were harvested and divided to be either frozen (at – 20 ℃) to lyse cells and release microcystins or refrigerated (at 4 ℃) to maintain cell-bound microcystins.

Sampler Designs and Calibration

Three sampler designs with were calibrated for the analysis of total microcystins, each with a different membrane material and pore size. Polyethersulfone (0.1 µm pores), nylon mesh (35 µm pores), or steel mesh (150 µm pores) were used to enclose 200 mg of Dowex L493 sorbent (Dow Chemical, Midland, MI). Dowex L493 was chosen as the passive sampler sorbent due to its suitability to a range of organic chemicals (Morrison and Belden 2016), and its large particle size which allows it to be used in samplers with large pore membranes. Prior to assembly, the sorbent was cleaned with methanol and dried in a desiccator. All membranes and sorbents were then enclosed in POCIS frames

made of steel rings with 5.5 cm diameter openings held together with steel bolts (Figure 2).

All samplers, regardless of design, were calibrated in the same manner using glass circulation tanks. Harvested microcystin contaminated water, which had previously been frozen to lyse cells, was used to spike the tanks in all initial calibration studies. The tanks were then filled to 3 L using activated carbon filtered tap water to dilute the microcystins. Three passive samplers (1 of each design) were placed in the tanks on steel bars to secure them in the middle of the water column. The tanks were then placed on stir plates, and flow was adjusted for each tank to 11 (± 1) cm/s (measured using a float and a timer). Tanks were renewed daily (100 % renewal) with microcystins, and paired water samples were taken randomly from 1 tank before and after renewals to confirm microcystin concentrations (n = 7). Tank water samples were then frozen for preservation and analyzed using ADDA-ELISA, which revealed that the average initial concentration was 0.726 (SD = 0.11) ng/mL. During calibration, the tanks had a decrease in microcystin concentration between each daily renewal, resulting in an average final concentration of 0.325 (SD = 0.15) ng/mL before the next renewal. Therefore, the water concentration used to calculate sampling rate was determined by calculating the average concentration of both the initial and final concentrations (0.526 ng/mL). Water temperature was also measured daily and maintained at 21 (± 2) ºC. Samplers were removed from the tanks and frozen (- 20 ºC) at days 2, 4, and 8 (n = 3 per time point). Sampling rate was determined with the equation $R_s = \frac{N}{C_w t}$ where R_s is the sampling rate (mL/day), N is the mass of the analyte on the passive sampler (ng), C_w is the water concentration (ng/mL), and t is the time deployed (days).

Measuring Dissolved versus All Microcystins

An additional study was conducted using nylon and polyethersulfone POCIS to explore the discrepancies between the uptake of dissolved microcystins versus cell-bound and dissolved microcystins (after referred to as "all" microcystins). Nylon and polyethersulfone samplers were placed in the same tank setup used in calibration (n = 3 per design, 2 samplers per tank) for 5 days. To keep *M. aeruginosa* cells intact, and thus maintain cell-bound microcystins, water used to spike the tanks was taken directly from live batches and refrigerated before use rather than being frozen. Tanks were again renewed daily and maintained in the same flow and temperature conditions as in calibration. To determine the amounts of dissolved and cell-bound microcystins in the water, paired samples were taken before and after each renewal (n = 5). One sample was filtered before freezing using 0.7 µm glass microfilters (Whatman plc, Maidstone, U.K.) to leave only dissolved microcystins, while the other was frozen before filtering to lyse cells and measure all microcystins. Cell-bound microcystins were calculated by subtracting dissolved from all microcystins. The 5-day average concentration for dissolved and all microcystins were then determined taking the average of the measurements before and after each renewal. The time weighted average water concentrations were determined from passive samplers with the equation $C_w = \frac{N}{R_s t}$ and compared to the concentrations determine by the analysis of water samples. Samplers and water samples were then analyzed using ADDA-ELISA.

Total Microcystins Analysis

To assess the practicality of measuring total microcystins from passive samplers, two methods of extraction and analysis were tested using ELISA and GC-MS. Both tests assessed the efficiency of microcystin extraction from a passive sampler sorbent followed by the analysis of the ADDA moiety as a measurement of total microcystins.

ADDA-ELISA was first assessed for measuring total microcystins from passive samplers. To determine the efficiency of the sorbent extraction, 200 mg of sorbent was placed into 10 mL of microcystin contaminated water (harvested from *M. aeruginosa*) in glass vials (n = 3). The mixture was then placed on a shaker for 30 minutes to allow the sorbent to collect the microcystins. To separate the sorbent from the water after mixing, the mixture was poured into an empty solid phase extraction (SPE) cartridge. The vials were then rinsed with deionized water to ensure that all the sorbent was collected in the cartridge. The sorbent was then dried under a vacuum and transferred to a second glass tube. 5 mL of methanol was placed in the tubes, which were then vortexed and placed in a freezer (- 20 ºC) overnight to extract. After soaking overnight in methanol, the sorbent and eluent were again passed through an empty SPE cartridge to isolate the eluent. A 50 µL aliquot of the eluent was then diluted with deionized water for analysis using ADDA-ELISA. All samples were at least diluted to less than 5% methanol per the ELISA manufacturer recommendations. The microcystin contaminated water used to spike the sorbent was also analyzed before and after mixing with the sorbent. Extraction efficiency was calculated by determining the amount of microcystins sorbed onto the sorbent (initial water concentration minus final) then dividing it by the amount extracted from the sorbent.

A method for analysis of total microcystins using GC-MS analysis of MMPB (formed from the ADDA moiety) was also assessed for its applicability for passive sampling. To determine the recovery efficiency of this method, microcystin-LR was placed into the passive sampler sorbent, mixed, then subjected to extraction (n = 5). Extraction was conducted by soaking the sorbent in 5 mL of methanol for 30 minutes, transferring the eluent to glass vials, then evaporating to dryness under a stream of nitrogen. Following the methods of Xu *et al.* (2013), the dried microcystin extract was oxidized for 1 hour using a 5 mL solution of 25 mM potassium permanganate, 50mM sodium periodate, and 100 mM potassium bicarbonate to cleave ADDA and form MMPB. The oxidation reaction was then stopped with the addition of sodium bisulfite, and the internal standard (4-PB) was added. To extract the MMPB and 4-PB from the oxidation solution, the mixture was passed through an HLB cartridge (Waters Corporation, Milford, MA) that was conditioned with methanol and water, and eluted with 4 mL of acetonitrile. The eluent was again evaporated to dryness in preparation for derivatization. Quality control samples included a microcystin spike (n = 3) directly into the oxidation solution to determine the oxidation efficiency, and an MMPB spike (n = 3) into the post-oxidation solution to determine the recovery efficiency of the HLB extraction.

Derivatization of all GC-MS samples and calibration standards was conducted by modifying the methods of Pu *et al.* (2014) using BSTFA-TSM. 50 µL of BSTFA-TMS was added to dried samples or standards in a 1.5 mL glass vial, followed by 50 µL of acetonitrile as a catalyst. The vials were then sealed and heated to 60 ºC for 2 hours. After heating, an additional 300 µL of acetonitrile was added to the solution and the newly

formed MMPB-TMS and 4-PB-TMS were subjected to GC-MS analysis (Agilent 5975c, Santa Clara, CA). The inlet temperature on the GC-MS was set to 280 ºC, and the column oven was programmed as follows: the initial temperature was 80 ºC and held for 2 minutes, followed by a ramp of 10 ºC/minute to 230 ºC, then a second ramp of 20 ºC/minute to 290 ºC, which was held for 4 minutes. Selected ion monitoring (SIM) parameters were 145, 189, and 248 m/z for MMPB-TMS and 91, 145, and 146 m/z for 4-PB-TMS. A five-point calibration curve was used for quantification with standards at 30, 100, 300, 1000, and 3000 ng/mL MMPB.

Statistical Analysis

Regression analysis was used to correlate growth parameters (cell count and phycocyanin content) to microcystin concentration as an indication of microcystin production by *M. aeruginosa*. Similarly, to confirm a linear uptake of microcystin onto the 3 sampler designs and to determine the sampling rate, regression analysis was used to correlate microcystin mass on the sampler to time. The slope of the linear regression line and the determined water concentration were used to calculate sampling rate. Regression analyses were conducted in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). To compare the 5-day average water concentrations as determined by measuring all or dissolved microcystins to those determined by passive samplers, F-tests were used to determine equal variances between the data sets. T-tests were then used to compare the averages assuming equal or unequal variances based on the F-tests.

CHAPTER III

RESULTS AND DISCUSSION

Production of Microcystin from Cyanobacteria

M. aeruginosa batches produced sufficient levels of microcystins to allow for calibration of passive samplers with water harvested from the batches. Based on regression analysis, both phycocyanin concentration and cell counts were correlated to microcystin concentrations in the batches (Figure 4). These growth parameters were used as indicators of sufficient microcystin production in each batch before harvest. However, they could not be used to replace measurement of microcystin concentration in the water entirely. While these parameters correlated to microcystins concentrations when the cyanobacteria were producing microcystins, numerous batches failed to produce microcystins altogether. Many studies have shown that microcystin production relies on the expression of certain genes, and the promotion of these genes is heavily influenced by environmental factors (Hisbergues *et al.* 2003; Schreidah *et al.*2020; Schwabe *et al.* 1988). While the growth conditions in the present study were kept as consistent as possible, slight variations may have influenced the expression of these genes over time. These growth parameters, therefore, only indicated when each batch has reached peak growth, and most likely peak microcystin production. Since peak production of microcystins varies between batches, ADDA-ELISA analysis was used to confirm microcystin concentrations before and during calibration studies.

Sampler Calibration and Designs

Based on linear regression analysis, all 3 sampler designs had linear uptakes of microcystin over time with R^2 values greater than 0.90 (Figure 5). Samplers with nylon and polyethersulfone membranes appeared to have the best linear uptake kinetics with R^2 values greater than 0.97. This indicates that they can be used to integrate total microcystin concentrations in water bodies over a given deployment period. To our knowledge, this is the first study to calibrate integrative passive samplers with microcystins harvested directly from cyanobacteria, which may represent a more realistic mixture of microcystins when compared to one derived from isolated microcystins or standards. Previous studies have also shown that sampling rate does vary among microcystin variants (Kohoutek *et al.* 2010; Jaša *et al.* 2019). This highlights the importance of calibration of mixtures of microcystins, particularly when concerned with total microcystins. This study calibrated passive samplers for analysis of total microcystins using ADDA-ELISA, which quantifies all microcystins variants present as total microcystin. As such, the composition of the mixture, (the specific variants present) was not determined. It is reasonable to assume that calibration of samplers for total microcystins with mixtures composed of different variants or different ratios might have slightly different sampling rates. This is one negative aspect of analyzing a mixture of compounds as a singular analyte (total microcystins), and it may be worth investigating the sampler uptake kinetics of different mixtures for analysis of total microcystins in the future. However, while sampling rate can vary among variants, traditional POCIS samplers typically have less than 50% relative standard deviation between sampling rates of different variants (Kohoutek *et al.* 2010; Jaša *et al.* 2019). Therefore, the differences in

sampling rates among microcystin variants may not be large enough to drastically affect the sampling rates calculated from measuring total microcystins.

The steel mesh design had a slightly lower R^2 value than nylon and polyethersulfone with regards to microcystin uptake over time, which may indicate a lag phase before the linear uptake phase (Figure 5). This might be explained by the interaction between microcystin and iron in the steel mesh. Iron has a relatively high affinity for microcystins, so it is possible that microcystins were binding to the iron before reaching the sampler sorbent (Gao *et al.* 2012; Schreidah *et al.* 2020). This would theoretically create a lag phase in the microcystin uptake, which would result in a slower uptake at earlier time points. Despite this potential lag phase, the steel mesh design still had a fairly linear uptake of microcystins over the full calibration period, and thus could be used to integrate microcystin concentrations with time.

Sampling rate generally increased with pore size of the membrane used to enclose the sorbent. Sampling rates for polyethersulfone, nylon, and steel membranes were 12.77, 82.55, and 255.19 mL/day respectively (Table 1). This is consistent with the work of Belles *et al.* (2014) who found that POCIS samplers with nylon membranes and larger pores had higher sampling rates for a range of organic pollutants. Theoretically, larger pores allow more water to reach the sorbent, which allows more of the analyte to accumulate there over time. There are no direct comparisons for microcystin calibrated for nylon or steel membranes, but for the polyethersulfone POCIS, the sampling rate determine in this study was relatively low compared to the rates determined for individual variants in previous studies (Kohoutek *et al.* 2010; Jaša *et al.* 2019). Kohoutek *et al.* (2010) found sampling rates of 87 and 90 mL/day for microcystin-RR and

microcystin-LR respectively for turbulent calibrations (i.e., continuous agitation during calibration). However, this study was not able to determine the actual velocity of the flow past the samplers. Under flow velocities of 1 cm/s (compared to 11 cm/s in the present study) Jaša *et al.* (2019) found sampling rates of 71, 80, and 151 mL/day for microcystin-YR, microcystin-LR, and microcystin-RR respectively. These discrepancies may largely be due to differences in the sorbent used. These mentioned calibration studies used Oasis HLB as a sorbent, whereas the present study uses Dowex L493. Dowex L493 was chosen because its larger particle size is ideal for use in samplers with larger pore sizes. However, Morrison and Belden (2016) determined that samplers with Dowex L493 had slightly lower sampling rates than those with Oasis HLB for the majority of organic pollutants tested. It could be possible that Dowex has a lower affinity for microcystins than HLB, which would describe the lower sampling rate. Morrison and Belden (2016) calibrated nylon POCIS with Dowex L493 under similar flow conditions to the present study (9 cm/s) for a variety of organic pollutants, including pesticides and poly aromatic hydrocarbons (PAHs), and determined sampling rates ranging between 305 to 1838 mL/day depending on the chemical. While these sampling rates are higher than the 82.55 mL/day determined for microcystins using nylon POCIS in the present study, it has been hypothesized that chemicals with larger molecular weights will have slower uptake rates than those with lower weights (Booij *et al.* 2003). Microcystin molecular weights range between 882 to 1117 Da (Spoof and Catherine 2016), while those chemicals measured by Morrison and Belden (2016) ranged from 188 to 450 Da. Therefore, it is possible microcystins simply have a lower sampling rate due to their large molecular weight. Despite the lower uptake rate, Dowex L493 sorbed enough microcystins to analyze using

ADDA-ELISA, even in samplers with the lowest sampling rate (polyethersulfone) in the shortest deployment period (2 days). This indicates that, while these sampler designs may have a lower affinity for microcystins than previous designs, all 3 designs can accumulate a sufficient mass of microcystins even in short deployments. Furthermore, this was the first study to calibrate samplers using microcystins contaminated water harvested directly from cyanobacteria (i.e., not isolated or purified beforehand). While the cells were lysed to release microcystins, this, in theory, only breaks the cell membrane to release internal microcystins to the water. There may still be a large matrix effect from any biological material from the cells that interacts with the microcystin molecules. If there is a matrix effect from the cyanobacterial cells, however, it would be important to account for than in a calibration study as these same effects could be expected in the environment.

Uptake of Dissolved versus All Microcystins

Based on the analysis of water samples, the 5-day average initial concentrations of dissolved and all microcystins were 0.871 (SD = 0.282) and 1.155 ng/mL (SD = 0.213) respectively. As expected, the concentration of microcystins decreased between daily renewals due to uptake from the passive samplers. Despite nearly 20% of the microcystins being initially bound to cells, both polyethersulfone and nylon samplers were able to accurately measure the time-weighted average water concentration over the 5 day deployment with concentrations calculated at 0.869 (SD = 0.132) and 1.508 (SD = 0.622) ng/mL respectively (Table 2). These concentrations determined by passive samplers were not statistically different from those determined by measuring either dissolved or all microcystins in water samples. Polyethersulfone and nylon samplers were chosen for further study specifically due to their exceptional linear uptake of microcystins

over time (as indicated by R^2 values greater than 0.97), which is further demonstrated with the accurate determination of these time-weighted average water concentrations. There were no statistical differences between microcystin concentrations determined by polyethersulfone samplers and water samples measuring either dissolved ($p = 0.991$, $t = 0.011$, $df = 11$) or all microcystins ($p = 0.053$, $t = 2.165$, $df = 11$). Similarly, there were no statistical differences between concentrations determined by nylon samplers and those determined by water samples measuring dissolved ($p = 0.227$, $t = 1.723$, $df = 2$) or all microcystins ($p = 0.435$, $t = 0.969$, $df = 2$). This may mean that some cell-bound microcystins were sorbed to the samplers while they were deployed. At the very least, this indicates that when the majority of microcystins are dissolved (free from cells), both polyethersulfone and nylon samplers can accurately determine the time-weighted average concentration of microcystins in contaminated water bodies. Typically, the ratio of dissolved to cell-bound microcystins shifts towards majority dissolved in the later stages of a bloom once growth slows and cells begin to release microcystins (Grützmacher *et al*, 2002; Peng *et al.* 2020). This means that outside of peak bloom periods, any microcystins in a water body will likely be majority dissolved. Therefore, both nylon and polyethersulfone passive samplers may prove useful for the purposed of long-term microcystins monitoring, particularly before and after cyanobacterial blooms. However, because cell-bound microcystins could not be measured directly, it was impossible to determine whether the samplers were, in fact, taking up cell bound microcystins. It is equally possible that during the study *M. aeruginosa* cells released a portion of their cell-bound microcystins after each renewal, allowing the microcystins to sorb to the samplers. For polyethersulfone samplers in particular, the 0.1 µm pores are likely to prevent any

interaction between the *M. aeruginosa* cells and the sorbent. The larger pores of the nylon samplers (35 µm) may allow some interaction, but it remains unclear whether this would lead to cell-bound microcystins sorbing to the sampler. Future investigations should explore the uptake kinetics of these passive sampler designs during different stages of cyanobacterial blooms, when the microcystins can be almost entirely cell bound (Grützmacher *et al*, 2002). However, for monitoring microcystins outside of peak bloom events, especially when the majority of microcystins are free from cells, both nylon and polyethersulfone samplers can provide low detection limits, and a more continuous and comprehensive analysis of total microcystins.

Total Microcystin Analysis from Passive Samplers

The extraction recovery from the passive sampler sorbent was first tested using ADDA-ELISA and microcystin contaminated water harvested from *M. aeruginosa*. Samples analyzed with ADDA-ELISA had good overall recoveries averaging 86.12% (SD = 6.57), even with the considerable dilution required to achieve less than 5% methanol in samples. Other studies have seen similar success measuring microcystins extracted from passive samplers using ELISA (Kudela 2011; Wiltsie *et al.* 2018) as ADDA-ELISA offers exceptional detection limits of 0.10 ng/mL (Eurofins Abraxis, 2021). Coupled with the extraction methods established in the present study and accounting for the required dilution and extraction efficiency, a theoretical mass of 11.6 ng microcystins could be detected on the passive sampler sorbent. Using this mass, and the sampling rates determine during calibration, exceptionally low water concentrations can be detected using passive samplers and ADDA-ELISA analysis of total microcystins. For the 3 sampler types tested, 8-day deployments coupled with ADDA-ELISA analysis

could detect concentrations of 0.114, 0.018 and 0.006 ng/mL for polyethersulfone, nylon, and steel samplers respectively (Table 1). For nylon and steel samplers specifically, these detection limits are much lower than what could be detected from a singular water sample using the same analysis (0.10 ng/mL).

The method for the derivatization of MMPB and 4-PB with BSTFA-TMS was successful and produced distinguishable peaks on the GC-MS chromatogram at 14.14 and 15.77 minutes respectively. The instrument detection limit for MMPB-TMS was established at 30 µg/L with a peak signal to noise ratio of 3.16. Extraction of MMPB from the oxidation solution (post oxidation) was relatively efficient with a recovery of 84.6% (SD = 9.73). However, the oxidative cleavage itself was not efficient. Samples spiked with microcystin into the oxidation solution had an average MMPB recovery of only 50.59% (SD = 9.69). Similarly, the sorbent samples spiked with microcystin had low recoveries of only 45.57% (SD = 3.40), which most likely resulted from a low oxidative cleavage efficiency based on the low recoveries of the microcystin spiked oxidation solution. The low efficiency of the oxidative cleavage of MMPB was unexpected, as some previous studies report recoveries greater than 85% (Wu *et al.* 2009; Xu *et al.* 2013). These studies also report, however, that slight variations in sample pH or reagent concentrations can greatly reduce the oxidation efficiency. Although the optimal conditions established by Xu *et al.* (2013) were matched as closely as possible, variations in these conditions may have led to a reduction in oxidation efficiency. Interestingly, a more recent and in-depth analysis of the permanganate oxidation kinetics of microcystins clarifies that the reaction has 17 potential products, and that the ADDA moiety itself has 2 potential oxidation sites (Kim *et al.* 2018). Therefore, it is also possible that oxidation

of microcystins in the present study resulted in the destruction of the ADDA moiety itself, leading to a lower yield of MMPB. Furthermore, the complexity of the method as a whole, including the multiple extractions and derivatization, presents numerous points where user error can reduce the recovery efficiency of MMPB. Despite the complexity of the method and the low efficiency of the oxidation step, GC-MS analysis of MMPB-TMS derived from microcystins bound to passive samplers is possible. With the established recovery efficiency and instrument detection limit, a theoretical mass of 125 ng microcystins (using MC-LR for the molar mass conversion) could be detected on a passive sampler sorbent using this method. As a result, 8-day deployments of passive samplers coupled with the present GC-MS analysis method could detect water concentrations of 1.2, 0.18, and 0.07 ng/mL microcystins for polyethersulfone, nylon, and steel samplers respectively. While future research could focus on improving the yield of MMPB from microcystins, coupling the present GC-MS methods with passive sampling can allow for the detection of microcystins below 1.6 ng/mL, which is the USEPA maximum safe drinking water concentration (USEPA 2015). Although ADDA-ELISA is the simpler and more efficient analysis as a whole, both ADDA-ELISA and GC-MS analysis of microcystins could be used for monitoring total microcystins below concentration limits when coupled with passive sampling.

Conclusion

The present study demonstrates the practicality of measuring total microcystins from integrative passive samplers. Two methods for extraction and analysis of total microcystins from passive samplers were established using ADDA-ELISA and GC-MS. While both methods had sufficiently low detection limits, ADDA-ELISA was chosen as

the more efficient and simpler analysis. Using ADDA-ELISA, calibration for total microcystins was successful for all 3 sampler designs tested. These designs, therefore, can be used to integrate total microcystin concentrations with time, and improve the detection limits of the analytical methods associated with analysis of total microcystins. The higher sampling rates of steel and nylon samplers may be ideal for short studies to allow for a sufficient accumulation of microcystin in a shorter period of time. Conversely, the lower sampling rate of the polyethersulfone may be better for long-term studies to allow the sampler to remain in the linear uptake phase for a longer period of time.

To our knowledge, this study represents the first attempt to calibrate passive samplers for analyses of total microcystins using water harvested directly from cyanobacteria. Microcystins harvested from cyanobacteria theoretically represent a more realistic mixture than isolated variants. It may be worth investigating how different mixtures of microcystin variants change sampling rates when analyzed as total microcystins. It will also be important to investigate how these samplers interact with different ratios of dissolved and cell-bound microcystins, particularly in studies concerned with active blooms. Regardless, the present study helps to lay the groundwork for analysis of total microcystins from passive samplers. Although it can certainly be beneficial to measure individual variants, analysis of total microcystins in water bodies ensures that no microcystin variants go undetected. Coupling this analysis with integrative passive sampling may offer a more comprehensive and time-integrated analysis of microcystins in the environment.

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APPENDICES

Table 1. Sampling rates (Rs) and theoretical detection limits for the three types of the samplers tested. Detection limits are calculated using the lowest mass of microcystin that is theoretically detectible on the passive sampler sorbent with ADDA-ELISA (11.6 ng), as established by the present study.

Sampler Material	Average Rs (mL/day)	8-day Detection Limit (ng/mL)
Polyethersulfone	12.77	0.114
Nylon	82.55	0.018
Steel	225.19	0.006

Table 2. Water concentrations as measured by water samples of dissolved and all microcystins (MCs) compared to those determined from polyethersulfone and nylon passive samplers. Water sample values represent 5-day averages combining values from immediately before and after each daily renewal. "All" microcystins refers to both dissolved and cell-bound microcystins. There were no statistical differences between values determined by passive sampler or measured by water samples ($p > 0.05$ in all cases).

Sample Type	MCs Concentration (ng/mL)	Standard Deviation
Dissolved MCs	0.871	0.282
All MCs	1.155	0.213
Polyethersulfone	0.869	0.132
Nylon	1.508	0.622

Figure 1. Chemical structures of microcystins, the ADDA moiety, and MMPB. The ADDA moiety can be cleaved from the microcystin molecule via oxidation to form MMPB.

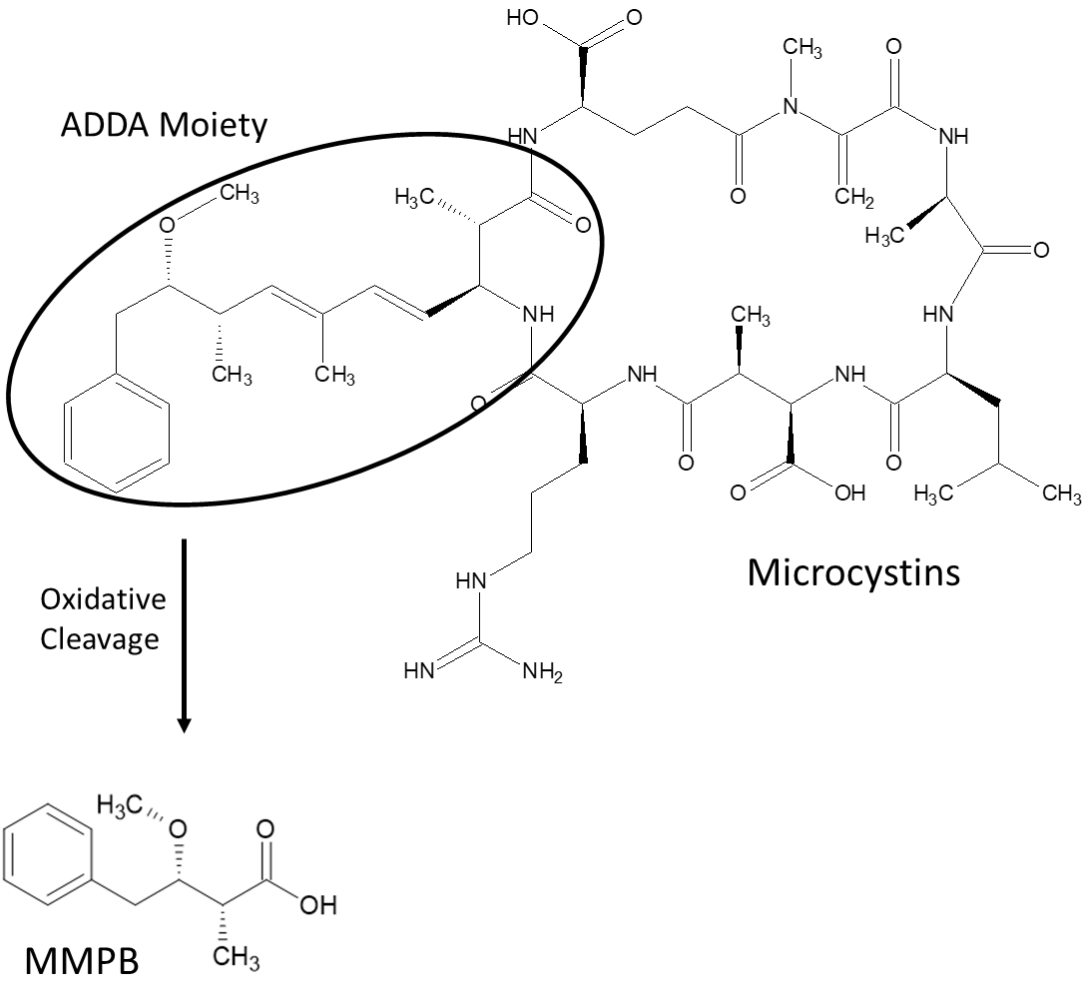


Figure 2. Expanded schematic of a POCIS style passive sampler. Sorbent and membranes are held together with two steel rings bolted together. Membranes were made from polyethersulfone, nylon, or steel mesh in the present study.

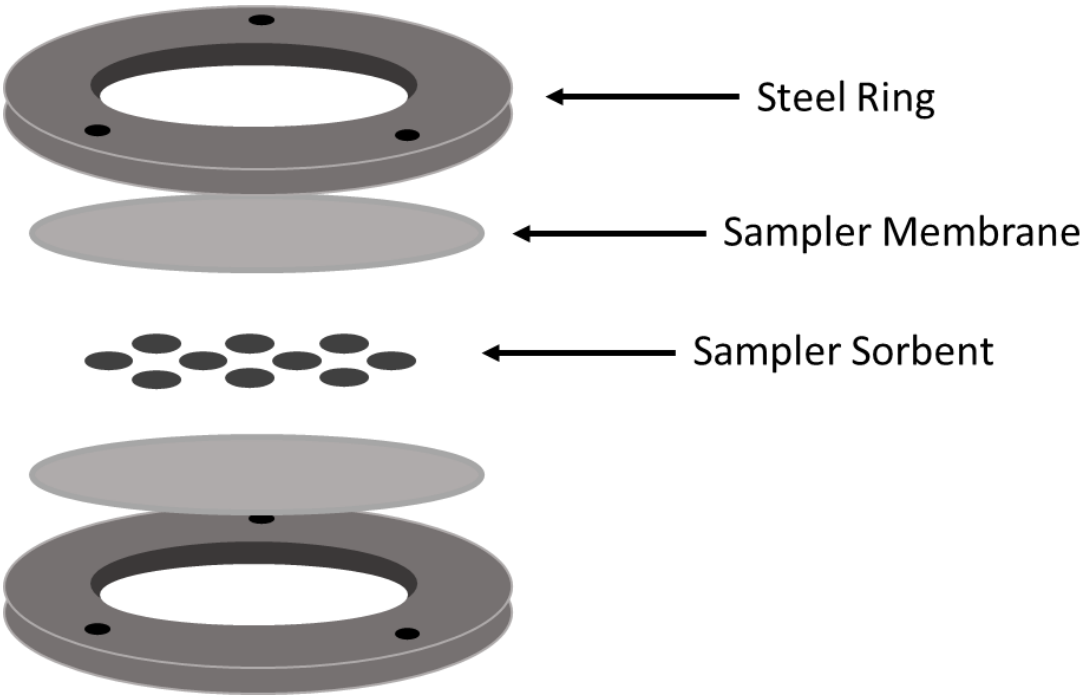


Figure 3. Theoretical uptake kinetics of a passive sampler in before reaching the equilibrium phase. Due to the continuous collection and linear uptake of the analyte to the sampler, low and fluctuating concentrations can be integrated with time.

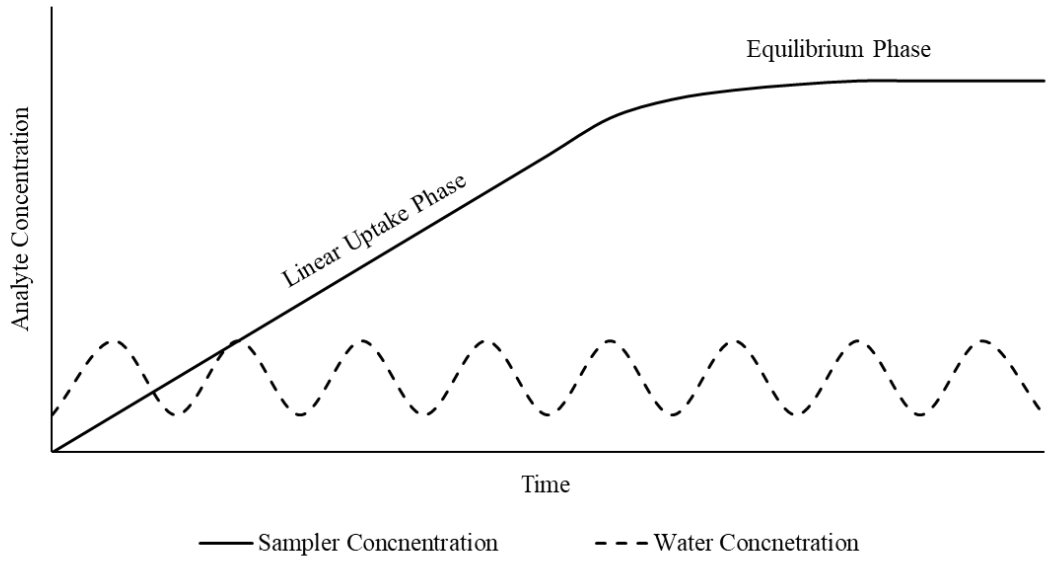


Figure 4. Regression analysis correlating phycocyanin concentration (top) and cell density (bottom) to microcystin water concentrations in *M. aeruginosa* batches sampled at 0, 4, 6, and 8 days after inoculation (n=4).

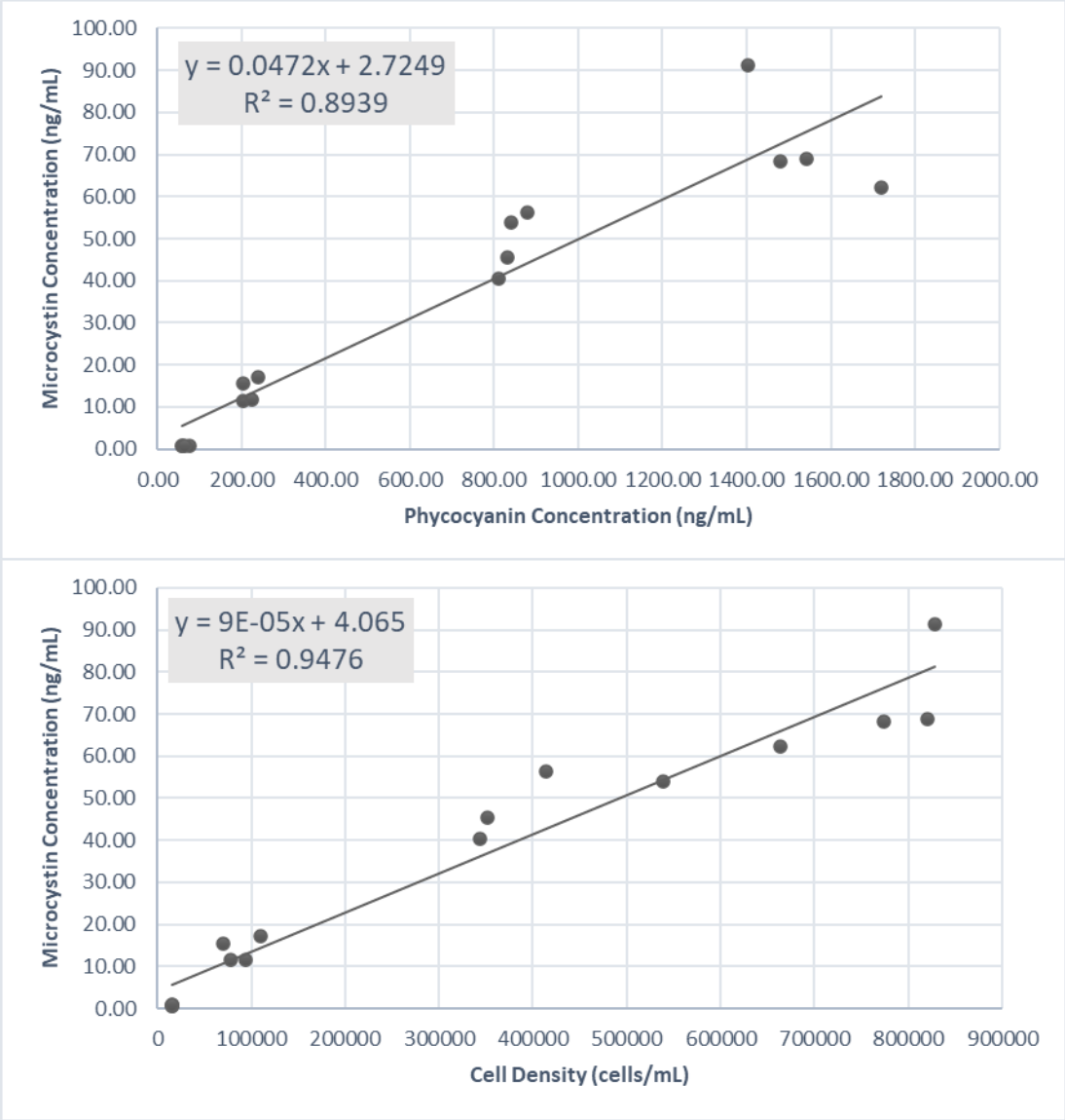
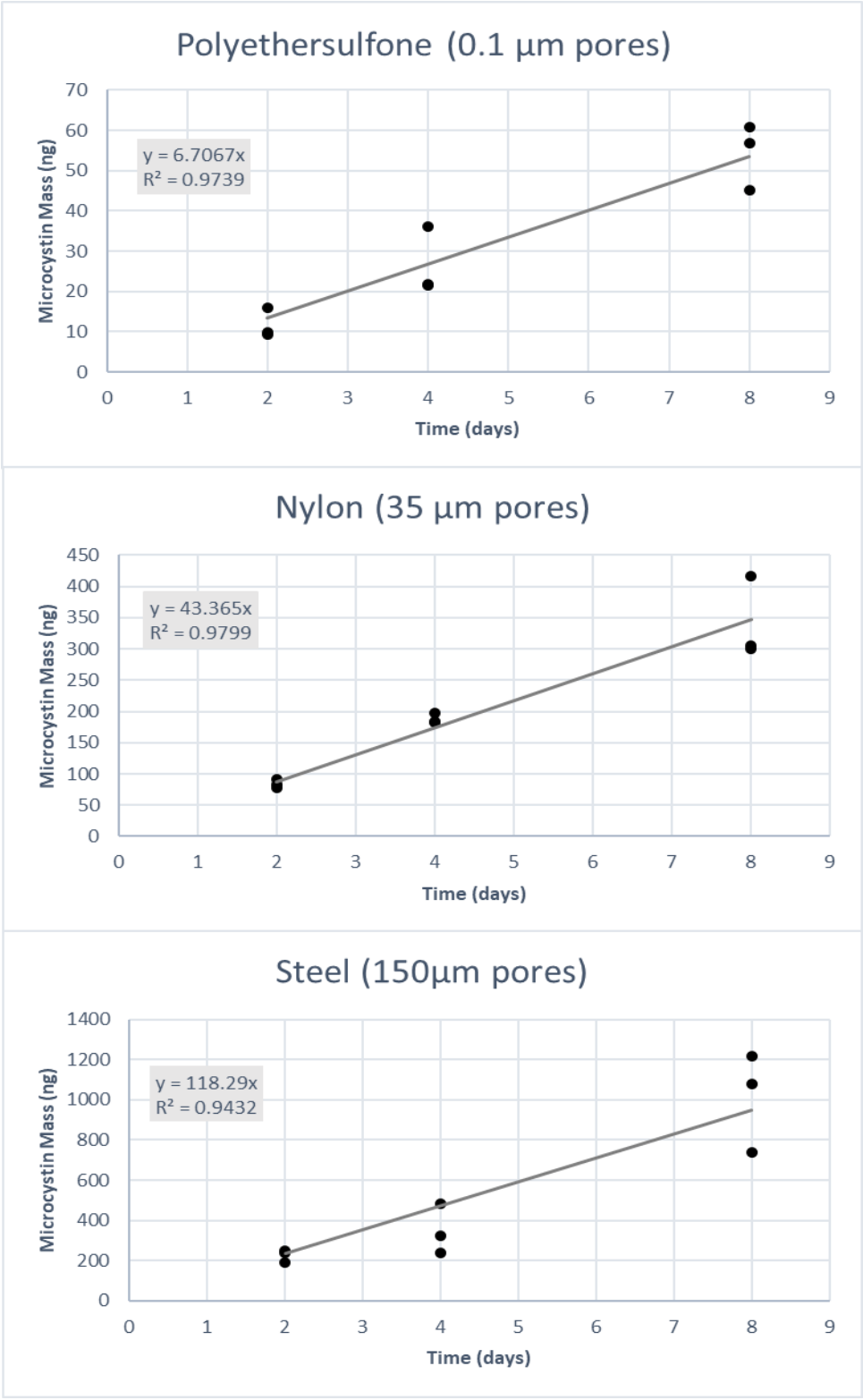


Figure 5. Regression analysis correlating microcystin mass sorbed to each sampler design over time at 2, 4, and 8 days. R^2 values greater than 0.90 were used as an indication of linear uptake of microcystins over time.



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