Novel Microfluidic Analytical Sensing Platform for the Simultaneous Detection of Three Algal Toxins in Water

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ABSTRACT: Globally, the need for “on-site” algal-toxin monitoring has become increasingly urgent due to the amplified demand for fresh-water and for safe, “toxin-free” shellfish and fish stocks. Herein, we describe the first reported, Lab-On-A-Disc (LOAD) based-platform developed to detect microcystin levels in situ, with initial detectability of saxitoxin and domoic acid also reported. Using recombinant antibody technology, the LOAD platform combines immunofluorescence with centrifugally driven microfluidic liquid handling to achieve a next-generation disposable device capable of multianalyte sampling. A low-complexity “LED-photodiode” based optical sensing system was tailor-made for the platform, which allows the fluorescence signal of the toxin-specific reaction to be quantified. This system can rapidly and accurately detect the presence of microcystin-LR, domoic acid, and saxitoxin in 30 min, with a minimum of less than 5 min end-user interaction for maximum reproducibility. This method provides a robust “point of need” diagnostic alternative to the current laborious and costly methods used for qualitative toxin monitoring.

1. INTRODUCTION

With the rapid incline of population growth, the global demand and pressure for clean water supplies has never been more apparent. The resultant anthropogenic environmental impacts are predicted to increase freshwater harmful cyanobacterial and algal bloom prevalence and duration. These impacts include both global warming and water quality degradation, particularly due to eutrophication. Human, ecological and economic health can all be negatively impacted by harmful cyanobacterial and algae blooms formed due to eutrophication. Human exposure transpired in Caruaru, Brazil, in 1996 in a haemodialysis clinic. It was reported that 86% of the 116 patients undergoing dialysis in this clinic experienced severe MC-LR related symptoms, including liver failure, resulting in the death of 52 patients.

Microcystis aeruginosa is one of the most recurrent toxigenic freshwater cyanobacteria and commonly prevails in fresh and brackish water. One of the most dangerous toxins produced from harmful algal blooms (HABs) is microcystin-LR (MC-LR), produced directly by M. aeruginosa, and is shown in Figure 1 left. MC-LR can cause significant health issues for both humans and animals, particularly by inducing hepatotoxicity and carcinogenesis through ingestion. In the EU, regulatory levels for MC-LR in water is 1 ng/mL for drinking water and bathing sites, with a >20 ng/mL concentration eliciting further action. One of the most extreme cases of human exposure transpired in Caruaru, Brazil, in 1996 in a haemodialysis clinic. It was reported that 86% of the 116 patients undergoing dialysis in this clinic experienced severe MC-LR related symptoms, including liver failure, resulting in the death of 52 patients.

A second documented marine toxin is domoic acid (DA), also demonstrated in Figure 1 center, which is a highly potent neurotoxin. Naturally manufactured by variant species of diatom, originating from the genus Pseudonitzschia, it can be responsible for eliciting the amnesic shellfish poisoning (ASP) illness. Following human exposure, some responses to ASP can include gastrointestinal distress, confusion, disorientation, seizures, permanent short-term memory loss, and in the most severe cases death. The earliest documented case of ASP contamination was in 1987 on the eastern coast of Prince Edward Island, Canada, where harvested mussels, contaminated with DA, were consumed. This case reported the death of 4 people, with a total of 143 people presenting similar ASP illness. Following the Canadian ASP event of 1987, and to protect seafood consumers, authorities established an action limit for DA of 20 μg DA/g shellfish tissue. As there is no well-defined action limit for free DA in drinking water, a method of extraction of DA from shellfish for detection using antibody technology, which involved a 250-fold dilution step, was used to translate the action limit for free DA to approximately 80 ng/mL in water.

A third highly prevalent marine toxin is saxitoxin (STX), also illustrated in Figure 1 right, which is another potent neurotoxin.

Supporting Information
It is most notably responsible for paralytic shellfish poisoning (PSP) and is produced by the genus *Alexandrium* spp. Some of the human responses following exposure to PSP can include gastrointestinal symptoms, numbness/tingling in mouth, dizziness, headache, fever, ataxia, respiratory distress, and death. Although there is currently no statutory guideline for STX contamination of drinking water, a suggested 3 ng/mL informal guideline concentration of for STX is currently used.

These relatively low guideline levels illustrate the importance of continuous environmental monitoring, and in particular, on-site algae-toxin monitoring.

Currently, there are several in situ-based systems with the potential to monitor toxin presence, and in particular MC-LR, where one such case was developed by MacKenzie et al. in 2004. This approach uses a technique, referred to as solid phase adsorption toxin tracking, which has since been adapted to allow the detection and monitoring of toxic algal blooms and shellfish contamination events. This technique, while beneficial, does require laboratory-based liquid chromatography—mass spectrometry analysis on the previously deployed and recovered materials. This limits the potential of the analysis system, preventing continuous autonomous analysis. Another disadvantage of this method is the low throughput capabilities, as well as the inability of real-time data capture, due to a weekly based deployment and recovery sampling regime. These limitations also illustrate the requirement for highly trained technicians who are capable of handling and characterizing weekly samples, consequently increasing the associated costs significantly. These increased costs would also negatively impact the ability of this approach to perform multiplexed, high density sampling in sites of interest. Other alternative biosensor-based methods, which have been used in the detection of MC-LR, have also been limited by this requirement of laboratory-based analysis. Chianella et al. detailed a novel molecularly imprinted polymer-based piezoelectric sensor for MC-LR with a low detection limit for 0.35 nM (0.35 ng/mL) using in-laboratory analysis. Similarly, electrochemical biosensors with reported sensitivities of 0.1 μg/L (0.1 ng/mL) for MC-LR and 9.0 × 10⁻¹¹ M (∼0.09 ng/mL) for a MC-LR specific gene sequence again require the use of laboratory-based instrumentation. A highly sensitive immunosensor for MC-LR has also been demonstrated, where a graphene–gold nanocomposite/functional conducting polymer/gold nanoparticle/ionic liquid composite film with electrodeposition achieved MC-LR detection limits as low as 3.7 × 10⁻¹⁷ M (∼4 × 10⁻³ ng/mL). Another immunosensor based-method by presented by Queiros et al. demonstrates the use of a Fabry–Pérot interferometer combined with an optical fiber, coated with a sol–gel imprinted sensing membrane, attained a MC-LR detection sensitivity of 12.4 ± 0.7 nm L/μg [∓0.08 nm L⁻¹(ng/mL)]. Finally, a cantilever-based immunosensor was also developed, which could assess MC-LR concentrations as low as 1 pg/mL (0.001 ng/mL) in varying water sources. While these methods achieve highly desirable detection sensitivities, they all suffer from the common requirement of expensive laboratory equipment, with specially trained personnel, to perform the analysis. However, an in situ based method has been previously reported by Long and colleagues, whereby a commercially available portable trace organic pollutant analyzer was used to detect MC-LR; however, the limit of detection (LOD) of the assay was significantly higher than any of the laboratory-based methods at 0.03 μg·mL⁻¹ (30 ng/mL), which is above the 1 and 20 ng/mL action limits for MC-LR in drinking water and bathing sites, respectively.

To facilitate toxin monitoring at the “site of interest”, in situ toxin detection can be alternatively achieved using Lab-On-A-Chip (LOC) technologies. This can be achieved through precisely controlling and manipulating of small quantities of liquids contained within a LOC platform, to process and analyze a sample as if in laboratory environments, even when in situ. These relatively new technologies are highly customizable and commercially viable alternatives to the current detection methods which have been previously employed for environmental monitoring. LOC-based platforms have been used to specifically target in situ environmental monitoring, including the detection of phosphate and *Escherichia coli* (*E. coli*), with the potential to also detect polycyclic aromatic hydrocarbons, endocrine disruptors (EDCs), inorganic ions, and heavy metals, by utilizing already developed microfluidic techniques. Whilst LOC platforms are highly convenient, cost-effective, and highly adaptive, the pumping technologies often required to drive sample progression through an LOC cartridge can be expensive. This is primarily due to the high costs associated with precise pumping mechanisms. A derivative of the LOC platform, circumventing these externally required expenditures is the Lab-On-A-Disc (LOAD) platform.

The LOAD centrifugal microfluidic platform replaces these previously required pumping mechanisms with a centrifugal driving force, utilizing a more cost-effective motor system. To date, on-chip water quality assessment systems have been primarily developed using LOC systems, with only a few examples reported on centrifugal disc platforms. There are multiple advantages associated with LOAD platforms, including automation of system through precisely-timed sample actuation using centrifugal forces, precise liquid handling, control of samples using valves, ability to multiplex assays using identical test conditions, and a myriad of detection techniques.
compatible on disc, making it an ideal technique for in situ environmental monitoring. In this paper, the first reported multi-analyte LOAD algal toxin sensor is described. The purpose of this sensor is to advance the first generation LOAD platform,\(^5\) whereby multitoxin detection of a single, prelysed sample was confirmed using a fluorescent microscope. The novelty of this system includes the use of highly specific recombinant antibody (rAb) technology, coupled with highly sensitive immunoaffinity purified antibodies (Abs) from chicken, which have far greater sensitivity than their commercial counterparts. The production of specific DA and STX Abs in chickens allowed for large quantities of highly sensitive immunoglobulin Y (IgY) to be purified with relative ease and minimum cost. The LOAD platform combines immunofluorescence with centrifugally driven microfluidic liquid handling to achieve a next-generation disposable device for in situ high-throughput sampling. rAb were selected over alternative antibody technologies. rAb ensure consistent biosensing detection, with high purity yielded from production and minimal batch-to-batch manufacturing variation. A low-cost, “LED-photodiode” based optical sensing system was tailor-made for the platform. This optical sensing system allows the fluorescence signal of the toxin-specific reaction to be quantified. This system can rapidly and accurately detect the presence of the targeted toxins in approximately 30 min, with a maximum of 5 min of user-interaction and high reproducibility. Because of the imminent requirement for a rapid and reliable qualitative assessment of waterbodies at the point of need, this sensor has the potential to provide an in situ alternative to the current laborious and laboratory-based methods used for multiple toxin detection.

2. RESULTS AND DISCUSSION

The system design was largely inspired by the limitations of current in situ environmental monitoring techniques. The system itself was specifically designed to prioritize some of these limiting factors, such as portability, reliability, and ease-of-use, where it is envisaged that future assembly line manufacturing will offer further reproducibility and cost-effectiveness improvements. This compact, rapid system was achieved through small hardware size, simultaneous multi-sample detection in separate assay form, and reduced assay runtime through minimal user interaction and incubation periods.

2.1. Microfluidic Disc Characterization. Reliable fluid manipulation is vital to conduct accurate assay protocols. For this reason, the on-disc fluidic dynamics were assessed, with the results demonstrated in Figure 2 and Supporting Video. The manual valve approach demonstrated excellent control of liquid, with minimal premature sample leakage between reservoir transitions. As this technique indirectly controls sample progression, via direct pneumatic air actuation, there was no additional cross-reactivity between the sample and microvalve material. This phenomenon occurs as the sample, while progressing within a microchannel with a \(\sim 7.5 \times 10^{-8}\) \(\text{m}^2\) cross-sectional area, experiences an increased surface tension force. This increased surface tension then prevents air-sample pass-through, resulting in an air compression proportional to the centrifugal force acting on the sample. Therefore, with the liquid acting as a cap for the succeeding reservoir, a pressure release through a valve is required to further sample progression. This pneumatic valving strategy can also be future automated, as described by Kim et al.\(^6\) It is expected that with the integration of a motor,\(^26,37\) sampler,\(^38\) and multidisc changer,\(^39–41\) long-term autonomous sensor deployment could be achievable.

2.2. Biosensor Characterization. Because of the potential human health hazard posed by MC-LR, DA, and STX (which shall be generically referred to as “toxin”), and the repercussions associated with a false qualitative screening of waterbodies, including human health, fishery, recreational, and economic impacts, a screening method needs to be capable of detecting toxins with a significant degree of confidence. An assessment of the screening data variability or coefficients of variation (CV) is critical in determining the reproducibility of the assay format. Initial experimental design approaches were investigated to evaluate and determine the optimal assay parameters for use with the system.

2.2.1. Biosensor Selection. To attain the low levels of relevant detection (ng/mL), highly sensitive biosensors were an essential component in achieving this detectability. All Abs (recombinant for MC-LR, and polyclonal for STX and DA) were produced in-house with MC-LR antibody fragments assessed (Figure 3). In the case of DA, it was also reported that the in-house produced scFv performed better that the commercial counterparts (sourced by Abcam Ltd.) (Figure 4).

2.2.2. Assay Optimization Studies. As the detection system was limited to an 11-bit ADC reference readout (in the form of “0-2047” fluorescence response units’ for the end-user), it was important to identify the system’s maximum fluorescent readability threshold. Therefore, the amount of fluorescent antibody required for this assay format was investigated by analyzing varying concentrations of primary antibody to ascertain the dynamic range of the system. There are several parameters that primarily influence direct planar assay performance; these include antibody/antigen concentration, contact time, and flow rate. These parameters were evaluated for this assay, while a rotational frequency of 35 Hz constant spin rate was used for the final assay procedure. Figure 5 illustrates the averaged results \((n = 3)\) of a direct binding assay performed on the disc to assess optimal antibody concentration and determined by the system via fluorescence at 430 nm.

It was observed that the optimum concentration of anti-MC-LR-Alexa-430 labeled-scFv to be used in the assay for MC-LR detection was approximately 40 \(\mu\text{g/mL}\) (1/75 dilution), as observed in Figure 5. This was subsequently calculated to be
approx. 12 and 20 μg/mL for anti-DA-Alexa-430 labeled-scFv and STX-Alexa-430 labeled-scFv, respectively. These Abs will be generically referred as “anti-toxin”-Alexa-430 labeled-scFv to avoid repetition. Once the assay conditions were established and direct binding of the antibody to the surface of the disc was successfully quantified, inter/intra assay studies were performed to develop a calibration curve. Method precision and accuracy for the assay were evaluated using validation standards in the same complex matrix as the study samples, that is, lake water samples.

2.2.3. Performance Studies. To assess the performance of the system with each of the toxins, a number of studies were conducted to identify the variation between assay readings on the same disc (intraday) and between separate disc readings on different days (interday). Six microfluidic discs, with six assay tests per disc, were manufactured for each toxin. Each of the six assays were set to be injected with varying toxin concentrates (0, 1, 10, 100, 1000, and 10 000 ng/mL), resulting in triplicate results (over two separate days) for each concentration. The poly(methyl methacrylate) (PMMA) discs were treated and functionalized at the test zone with “toxin”-conjugate and the control zone with anti-chicken antibody. The incubation zone was coated via passive adsorption with anti-toxin-Alexa-430 labeled-Abs. A silanization approach was also deemed the most effective in coating the disc surfaces with the detection molecule of interest, namely, a toxin−BSA conjugate. Issues arose with the level of background noise observed from the uncoated discs. The background noise was circumvented by coating the perimeter of each well with black acrylic paint to negate any inherent optical interference generated from the PMMA discs.

To determine the interday variation of results, calibration curves were generated for each toxin in the range 0−10 000 ng/mL. The CV values between the interday batches were then calculated via calibration curve analysis. For the intraday studies; lake water aliquots (1 mL) were spiked with toxin at concentrations of 0, 1, 10, 100, 1000, and 10 000 ng/mL. Sample progression, through each assay step (see Figure 8), included pass-through of the incubation chamber, where the anti-toxin-Alexa-430 labeled-scFv was coated. This was resuspended by incubation for 5 min with the toxin sample prior to passage over the test chamber, whereby toxin in the sample and immobilized toxin on the disc compete for binding to the anti-toxin scFv. Following resuspension of the Abs at the incubation-zone (see Figure 8), the sample was centrifuged to
propel the sample through the test, control, and waste chambers.

Binding was fluorescently observed in both test and control chambers separately. Bound scFv was quantified via fluorescence measurement with a 430 nm excitation light-emitting diode (LED), with the prefiltered optical range above 475 nm collected, including the emission peak at 545 nm. Fluorescence measurement occurred after assay completion, with each single fluorescence value programmed to consist of an averaged \( n = 640 \), with \( C \) sampled every 8 \( \mu s \), reading generated every \( \sim 0.5 \) s, with a further averaging of data captured over a 30 s run. The signal generated from the bound scFv was found to be inversely proportional to the amount of toxin present in the test sample. The sample was then passed from the test chamber to the control chamber where scFv was picked up by the control antibody. Once signal could be observed in this control chamber, it was used as a qualitative measure to ensure that the sample had successfully passed through all zones of the disc, thus signifying cessation of the assay. The readings generated from assessment of these lake water-spiked standards formed the calibration curves for each toxin whereby the system’s performance could be determined.

To determine the detection capabilities of the system, equal volume lake water samples were spiked with varying concentrations of “toxin” (0, 1, 10, 100, 1000, and 10,000 ng/mL). The spiked lake water samples were individually applied and run through the prefunctionalized PMMA disc. The fluorescent response units (R) of the system were then normalized by dividing the max achievable fluorescence of the blank (0 ng/mL) response (R0). The intra-assay analysis of each of the toxins, shown in Figure 6, was performed in triplicate within the same assay. The observed CV for this assay was <0.05%, proving considerable quantitative agreement between the intra-assay standards. A best-fit calibration curve was applied to the mean data, with assay accuracy determined by comparing the error of predicted concentrations against known standards. These results are shown in Table 1.

The precision analysis shown in Table 1 demonstrates excellent correlation <20% for high concentrations of MC-LR when estimated from the calibration curve. This is evident by the estimated concentration error recorded, from use of the calibration curve for validation standards, down to as low as 100 ng/mL of MC-LR. However, it was noted that while the overall reported value difference reduced between decreasing MC-LR levels, the relative percentage deviation to the expected value increased. This may be due to steric hindrance effects during the binding event, when low or noncompeting levels of MC-LR are present, and full capacity binding of the Abs at the test zone is attempted.42 Ng et al.43 discuss the use of long flexible linkers such as polyethyleneimine and dextran being more favorable for covalent attachment than shorter linkers such as 3-aminopropyltriethoxysilane (APTES), as the long flexible linkers circumvent problems associated with antibody accessibility and steric limitations, which could be a contributing factor to the low sensitivity observed within the assay. Taking these hindrance effects into consideration, and the lower optimum concentration of anti-toxin-Alexa-430 labeled-Ab used for the DA and STX assays, an increased averaged error correlation (Table 1) of <26 and <32%, respectively, was not unsurprising.

Quantitative immunoassays rely on calibration correlations to determine the analyte concentration in samples from the

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**Figure 6.** Intra-assay analysis \( (n = 3) \) for MC-LR, DA, and STX measured on the microfluidic sensor. Spiked concentrations of “toxin” were incubated with anti-toxin-Alexa-430 labeled-scFv. The sample was passed to the test zone to interact with coated toxin conjugate. Signal was observed by fluorescent measurement at the test zone at 430 nm.

**Table 1.** Precision Determination of “Back-Calculated” Concentration Percentage Error Extrapolated from the Calibration Curve by Estimation of the Actual Concentrations of Free “Toxin” (ng/mL)

| actual toxin concentration (ng/mL) | microcystin recovery variance (expected value ± reported value difference) (ng/mL) \( (n = 3) \) | DA recovery variance (expected value ± reported value difference) (ng/mL) \( (n = 3) \) | STX recovery variance (expected value ± reported value difference) (ng/mL) \( (n = 3) \) |
|----------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| 10,000                           | 10,000 ± 1560                                   | 10,000 ± 181                                    | 10,000 ± 3640                                   |
| 1,000                            | 1000 ± 192                                      | 1000 ± 221                                      | 1000 ± 350                                      |
| 100                              | 100 ± 15.5                                      | 100 ± 43.6                                      | 100 ± 17.6                                      |
| 10                               | 10 ± 3.28                                       | 10 ± 4.69                                       | 10 ± 4.2                                        |
| 1                                | 1 ± 0.368                                       | 1 ± 0.131                                       | n/a                                             |
strength of the signal produced. Figure 7 illustrates how the log of analyte concentration is plotted against the percentage antibody bound. The latter is the signal level expressed as a normalized function of the total signal response units per response in the presence of no competitive agent (R/R0). The graphs shown in Figure 6 illustrates the assessment of toxin detection down to 1 ng/mL in spiked lake water samples. Intraday CV (n = 3) were calculated as <0.1% for replicates within the same assay (Figure 6), and the interassay (Figure 7) CV (n = 2) were calculated as <0.27% for the average calibration curve of MC-LR. These CV values confirm the reproducibility of the assay. The detection capacity of this assay as observed from the validated standards is approximately 7.2 ng/mL.

The LOD for the system was observed by using eq 1. The LOD was calculated to be 7.2 ng/mL for MC-LR for this assay. This LOD meets the need for an early warning system capable of providing an early warning system for the qualitative assessment of recreational or bathing waters. Because of the low LOD, the system shows detection of low levels of toxins, which makes it highly applicable for use as a warning system for water quality, with results in 30 min with minimal end-user interaction. This assay represents several notable advantages; first, the system is made of inexpensive materials suitable for mass replication and second, the simple design offers the potential to become a rapid and easy-to-use instrument for direct use in the field at the point of need. It has a high within-assay response-reproducibility and a negligible assay turnaround time. The limitations of the proposed assay relate to potential deviations induced to individual discs because of each of the discs currently being manufactured and assembled by hand, which it is hoped in the future can be produced using industry standard assembly lines. Also, while the biosensor application within the disc is initially complex, it is hoped that through mass production breakthroughs such as automated assembly line production, this platform could become significantly faster and more cost-effective to produce. This proof of concept system shows detection of low levels of toxins, which makes it highly applicable for use as a warning system for the qualitative assessment of recreational or fishing waters. Because of the system being designed for the detection of “free toxin”, the addition of a lysing step for extracting toxin from the species for improving the sensitivity of the system requires would be beneficial and has been previously demonstrated either on disc or through a preloading mechanism. The system will be coupled with a wireless communications platform, motor, and automatic sampler for use as an automated rapid warning, integrated system. This would include monitoring, analyzing, interpreting, and distribution monitoring data, in which continuous real-time detection can be performed for offering generic warning or trigger an alarm as required, similar to the deployable MARIABOX system.
3. CONCLUSION

Intoxication events due to consumption of contaminated seafood and water supplies due to prevailing marine toxins is becoming a global problem. Ideally, the monitoring of harmful toxins like MC-LR, DA and STX should be performed on a device in situ, which is sensitive enough to perform accurate detection toxin outbreaks in a complex aquatic matrix. Currently, there is a high priority for the development of easy-to-use, rapid, robust, and nonexpensive devices for monitoring of the required low action level concentrations of toxins. Herein, a rapid system capable of providing an early warning system for the presence of toxic residues in water samples in situ is described. This system is capable of detecting algal toxin residues in lake water matrices using rAb technology and immuno-affinity-purified Abs with fluorescent-labeling. The advantages of the system are the incorporation of these elements into a rapid assay for the concurrent detection of three algal toxins at the point of need. The Abs are produced under minimum cost conditions and are made in very large quantities. The instrument provided reproducible dynamic linear ranges and rapid assay times. The rapid fabrication techniques used in disc generation offered a platform, whereby tailoring of the prototype’s size, shape, reservoir volume, and surface structure for rapid assay integration became feasible. This allowed facilitation of a fully enclosed platform, specifically optimized for minimal end-user interaction. In addition, the disposable discs can be mass produced, allowing convenient and safe “on site” toxin handling, with future possibilities aimed at fully autonomous system deployment. While these toxin analytes were used as a “proof of concept” to determine the functionality of the system, the system is designed generically to allow for future multiplexing and with testing of numerous toxin variants. This method could alleviate the costly laboratory procedures associated with frequent toxin monitoring.

4. MATERIALS AND METHODS

4.1. Chemical and Biological Reagents Used. Bis[3-(trimethoxysilyl)propyl]amine (cat no. 413356), Micro90 (cat no. Z281565), ethanol, isopropanol, and phosphate-buffered saline (PBS) were all obtained from Sigma-Aldrich, Ireland Limited, Arklow, County Wicklow, Ireland. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide bovine serum albumin (Imject EDC BSA) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide keyhole limpet hemocyanin (Imject EDC KLH) spin conjugation kits were obtained from Fisher Scientific (UK). An Alexa Fluor 430 (Alexa-430) fluorescent kit (cat no. A10171) was obtained from Bio-Sciences Ltd., Charlemont Terrace, Crofton Rd, Dún Laoghaire, Dublin, Ireland. MC-LR (cat no. ALX-350-012-M001) was obtained from Enzo life sciences (UK) Ltd. DA polyclonal antibody was obtained from Abcam Ltd. Cambridge, United Kingdom. Donkey anti-chicken IgY Fab was obtained from Gallus Immunotech (Antibodies-online GMBH). Lake water samples were obtained from Leixlip reservoir in Dublin, Ireland, and stored in RNase and DNAse free tubes (Fisher Scientific, UK) at 4°C. Black acrylic paint (obtained from Tiger Direct) was also used for biosensor signal enhancement.

4.2. Preparation of Conjugates and Fluorescent Antibodies. MC-LR–BSA conjugates and the recombinant anti-MC-LR-Alexa-430 were prepared, as per the method by Murphy et al. All animal models used were approved by the
Department of Health and Children, Ireland licensing authority under the Cruelty to Animals Act 1876 as amended, for Dublin City University under license ref no. B100/2705. DA and STX conjugates were prepared using an Imject EDC KLH spin conjugation kit (Fisher), as per the manufacturer’s instructions. A Leghorn chicken was initially immunized subcutaneously with of equal parts solution of DA-KLH/STX KLH conjugate and Freund’s complete adjuvant. The final concentration of the initial immunization was 200 µg/mL. The first boost (day 14) was then administered using 100 µg/mL of each conjugate in PBS, mixed in a 1:1 ratio with Freund’s incomplete adjuvant, in a final volume of 1 mL. The final 4 boosts that followed (days 36, 52, and 66) all contained 100 µg/mL of each conjugate and were administered in the same manner as the first boost using an incomplete adjuvant. Serum Abs were isolated from the chicken and subsequently labeled with Alexa-430.

4.3. Microfluidic Disc Manufacturing. The microfluidic disc was designed and developed as a single-assay version of the previously reported multitoxin detection disc. The previously reported multitoxin detection disc was fluidically optimized for sample metering between three separate assays via pneumatic-based valving with detection performed using a fluorescent-based microscope, whereas this single assay format, shown in Figure 8, was fabricated primarily to fully characterize a toxin detection performance on a microfluidic Disc. The previously reported multitoxin detection disc was designed and developed as a single-assay version of the disc. The previous multitoxin detection disc was fluidically optimized for sample metering between three separate assays via pneumatic-based valving with detection performed using a fluorescent-based microscope, whereas this single assay format, shown in Figure 8, was fabricated primarily to fully characterize a toxin detection performance on a microfluidic disc platform using an in-house built fluorescence-based complementary system. The microfluidic disc platform was manufactured from PMMA sheets and “pressure sensitive adhesive” (PSA) sheets (ARSeal90880), obtained from Radionics and Adhesives Research, respectively. The discs were manufactured through assembling consecutive layers of PMMA and PSA. The roof was additionally covered using PSA to act as a manual active microfluidic valve layer. The reservoir floor of the biosensor was laser etched to improve the binding process (70% power, 60% using Epilog Zing 16, Epilog Laser USA) with the surrounding reservoir walls painted black, using acrylic paint, before full disc assembly.

4.4. Preparation of Reservoir Surface for Bioactivation. The PMMA layers of the disc were thoroughly surface cleaned in a class-1000 clean room with the assistance of ultrasonication; a 2% (v/v) solution, consisting of Micro-90 in deionized water, followed by isopropanol. Finally, the PMMA layers were each rinsed three times with deionized (DI) water, followed by isopropanol. Following this, the PMMA and PSA disc layers were assembled as two halves [the lower half (LH) and upper half], with a center point at the floor of the reservoirs, leaving the reservoir floors exposed on the LH for antibody immobilization treatment. After cleaning and assembly, the two halves of the discs were then placed in heat-sealed bags for storage, while the biosensor components were being integrated to the LH of the disc. Following this integration, the two halves were then combined to form a single disc.

4.5. Amine Surface Functionalization of Biosensor Reservoir Floor by Liquid Phase. Following the cleaning process, the LH of several discs were then exposed to a microwave-induced O2 plasma, allowing multiple reservoir floor surfaces to be treated simultaneously, using the plasma cleaner (Harrick plasma, USA). The O2 gas stream was regulated by the integrated mass flow controller until the required operating pressure within the chamber adjusted to approximately 1000 mT. The LH of the discs was exposed to the O2 plasma for 10 min on the “high setting”. A 3% APTES solution was prepared in 98% ethanol to functionalize the PMMA disc reservoirs. This solution was then immediately applied to the detection reservoirs (reservoir 3 “Test” and reservoir 4 “Control”) of the surface-activated discs to provide a binding bridge between the surface oxidation of the reservoir floor and hydroxylation (–OH groups) of the toxin conjugates and toxin-specific Abs. The discs were then incubated at room temperature for 1 h in a fume hood in a 15 cm diameter Petri dish with cover. Following functionalization, the discs were then cleaned twice, using a solution of 98% ethanol under ultrasonication for 15 min. The discs were baked at 60 °C for 2 h in an oven, where significant cracking of the PMMA discs was noted at high temperatures. The discs were then allowed to cool to room temperature, at which point the toxin conjugate/Abs were added to reservoir floor.

4.6. Microfluidic Disc Characterization. The fluidic motion on the microfluidic discs was assessed using a customized, in-house developed “spin stand” (shown in Figure 9), as previously demonstrated by Kirby et al. Briefly, the centrifugation of the discs was regulated using a computer-operated spindle motor (Faulhaber Minimotor SA, Switzerland). A highly sensitive, short-exposure camera (Pixelify, PCO, Germany) was combined with a stroboscopic-programmed light source (Drelloscop 3244, Drello, Germany), which was synchronized with the spindle motor using in-house built electronics, for visualizing on-disc fluidic performance under centrifugation. Furthermore, as the roof was covered using PSA to act as a manual active microfluidic valve layer, the microvalves was attained by pin puncture, allowing pneumatic pressure release which had prevented sample progression to occur. In summary, the discs were spun at 35 Hz for 30 s to transport the sample to the subsequent reservoir, followed by halting of the disc for the required incubation period per reservoir. After this wait period, the sequential manual microvalve was opened. This cycle was repeated until the sample had successfully completed the full assay protocol.

Figure 9. “Spin stand” used for microfluidic characterization. This observation setup had a triggering mechanism for actuation of camera and pulse light every 2π rotations (whereby n is a natural number and dependant on rotational speed). This gives the viewer the perspective of a stationary disc with fluidic motion, giving precise liquid characterization whilst simultaneously running assay to be tested in the detection system.
4.7. Optimization of Antibody Concentration. Preliminary studies were performed to determine the optimal labeled-antibody concentration for use in the final assay. An optimal antibody concentration will provide sufficient signal observation when interacted with the immobilized toxin conjugate on the surface, and subsequently measured fluorescently at 430 nm on the system. Varying dilutions of each antibody from 1/35, 1/75, 1/150, and 1/300 were assessed fluorescently, by applying 100 μL of each to the inlet channel on a functionalized disc and spinning the disc on a spin stand at 35 Hz for 1 min. A depletion sample which included 10 μg/mL of free toxin with 1/75 dilution of antibody at equal volumes was assessed also. The amount of bound antibody was determined via fluorescent measurement at 430 nm.

4.8. Toxin Conjugate and Control Antibody Binding to Biosensor Surfaces. The Alexa-430 labeled specific Abs were coated on the 2nd radially aligned “incubation zone” reservoir (see Figure 8) where 100 μL of optimally determined antibody concentration was added (40 μg/mL). As this reservoir was not functionalized prior to coating, the antibody was passively adsorbed onto the reservoir floor of the incubation zone. The sequential release of the sample was then required for antibody resuspension within the incubation zone. MC-LR, conjugated to BSA (1 μg/mL), was prepared in a PBS solution, where 100 μL was then added to each of the 3rd radially aligned “test” reservoir (see Figure 8). Commercially available anti-chicken IgY (H + L) (100 μL), at approximately 10 μg/mL (Gallus Immunotech Inc., Canada), was added to each of the 4th radially aligned “control” reservoir (see Figure 8). The coated discs were stored at 4 °C covered for 12 h. To avoid any nonspecific binding from occurring on the biosensor reservoir floor, the remaining activated amine sites were then subsequently blocked with 200 μL of 3% BSA (in PBS solution) added to each reservoir. The blocking agent was left to incubate at 37 °C for 1 h, after which it was aspirated. Finally, the disc assembly was completed and the prepared disc was ready for toxin determination.

The exact same procedure to determine the calibration curve for DA was followed as was outlined previously for the MC-LR calibration curve. The variables used for DA include approximately 12 μg/mL of antibody as assessed via ELISA. The conjugate coated on the test well was 1 μg/mL of DA–BSA conjugate prepared in-house in DCU. The control antibody used in the control well was the anti-chicken IgY (H + L) 100 μL, a commercial antibody from Gallus Immunotech Inc., Canada.

Similarly, the exact same procedure to determine the calibration curve for STX was followed as was outlined in section for the MC-LR calibration curve. The variables used for STX include approximately 20 μg/mL of antibody as assessed via ELISA. The conjugate coated on the test well was 1 μg/mL of STX–BSA conjugate prepared in-house in DCU. The control antibody used in the control well was the anti-chicken IgY (H + L) 100 μL, a commercial antibody from Gallus Immunotech Inc., Canada.

4.9. Manufacture of the Detection Platform. The detection system shown in Figure 10 was 3D-printed from acrylonitrile butadiene styrene, to hold the disc, with an incorporated fluorescent detection apparatus to separately capture fluorescence values at both reservoirs three and four on the disc (Figure 8). The detection system utilized a top-down detection configuration with a 405 nm excitation LED (cat no. 713-4898, Radionics Ltd Ireland), a 475 nm long pass filter (cat no. 64-617, Edmund Optics Ltd.), and a photo-LED emission detector sensitive from 420–675 nm (cat no. 708-2813, Radionics Ltd., Ireland). This in-house developed system was controlled by a Pololu Wixel microcontroller (cat no. 785, Cool Components Ltd.), which transmits communications to a user interface via a universal serial bus (USB) and/or wirelessly (using a second Pololu Wixel). While this model does not contain a motor, as microfluidic characterization was done using the previously described “spin stand”, motor incorporation will be done in future models similar to those described by Duffy et al.26,27

4.10. Measurement of Toxin Binding. The preliminary assay was performed at room temperature in the laboratory using the developed system box connected to a portable laptop facilitating analysis via the Pololu Wixel software. The analysis was performed by adding 100 μL of the lake water sample via a pipette to the sample load zone on the disc. When spun at 35 Hz, the water sample travels from the sample loading zone into the incubation zone. The water sample is allowed to interact with the now reconstituted antibody in the incubation zone for 10 minutes and the disc is spun again at 35 Hz. The water sample with potential toxin and resuspended antitoxin antibody then advanced to the test zone, whereby toxin in the water sample and toxin coated on the wells of the test zone compete for binding to the fluorophore conjugated antibody. Bound antibody on the wells of the test zone is inversely proportional to the amount of toxin present in the water sample. The water sample continues through to the control zone, whereby anti-chicken Abs capture the fluorophore-labeled anti-chicken Abs; this signifies successful transition of the water sample from the loading zone through the incubation and test zones and to the control zone. The water sample then flows from the control zone to the waste zone, and the fluorescent readings are measured. Readings from the system are taken every 0.5 s; these values are exported into excel and an average of 10 measurements is taken for each fluorescent analysis.
**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00240.

Additionally, a Supplementary Video is included to demonstrate visualization of fluidic motion on disc platform using the “spin stand” method (AVI)

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**Notes**

The authors declare no competing financial interest.

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