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Article

Fluorescence Correlation Spectroscopy (FCS) Unlocks the Presence of Microcystin-LR in Fresh Water

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Abstract

Water is essential for human life, and access to clean water is considered a basic human right by the United Nations. Around the world, a high proportion of the population still does not have access to safe fresh water, with a high impact on health. This situation perpetuates a cycle of poverty, hindering economic development and exacerbating inequality. The water is considered unsafe to drink if it is contaminated. The contamination can be categorized into three types: physical, chemical, and biological. Biological contamination arises from the presence of living organisms in water, such as bacteria, viruses, algae, fungi, and parasites. Recently, the scientific community has raised the alarm on contamination caused by a large group of bacteria known as *Cyanobacteria*, which can release harmful toxins in water, including mycotoxins like Microcystin-LR (MC-LR). In this context, we present the application of fluorescence correlation spectroscopy (FCS) to develop a competitive assay for detecting the presence of traces of the MC-LR toxin in fresh water.

Keywords: *Cyanobacteria* bloom; fluorescence correlation spectroscopy (FCS); immunoassay; microcystin

1. Introduction

Water is a finite resource essential to human life, agriculture and industry. Access to safe and uncontaminated water is essential for human life. However, in different parts of the world, many communities still have problems of lacking water, or they have access only to contaminated water with serious health risks, environmental impacts, and economic consequences [1].

The water contamination can arise from various sources, such as industrial and agricultural waste, sewage, and natural events like hurricanes and floods. The contamination is generally classified based on its origins: physical, chemical, and biological. All these types of contaminations produce unsafe drinking water. Biological contamination is originated by the presence of living organisms in water, like bacteria, viruses, algae, fungi, and parasites. These contaminants can lead to severe health issues, including gastrointestinal illnesses, skin infections, and respiratory diseases. Recently, the scientific community has raised alarms about contamination caused by a group of bacteria known as *Cyanobacteria* [2]. The *Cyanobacteria* are photosynthetic prokaryotic microorganisms that live in diverse range of environments, from freshwater to marine ecosystems. The uncontrolled growth of this bacterium due to anthropogenic factors (nutrient over-enrichment of water ecosystem degradation, increase in water temperature, and increase in the CO₂ level in the atmosphere) led to the *Cyanobacteria* bloom phenomenon [2]. *Cyanobacteria* belonging to the genera *Microcystis*, *Anabaena*, *Planktothrix*, and *Nostoc* can produce and release in water harmful toxin molecules named Microcystins (MCs).

Belonging the MCs family, the most toxic molecule is the microcystin-LR (MC-LR). Due to its high toxicity the World Health Organization (WHO) has identified the provisional value of the maximum amount of MC-LR assumption. It has been set for drinking water at 1.0 µg/L and a daily intake value (TDI) of 0.04 µg/kg MC-LR/body weight [2].

The most common methods used to detect MC-LR include chromatographic methods (HPLC, LC/MS, GC/MS), immunological methods (ELISA), and protein phosphatase inhibition assays (PIIA) [3]. Recently, a competitive Förster resonance energy transfer (FRET) to detect the presence of MCs in solution has been developed [4].

Several studies have shown the effectiveness of Fluorescence correlation spectroscopy (FCS) to address fundamental questions in biology [5–12]. Furthermore, some studies have used this technique to identify the presence of specific molecules, such as allergens, toxins and antibiotics, in various matrices [13,14].

In this work, we present a competitive FCS assay to detect the presence of MC-LR traces in fresh water.

2. Materials and Methods

2.1. Materials

MC-LR mouse monoclonal antibody (clone MC10E7; ref. #ALX-804-320-C200) and t Microcystin-LR (ref. # ALX-350-012-M001) were purchased from Enzo Life Sciences (Enzo Life Sciences Inc., Farmingdale, NY, USA). Bovine serum albumin microcystin-LR (MC-LR BSA) conjugate (ref. # DAGHY010) was purchased from Creative Diagnostics (Creative Diagnostics, Shirley, NY, USA), while CF488 fluorescent dye was purchased from Biotium (Biotium Inc., Fremont, CA, USA). The microplates (96-well), C8 lock-well maxisorp Nunc (ref. cod. 446469), were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Goat polyclonal IgG-HRP secondary antibody used in the ELISA experiments was purchased from Abcam (Abcam plc., Oregon, OR, USA). Polyclonal antibody anti-Ovalbumin, used as a control in the experiments, was purchased from Covalab (Bron, France). Sephadex G25 chromatographic resin was purchased from Sigma-Aldrich (Sigma-Aldrich S.r.l., Milan, Italy). All other chemicals were commercial samples of the purest quality.

2.2. Preparation of the MC-LR BSA Labeling with CF488 Dye

MC-LR BSA was labeled with the fluorescent probe CF488 following the method described by Capo [4]. In brief, the conjugate was dissolved in 0.10 mL of 20 M sodium phosphate buffer, pH 7.4, at a concentration of 2.0 mg/mL and mixed with CF488 (molar dye-antibody ratio: 12:1) dissolved in dimethyl sulfoxide (DMSO). The obtained mixture was adjusted to pH 8.3, and the reaction was stirred in the dark at room temperature for 1 hour. After the incubation period, the labeled conjugate, MC-LR BSA 488, was purified from any unreacted CF488 dye using a Sephadex™ G25 column. The chromatography was performed with a solution of 20 mM sodium phosphate and 150 mM NaCl at pH 7.4. Fractions of 50 µL each were collected and analyzed using a Jasco V-550 spectrophotometer (wavelength range: 220 nm-500 nm). The fractions containing a peak corresponding to the CF488 dye were pooled, filtered, and concentrated using a Vivaspin® 500 (Sartorius AG, Göttingen, Germany).

2.3. Indirect ELISA Test

To evaluate the effect of the labeling process on the anti-MC-LR antibody, their binding capability was investigated by indirect ELISA test. The test was performed according to El Kojok H et al [15]. In brief, 50 µL of the MC-LR BSA 488 solution, diluted in the coating buffer at pH 9.6, was added to each well and incubated overnight at 4 °C. After overnight incubation and washed step (three times with a solution of 200 µL TBS at pH 7.4 for 10 min), the plate was incubated with a 100 µL blocking solution (TBS, 5% skim milk) for 1 h at 37 °C. After this incubation, the wells were washed three times with a solution of 200 µL TBS-0.05% Tween 20. A solution of anti-MC-LR at 1.0 µg/mL (50 µL) was added to each well followed by incubation at 37 °C for 1 h. The wells were washed three-times with a solution of 200 µL TBS-0.05% Tween 20 at pH 7.4 for 10 min, then a solution of 50 µL of diluted anti-mouse secondary antibody, at a concentration of 1 µg/mL, was added to each well and incubated at 37 °C for 1 h. A solution of 100 µL of the 3,3', 5,5'-tetramethylbenzidine (TMB) per

individual well was added and incubated at room temperature for 10 min. Finally, a 100 μ L stop solution was added to the wells (2.5 M HCl), and the absorbance at 450 nm was measured by Tecan Infinite 200 PRO plate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.4. Fluorescence Steady-State Measurements

Fluorescence steady-state experiments were performed using the ISS K2 fluorometer (ISS Inc., Champaign, Illinois, USA) equipped with a temperature-controlled sample holder. The stock solution of MC-LR BSA 488 was diluted to a final optical density of 0.07 O.D. at 488nm to prevent the inner filter effect [16]. Excitation was fixed at 488 nm, and emission spectra were recorded from 500 nm to 600 nm in 1.0 nm intervals at a scan speed of 100 nm/min, using a 1.0 cm light path fluorescence quartz cuvette. Both the excitation and emission slit widths were set at 2 nm. The MC-LR BSA 488 emission spectra were carried out at 25 °C, in PBS buffer at pH 7.4, with a final volume of 750 μ L.

2.5. Fluorescence Correlation Spectroscopy (FCS) Experiments

The FCS measurements were performed on Alba V, the dual-channel fluorescence correlation instrument from ISS (ISS Inc., Champaign, Illinois, USA). The instrument combines a confocal scanning microscope with FCS, and it uses avalanche photodiodes as detectors. It is equipped with a Nikon inverted microscope and with a laser launcher with two lasers (488 nm and 640 nm); the output of each laser is aligned to a single-mode fiber to produce a Gaussian profile laser beam to be delivered to the instrument. Nikon high-numerical-aperture (NA) water objective (60 $\times$; NA 1.2) and focused on the solution sample. The resulting fluorescence was collected through the same objective and separated from the laser light by a dichroic mirror (Croma). A 50- μ m pinhole was used in the confocal detection channel. All pinhole adjustments, shutters, optics, filter wheels, XYZ-fine positioning of the stage, and positioning of the objective are computer-controlled through the Vista Analysis Software program (ISS Inc., Champaign, Illinois, USA). The alignment of the instrument and the characteristics of the confocal beam were validated using rhodamine 110, which has a known diffusion coefficient of 430 μ m²/s [17,18]. All measurements were performed at room temperature, and the obtained data were analyzed with the Vista Analysis Software program (ISS Inc., Champaign, Illinois, USA).

2.5.1. FCS Binding Analysis

The antibody binding measurements were performed by diluting MC-LR BSA 488 in PBS buffer at pH 7.4 to a final volume of 100 μ L, then incubating with increasing concentrations of anti-MC-LR (0.0-125 nM). For each concentration, before performing the FCS measurements, the samples were incubated in the dark condition and at room temperature for 30 minutes.

2.5.2. FCS Competitive Assay

The monoclonal antibody anti-MC-LR (125 nM) was incubated for 30 minutes with increasing concentration of unlabeled MC-LR (from 0.1 to 20 nM). At the end of this incubation step, BSA-MC-LR 488 was added, incubated for 30 minutes at room temperature, and then FCS measurements were performed.

2.5.3. Specify of FCS Assay

To evaluate the specificity of the assay, the measurement was performed using a different antibody. For this purpose, the polyclonal antibody anti-ovalbumin (125 nM) was incubated for 30 minutes with 20 nM of MC-LR. At the end of this incubation step, MC-LR BSA 488 was added, incubated for 30 minutes in dark conditions at room temperature, and then the FCS measurements were performed.

2.5.4. FCS Data Analysis

All collected data were analyzed using the Vinci Analysis Software program (ISS Inc., Champaign, Illinois, USA). Specifically, a fitting model based on a three-dimensional Gaussian and two species observation volume was employed (Equation 1).

$$G(\tau) = \left(\frac{2\sqrt{2}}{\pi\sqrt{\pi}\omega_0^2 z_0 C} \right) \times \sum_{i=1}^n \frac{f_i}{\left(1 + \frac{8D_i\tau}{\omega_0^2}\right) \sqrt{1 + \frac{8D_i\tau}{z_0^2}}} \exp \left[\frac{-(V_i\tau)^3}{\omega_0^2 z_0 \left(1 + \frac{8D_i\tau}{\omega_0^2}\right) \sqrt{1 + \frac{8D_i\tau}{z_0^2}}} \right] \quad (1)$$

In this equation, $G(\tau)$ represents the autocorrelation function, C is the concentration of the fluorescence molecule, w_0 is the beam waist, z_0 is the beam height, D is the diffusion coefficient, V is the excitation volume, Π is Archimedes' constant, and τ is the time diffusion.

2.6. Statistical Analysis

Fluorescence measurements and ELISA experiments were performed in triplicate; then, means and standard deviation (SD) were calculated. For the assay performance was determined according to Shrivastava (LoD = 3.3 S/b) [19]. The graphs were realized by Excel 2016 Microsoft® and/or by Origin Pro® 8.0 software.

3. Results and Discussion

MCs are cyclic heptapeptides released in water from *Cyanobacteria* MC-LR is extremely toxic to human health. The chemical structure of MCs consists of a constant region made up of five amino acid residues and a variable region containing two amino acid residues [2]. In the case of MC-LR, the variable region contains arginine and leucine residues. We used a specific monoclonal antibody anti-MC-LR, which can recognize all the variants of the four-Arg microcystins. We focused our attention on the MC-LR due to its high prevalence in freshwater and its significant toxicity on health.

Figure 1 shows the FCS optical setup. It includes the confocal volume size (Figure 1A) and two analysis methods: autocorrelation analysis (AC) and photon counting histogram analysis (PCH) (Figures 1B and 1C).

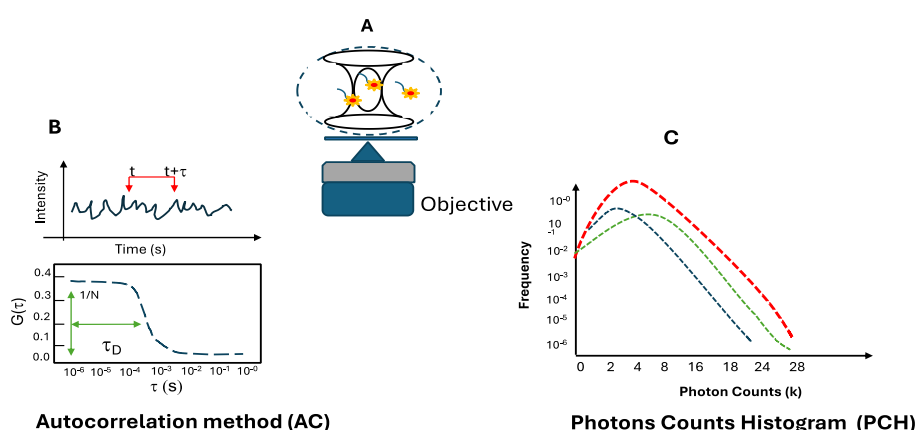


Figure 1. Fluorescence correlation spectroscopy (FCS) setup. In the general FCS optical setup, a laser is focused to a diffraction-limited spot using a high-numerical-aperture objective lens. Emitted light is collected through a confocal pinhole, defining a small observation volume of approximately 0.2 femtoliters (A). Fluctuations analysis using autocorrelation (AC) analysis (B) and photon counting histogram (PCH) analysis (C).

MC-LR was conjugated to BSA and the MC-LR BSA complex was labeled with the fluorescence dye (CF488). The degree of labeling (DoL) of MC-LR BSA 488 was calculated from the absorption

spectrum (data not shown) according to Varriale [12]. It was possible to estimate a labeling of three molecules of CF488 for one molecule of MC-LR BSA.

To evaluate the effect of the labeling process on the binding capacity of the anti-MC-LR to the MC-LR BSA 488, an indirect ELISA test was performed (Figure 2B). The results show that the anti-MC-LR bind the labeled conjugate MC-LR BSA 488 from 0.31 $\mu\text{g/mL}$ to 10.0 $\mu\text{g/mL}$.

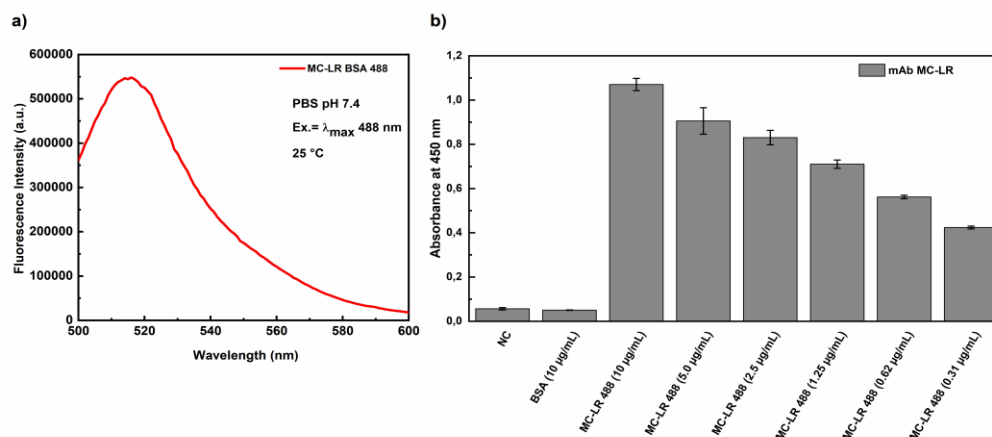


Figure 2. Emission spectrum of the MC-LR BSA 488 (a). The fluorescence emission spectrum was acquired upon excitation at 488 nm, at 25 °C in PBS buffer pH 7.4. Indirect ELISA test monoclonal anti-MC-LR vs MC-LR BSA 488 (b). The experiments were performed in triplicate at 25 °C. A BSA solution and a no-coating (NC) well were used as a negative control.

3.1. FCS Measurements: Binding Characterization

The interaction between MC-LR BSA 488 and anti-MC-LR antibodies was carried out in different steps. We investigated the diffusion of the MC-LR BSA 488 alone (Figure 3A black dots) and analyzed the obtained fluctuation data using the equation reported in the section 2 (3D Gaussian-2 species). We estimated a diffusion coefficient (D) value of 73.02 mm^2/s . This value is consistent with the molecular weight of the BSA molecule as reported in literature and slightly different from the theoretical value considering the presence of MC-LR molecules [20].

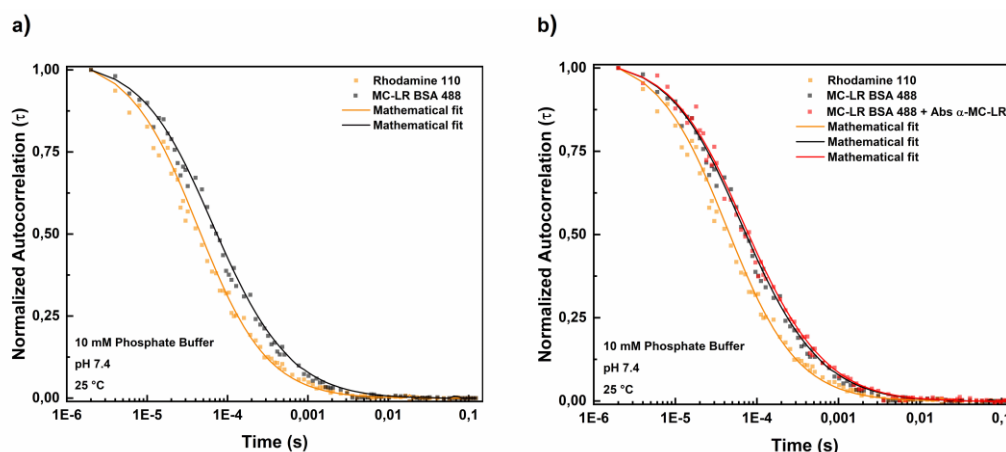


Figure 3. Fluorescence correlation spectroscopy measurements of MC-LR BSA 488 in the absence and in the presence of anti-MC-LR. Normalized autocorrelation curve of MC-LR BSA 488 (black dots) in PBS pH 7.4 compared with the autocorrelation spectrum of Rhodamine 110 (orange dots) (a). Normalized autocorrelation MC-LR BSA 488 in the absence (black dots) and in the presence of 125 nM of mAb anti-MC-LR (red dots), compared with the autocorrelation spectrum of Rhodamine 110 (orange dots) (b). The measurements were performed in PBS buffer at pH 7.4 and at 25 °C.

Furthermore, we evaluated the formation of the complex anti-MC-LR/MC-LR BSA 488. For this purpose, the MC-LR BSA 488 was incubated with a higher concentration of mAb anti-MC-LR. Figure 3B shows the normalized autocorrelation curves obtained of the MC-LR BSA 488 in the absence and in the presence of anti-MC-LR (125 nM). The diffusion coefficient values, determined with the autocorrelation function, show a reduction of the diffusion coefficient value. Specifically, the diffusion coefficient values change from 73.02 $\mu\text{m}^2/\text{s}$ to 49.52 $\mu\text{m}^2/\text{s}$ when the antibody concentration reaches 125 nM. This change is consistent with the formation of a molecular complex similar in size to the anti-MC-LR/MC-LR BSA 488 complex (Table 1).

Table 1. Diffusion coefficient in the absence and in the presence of an increased concentration of monoclonal anti-MC-LR.

[anti-MC-LR] nM	Diffusion coefficient $\mu\text{m}^2/\text{s}$	Standard Deviation +/-	Chi-square
0.00	73.02	0.87	0.83
17.0	65.58	0.53	1.15
41.0	59.20	0.70	0.75
125.0	49.52	0.62	0.77

Figure 4 show the variation of the diffusion coefficient values (Table 1) associate with the increased concentration of anti-MC-LR in solution (from 0.0 to 125 nM).

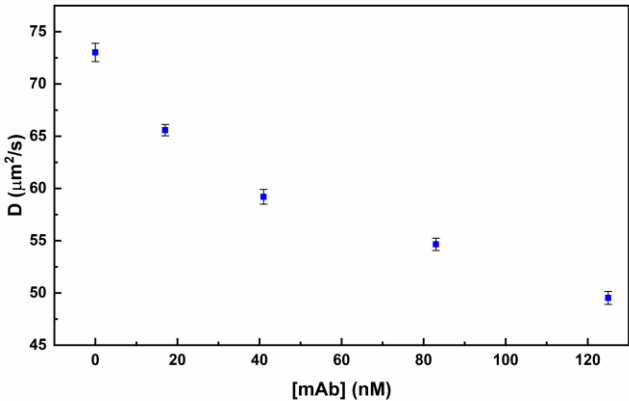


Figure 4. Binding of anti-MC-LR to MC-LR BSA 488. Variation of the diffusion coefficient values of the MC-LR BSA 488 in the absence and in the presence of increased concentration of anti-MC-LR (0.0 - 120 nM). The measurements were performed in PBS buffer at pH 7.4, 25 °C.

3.2. FCS Measurements: Competitive Assay

Figure 5A shows the plot of the change of the diffusion coefficient value versus MC-LR concentration. The increase of MC-LR concentration results in an increase of the value of the diffusion coefficient that becomes close to the value calculated in the absence of the MC-LR (Table 2). In particular, the diffusion value changes from 48.82 $\mu\text{m}^2/\text{s}$ to 77.56 $\mu\text{m}^2/\text{s}$. This behavior suggests the complete dissociation of the anti-MCL-LR/MC-LR BSA 488 complex in the presence of the MC-LR.

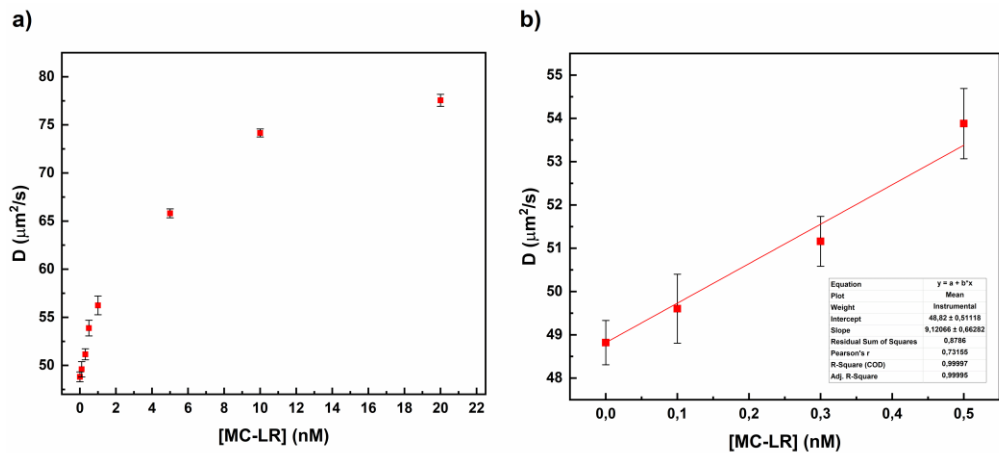


Figure 5. Fluorescence correlation spectroscopy competitive assay. Variation of the diffusion coefficient value in the presence of increasing concentration of MC-LR (a). The calibration curves were obtained through a linear fitting function in the range 0.0 to 0.5 nM of MC-LR (b).

Table 2. Diffusion coefficient values of anti-MC-LR/MC-LR BSA 488 in the absence and in the presence of increasing concentration of MC-LR.

[anti-MC-LR]	[MC-LR]	Diffusion coefficient	Standard Deviation	Chi-square
	nM	$\mu\text{m}^2/\text{s}$	+/-	
125 nM	0.0	48.82	0.51	0.75
	0.1	49.60	0.79	0.94
	0.3	51.16	0.57	0.74
	0.5	53.88	0.81	1.02
	1.0	56.24	0.97	0.85
	5.0	65.81	0.46	0.92
	10.0	74.16	0.41	1.12
	20.0	77.56	0.62	1.09

A linear correlation was observed over the range of 0.0 to 0.5 nM, whereas at higher concentrations (0.5 to 20 nM), no linear trend was observed. Therefore, the calibration curve was calculated in the linear range to determine the limit of detection (LoD). The LoD, calculated by $3.3 S/b$, where S is the standard deviation of the y-intercept of the linear regression and b is the slope of the linear range [19], was estimated to be 0.18 nM (0.18 $\mu\text{g/L}$) (Figure 5B). These results suggest that the use of high-avidity monoclonal antibodies with the innovative fluorescence immunoassay allows for the detection of the presence of traces of MC-LR in fresh water.

4. Conclusions

In this work, we explored the application of FCS to determine the presence of traces of MC-LR in fresh water. The developed assay showed a LoD value of 0.18 nM (0.18 $\mu\text{g/L}$). This value is lower than the provisional limit value (1.0 $\mu\text{g/L}$) established by WHO.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, S.D. and A.V.; methodology, G.F.; software, A.V.; validation, G.F., A.V.; formal analysis, A.V.; investigation, A.V.; resources, S.D.; data curation, A.V.; writing—original draft preparation, A.V.; writing—review and editing, S.D.; project administration, A.V.; funding acquisition, S.D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

FCS	Fluorescence Correlation Spectroscopy
MC-LR	Microcystin-LR
BSA	Bovine Serum Albumin
WHO	World Health Organization
AC	Autocorrelation analysis
PCH	Photon Counting Histogram Analysis
OVA	Ovalbumin
ELISA	Enzyme-Linked Immunosorbent Assay

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