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Abstract:	Global warming and eutrophication make some aquatic ecosystems behave as true bioreactors that trigger rapid and massive cyanobacterial growth with relevant health and economic consequences. Many cyanobacterial strains are toxin producers and few cells are necessary to induce irreparable damages to the environment. Therefore, water body authorities and administration require rapid and efficient early warning systems providing reliable data for supporting their preventive or curative decisions. This manuscript reports an experimental protocol for in field detection of toxin-producing cyanobacterial strains by using an antibody microarray chip with 17 antibodies (Abs) with taxonomic resolution (CYANOCHIP). Herein, a multiplex fluorescent sandwich microarray immunoassay (FSMI) for simultaneous monitoring of 17 cyanobacterial strains frequently found in blooming in freshwater ecosystems, some of them toxin producers is described. A microarray with multiple identical replicates (up to 24) of the CYANOCHIP onto a single microscope slide was printed to simultaneously test a similar number of samples. Liquid samples can be tested either by direct incubation with the Abs or after cell concentration either by filtration through 1-3 µm filter. Solid samples as sediments and ground rocks are firstly homogenized and dispersed by a hand-held ultrasonicator into an incubation buffer, filtered (5-20 µm) to remove the course material, and the filtrate incubated with the Abs. Immunoreactions are revealed by a final incubation with a mixture of the 17 fluorescence -labelled Abs and read by a portable fluorescence detector. The whole process takes around 3 h, most of it corresponding to 2 1-hr periods of incubation. The output is an image where bright spots correspond to the positive detection of cyanobacterial markers.
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TITLE:
Experimental protocol for detecting cyanobacteria in liquid and solid samples with an antibody microarray chip

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SHORT ABSTRACT:
The presence of cyanobacterial toxins in fresh water reservoirs for human consumption is a major concern for water management authorities. To evaluate the risk of water contamination, this article describes an protocol for the in-field detection of cyanobacterial strains in liquid and solid samples by using an antibody microarray chip.

LONG ABSTRACT:
Global warming and eutrophication make some aquatic ecosystems behave as true bioreactors that trigger rapid and massive cyanobacterial growth; this has relevant health and economic consequences. Many cyanobacterial strains are toxin producers, and only a few cells are necessary to induce irreparable damage to the environment. Therefore, water-body authorities and administrations require rapid and efficient early-warning systems providing reliable data to support their preventive or curative decisions. This manuscript reports an experimental

protocol for the in-field detection of toxin-producing cyanobacterial strains by using an antibody microarray chip with 17 antibodies (Abs) with taxonomic resolution (CYANOCHIP). Here, a multiplex fluorescent sandwich microarray immunoassay (FSMI) for the simultaneous monitoring of 17 cyanobacterial strains frequently found blooming in freshwater ecosystems, some of them toxin producers, is described. A microarray with multiple identical replicates (up to 24) of the CYANOCHIP was printed onto a single microscope slide to simultaneously test a similar number of samples. Liquid samples can be tested either by direct incubation with the antibodies (Abs) or after cell concentration by filtration through a 1- to 3-µm filter. Solid samples, such as sediments and ground rocks, are first homogenized and dispersed by a hand-held ultrasonicator in an incubation buffer. They are then filtered (5-20 µm) to remove the coarse material, and the filtrate is incubated with Abs. Immunoreactions are revealed by a final incubation with a mixture of the 17 fluorescence-labeled Abs and are read by a portable fluorescence detector. The whole process takes around 3 h, most of it corresponding to two 1-h periods of incubation. The output is an image, where bright spots correspond to the positive detection of cyanobacterial markers.

INTRODUCTION:

The detection and monitoring of microorganisms in complex natural microbial communities are crucial in many fields, including biomedicine, environmental ecology, and astrobiology. Cyanobacteria are prokaryotic microorganisms well-known for their ability to form blooms (excessive proliferation) of cells in fresh water. They are ubiquitous, and many species are able to produce toxins, leading not only to a potential risk for human health, but also to an ecological impact. In this regard, it is essential to develop rapid and sensitive methods for the early detection of cyanobacteria and/or their toxins in soil and water. For this purpose, a multiplex fluorescent sandwich microarray immunoassay (FSMI) has been developed as a tool for water managers to help them in making decisions and, consequently, in implementing proper water management programs.

A diverse range of methods has been developed to detect and identify cyanobacterial cells and cyanotoxins in soil and water, including optical microscopy, molecular biology, and immunological techniques. These methods can vary greatly in the information they provide. Microscopic techniques are based on cell morphology and the detection of *in vivo* fluorescence from cyanobacterial pigments, such as phycocyanin or chlorophyll a[1]. Although they are quick and cheap methods for real-time and frequent monitoring that inform about the type and number of cyanobacteria present in a sample, they do not give information about the potential toxicity. In addition, they require a certain level of expertise, considering that it is often very difficult to distinguish between closely-related species[2]. To overcome these limitations, light microscopy must be accompanied by both biological and biochemical screening assays and physicochemical methods for the identification and quantification of cyanotoxins.

Enzyme-linked immunosorbent assays (ELISA), protein phosphate inhibition assays (PPIA), and neurochemical tests in mice are examples of biochemical screening assays for the detection of cyanotoxins. While the first two are rapid and sensitive methodologies, false positives have been described when using ELISA and PPIA tests are restricted to three types of toxins. The

mouse bioassay is a qualitative technique with low sensitivity and precision, and special licensing and training is required. In addition, it does not give information about the type of toxins present in a sample. Cyanotoxins can be identified and quantified by other analytical methods, such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), or matrix-assisted laser desorption/ionization time of flight (MALDI-TOF). However, this is only possible if reference standards, which are needed to determine individual toxin concentrations in complex samples, are available[3,4]. Moreover, these methods are time-consuming; require costly equipment, supplies, and sample preparation; and must be performed by experienced and specialized staff.

Molecular-based methods have been applied for decades to detect, identify, and quantify cyanobacteria and their corresponding cyanotoxins thanks to the sequence information published in the genome databases (*e.g.,* National Center for Biotechnology Information, NCBI). Among these methods are those based on the polymerase chain reaction (PCR), which requires the design of sets of primers for DNA amplification and depend on previous knowledge of DNA sequences of different cyanobacterial species. While gene detection, like the phycocyanin operon, leads to accurate identification at the genus level, some species or strains are undetected with this method. However, toxin-encoding genes, such as those belonging to the microcystin operon, facilitate the identification of toxins in samples where the producers are scarce[5]. Nonetheless, the detection of toxin markers by PCR does not necessarily imply toxicity in the environment. Furthermore, the set of primers developed to analyze the whole range of species of cyanobacteria and toxin producers present in a sample is still incomplete, and further studies must be done to identify unknown species. Other molecular techniques are non-PCR-based, such as fluorescence *in situ* hybridization (FISH) and DNA microarrays.

In the last two decades, microarray technology has gained importance in many fields of application, particularly in environmental monitoring. DNA microarrays allow for discrimination between species and analytes[4, 6-10], but they are considered very laborious and time-consuming tasks that involve multiple steps (*e.g.,* microarray performance, DNA extraction, PCR amplification, and hybridization). For that reason, less time-consuming assays based on antibodies, such as sandwich and competitive immunological microarrays, have become an essential and reliable high-throughput method for the detection of multiple environmental analytes[11-13]. The capability of antibodies to specifically recognize their target compounds and to detect small amounts of analytes and proteins, along with the possibility of producing antibodies against almost any substance, make antibody microarrays a powerful technique for environmental purposes. In addition, the capability of achieving multiple analyses in a single assay, with limits of detection ranging from ppb to ppm, is one of the main advantages of this method[14].

Antibody-based biosensors have proved to be sensitive and rapid tools for the detection of a wide range of pathogens and toxins in environmental monitoring[15-21]. While DNA methods involve several steps, the antibody-based microarrays only require a small sample preparation that is mainly based on a short lysis step in an appropriate solution buffer. Delehanty and

Ligler[15] reported the simultaneous detection of proteins and bacterial analytes in complex mixtures based on an antibody sandwich immunoassay capable of detecting a protein concentration of 4 ppb and 10^4 cfu/mL of cells. Szkola *et al.*[21] have developed a cheap and reliable multiplex microarray for the simultaneous detection of proteotoxins and small toxins, compounds that might be used in biological warfare. They detected concentrations of ricin toxin, with a limit of detection of 3 ppb, in less than 20 min. Recently, the CYANOCHIP, an antibody microarray-based biosensor for the *in situ* detection of toxic and nontoxic cyanobacteria, has been described[22]. This microarray allows for the identification of potential cyanobacterial blooms, mostly in aquatic environments, which are difficult to identify microscopically. The limit of detection of the microarray is 10^2-10^3 cells for most species, turning this biosensor into a cost-effective tool for the multiplex detection and identification of cyanobacteria, even at the species level. All these properties make the antibody microarray technique, and particularly the method presented in this work, a quicker and simpler method compared to the aforementioned techniques.

This work presents two examples of experiments that use an antibody microarray-based biosensor to detect the presence of cyanobacteria in soil and water samples. It is a simple and reliable method based on a sandwich immunoassay format that requires very small sample volumes and very basic sample preparation. The method requires a short time and can be easily performed in the field.

PROTOCOL:

1. Preparation of the immunogens

1.1. Grow each cyanobacterial strain in the corresponding culture medium under conditions described in Table 1.

NOTE: Growth medium and culture conditions for each cyanobacteria are listed in Table 1. All the cyanobacterial strains, with the exception of K17, belong to Antonio Quesada's group from Autonoma University (Madrid, Spain). The antibody against *Planktothrix rubescens* was generated from a natural sample of the monospecific bloom of this cyanobacterium from Vilasouto reservoir (northern Spain).

1.2. Quantify the number of cells using a cell counting chamber by optical microscopy to obtain approximately 10^8 cells/mL from a late exponential or stationary growth phase culture.

1.3. Harvest cells from 5 mL of culture by centrifugation at 2,000 x g for 5 min.

1.4. Discard the supernatant and resuspend the cells in a 10-mL tube with 5 mL of 1X phosphate-buffered saline (1X PBS) to obtain around 10^8 cells/mL.

1.5. Homogenize and lyse the cells of the suspension by sonication for 5 cycles, 30 s each, with a 30- to 60-s pause on ice, using a portable, hand-held ultrasonic processor or by dipping the tube into the water bath of a cell disruptor at maximum amplitude (30 KHz).

1.6. Repeat steps 1.3-1.5 for each strain of cyanobacterium.

NOTE: Sonication produces cell inactivation and cellular content release. While proteins and polysaccharides are good immunogens, specific molecules, such as lipids and nucleic acids, do not induce a humoral immunogenic response by themselves. Therefore, they must bind to carriers, such as polysaccharides or proteins, to increase the molecular complexity. In addition, sonication releases intracellular material that can trigger antibody production.

2. Production of polyclonal antibodies

2.1. Prepare the first immunogen dose by mixing 0.5 mL of the ultrasonicated cell lysate obtained in step 1.5 with 0.5 mL of complete Freund's adjuvant. Deliver it to the operator of an animal facility for polyclonal rabbit antibody production.

2.2. Prepare three more doses as before for further use as memory boosts in the antibody production process by mixing 0.5 mL of the same homogenate/lysate obtained in step 1.5 with 0.5 mL of incomplete Freund's adjuvant. Give them to the animal facility to fulfill the antibody production process.

2.3 Repeat steps 2.1 and 2.2 for each new antibody production process.

NOTE: Normally, antibody production is entrusted to specialized animal facilities or companies, because appropriate licensing and training are required to work with animals. The companies supply a relative enzyme-linked immunosorbent assay (ELISA) measurement of the quantity of antigen-specific antibodies present in the serum sample.

3. Antibody purification

3.1) Purify the immunoglobulin G (IgG) fraction from both the immune and the pre-immune serum collected in step 2 by protein-A affinity chromatography. Some commercial purification kits, based on cartridge systems, work well; follow the provider instructions.

3.2) When the purification kit does not provide a desalting system, change the buffer after the elution of the purified antibodies to 0.1X PBS, either by dialysis or by using centrifugal filter devices with a 100-kDa (or lower) membrane pore size.

3.3.) Determine the antibody concentration by measuring the absorbance at 280 nm or by using colorimetric methods, such as Bradford[23], Lowry[24], or Bicinchoninic acid (BCA)[25].

NOTE: It is important to avoid including amine groups in the elution buffer (*e.g.,* Tris buffers) because they compete with the antibody for binding to solid surfaces activated with epoxy groups. After purification, it is important to test the antibody activity with ELISA.

4. Fluorescence antibody labeling

4.1) Label the purified antibodies obtained in section 3 with a fluorochrome (*e.g.,* a far-red

fluorescent dye) by dissolving a commercial vial containing dye for labeling 1 mg of protein into 100 µL of dimethyl sulfoxide (DMSO). Add 2 µL of the dissolved dye to each antibody preparation at a concentration of 2 mg/mL in a final volume of 50 µL in 50 mM phosphate-buffered saline (pH 8.5).

4.2) Maintain the labeling reactions under continuous agitation for 1 h at ambient temperature and 1,200 rpm on a vibrating platform.

4.3) Purify the labeled antibodies by size exclusion chromatography (*e.g.,* using a gel with a fractionation range between 1.5 and 30 kDa trapped into a column), following supplier recommendations.

4.4) Measure the absorbance at 280 nm and at 650 nm in eluates and calculate the labeling efficiencies following supplier recommendations.

NOTE: Up to 50 x 50-µL antibody-labeling reactions can be done with a single vial of the fluorescent dye for labeling 1 mg of protein. It is recommended to cover the tubes with aluminum foil or, alternatively, to use opaque 0.5-mL tubes to avoid quenching processes after step 4.3. For IgG antibodies, optimal labeling is achieved with 3-7 mol of the dye per mol of antibody.

5. CYANOCHIP production

5.1) Antibodies and controls in printing solution

5.1.1) Prepare 30 µL of each purified antibody in printing solution by mixing each antibody at 1 mg/mL in a commercial 1X protein printing buffer with 0.01% (v/v) Tween 20 (a non-ionic detergent), all as final concentrations.

NOTE: Alternatively, an antibody solution may contain 20% glycerol, 1% (w/v) sucrose (or trehalose, as a preservative), and 0.01% (v/v) Tween 20 in carbonate buffer (pH 8.5).

5.1.2) Prepare 30 µL of the printing solution as controls: (a) 1X protein printing buffer with 0.01% (v/v) Tween 20, (b) protein-A purified pre-immune serum at 1 mg/mL in 1X protein printing buffer with 0.01% (v/v) Tween 20, and (c) bovine serum albumin (BSA) at 1 mg/mL in 1X protein printing buffer with 0.01% (v/v) Tween 20.

5.1.3) Prepare 30 µL of a fluorescently-labeled, purified pre-immune serum at different concentrations (*e.g.,* from 50 µg/mL to 1 µg/mL) in 1X protein printing buffer with Tween 20, as in step 5.1.1. These samples will be used as fluorescent frame markers and for relative fluorescence quantification after spotting.

5.1.4) Add 30 µL per well of the printing solutions prepared in steps 5.1.1, 5.1.2, and 5.1.3 to the highest-quality 384-well microplates for microarray manufacturing, such as polypropylene.

NOTE: Protein printing buffer increases the quality and stability of the antibodies, and Tween 20 homogenizes spot morphology and builds up protein coupling up. It is recommended to

maintain the 384-well microplate at 4 °C before use. Store it at -20 °C for long periods of time. Polypropylene has low DNA, protein, cell extract, and small-molecule intrinsic binding.

5.2) Antibody printing onto a microscope slide

NOTE: Print the antibodies onto activated microscope slides by using different array platforms, like contact, split-needle, or non-contact devices, such as piezoelectric printers or "ink jet" technologies. In this work, the CYANOCHIP has been routinely printed by contact using a robotic system (arrayer) capable of spotting nL quantities of the antibodies at the µm scale.

5.2.1) Set the environmental conditions of the printing room to 20 °C and 40-50% relative humidity.

5.2.2) Set up the slide substrates (*e.g.,* 75- x 25-mm epoxy-activated microscope glass slides) to perform several identical antibody arrays on each slide.

5.2.3) Spot each purified antibody, including controls and the reference frame, in a triplicate spot pattern; under these conditions, the spots are 180-200 µm in diameter.

5.2.4) After printing, leave the slides for at least 30 min at ambient temperature to let them dry, and then store them at 4 °C; for working in the field, the slides can be transported and stored at ambient temperature for several months.

NOTE: The CYANOCHIP is printed in a triplicate spot microarray format with 3 x 8 identical microarrays per slide or 9 identical arrays in a 1 x 9 microarray format. Each array size must not be higher than the reaction chamber dimensions for a 24-well gasket (usually 7.5 x 6.5 mm in a 3 x 8 hybridization chamber).

5.2.5) Before using the microarray to analyze environmental samples, use FSMI to determine the working dilution for each antibody in a titration curve. For each antibody, use a standard concentration of the corresponding immunogen (10^3-10^4 cells/mL) and serial dilutions of the fluorescent antibody (between 1:500 and 1:32,000). The optimal antibody concentration corresponds to 50% of the maximum signal intensity obtained in the titration curve. Also, the sensitivity and specificity for each antibody must be determined, as described in Blanco *et al*[22].

6. Preparation of environmental multianalyte extracts for the fluorescent sandwich microarray immunoassay (FSMI)

6.1) Multianalyte extract from a liquid sample

6.1.1) Take 1-100 mL of the liquid sample with a sterile syringe (*e.g.,* water from the shore of a water reservoir); the amount of sample is greatly dependent upon the potential concentration of the targets.

6.1.2) Concentrate the cells by passing the water sample through a 3-µm pore size, 47-mm diameter polycarbonate filter; a cellular concentration between 10^3-10^8 cells/mL is desirable for positive detection, but the actual concentration is unknown.

6.1.3) Recover the biomass collected in the filter with 1 mL of a modified Tris-buffered saline, Tween 20-reinforced buffer (TBSTRR; 0.4 M Tris-HCl (pH 8), 0.3 M NaCl, and 0.1% Tween 20) by scraping it with a spatula into a 15-mL tube.

6.1.4) Homogenize and disaggregate by using a hand-held ultrasonic processor, as described in step 1.5, or just by pipetting up and down multiple times; this prepares the sample for analysis by the microarray .

6.2) Multianalyte extract from a solid sample

6.2.1) Weigh up to 0.5 g of the solid sample (*e.g.,* rock, soil, or sediments) into a 10-mL tube and add up to 2 mL of TBSTRR.

6.2.2) Sonicate by immersing the sonicator probe in the tube, by dipping the tube into the water bath of a powerful sonicator horn, or by using a hand-held sonicator. Perform at least 5 x 30-s cycles at 30 kHz, stopping for 30 s while on ice .

6.2.3) Filter to remove sand, clay, and other coarse material with a 10-mL syringe coupled to a 10- to 12-mm diameter, 5- to 20-µm pore size nylon filter holder. Push the sample through the filter into a 1.5-mL tube. If the filter saturates, agitate the suspension in the syringe and take it to a new one; this prepares the filtrate material for the immunoassay (step 7).

NOTE: The buffering capacity of TBSTRR depends on the type of sample. It is important to carry out the immunoassay immediately after the preparation of the environmental extract to avoid the effect of enzymatic degradation on the analytes. Alternatively, add protease inhibitors into the environmental extract and freeze at -80 °C until the next step.

7. Fluorescent sandwich microarray immunoassay (FSMI)

7.1) Blocking the CYANOCHIP

NOTE: Immediately before use, treat the printed slides to block all free epoxy groups on the slide and to remove the excess of non-covalently-bound antibodies.

7.1.1) Immerse the microarray into 0.5 M Tris-HCl (pH 9) with 5% (w/v) BSA solution on a clean surface (*e.g.,* Petri dish or a 50-mL tube) with mild agitation from a rocker platform for 5 min. Alternatively, lay the slide down onto a 100- to 200-µL drop of the above solution with the microarray spots facing it. Leave for 3-5 min and then proceed.

7.1.2) Carefully pick up the slide using plastic-tipped forceps; try to avoid touching microarray zones. Eliminate the excess of liquid by softly knocking the slide onto a paper towel. Immerse it in 0.5 M Tris-HCl (pH 8) with 2% (w/v) BSA solution for 30 min with mild agitation from a rocker platform.

7.1.3) Dry the slide by performing a short centrifugation (200-300 x g for 1 min) using a commercial microcentrifuge adapted for microscope slides. Alternatively, dry the slide by softly

knocking onto a paper towel.

7.2) Incubation of the multianalyte sample extract with the microarray
7.2.1) Set up the slide in a commercial microarray hybridization cassette with 24 wells for multiple microarrays; follow the provider instructions.

7.2.2) After slide and cassette assembly, pipette up to 50 µL of the sample extract or a dilution of it in TBSTRR into each well of the cassette.

7.2.3) Repeat step 7.2.2 for each sample to be analyzed.

7.2.4) As a blank control, pipette 50 µL of TBSTRR buffer into at least two separate wells of the cassette.

7.2.5) Incubate at ambient temperature for 1 h with mixing by pipetting every 15 min or by leaving it under mild shaking. Alternatively, incubate for 12 h at 4 ^{o}C.

NOTE: Use other incubation gaskets as a function of the microarray pattern. The time and temperature of the incubation in step 7.2.5 are empirical parameters that normally depend on the affinity and binding kinetics of each paired antigen-antibody.

7.3) Washing
7.3.1) Remove the samples by putting the cassette down and carefully knocking it onto a clean, absorbent paper.

7.3.2) Wash the wells by adding 150 µL of TBSTRR to each one, and eliminate the buffer, as above.

7.3.3) Repeat step 7.3.2 three more times.

7.4) Incubation with fluorescent detector antibodies
7.4.1) Add 50 µL of an antibody mixture containing the 17 anti-cyanobacterial-strain antibodies, each labeled with the fluorochrome in TBSTRR with 1% (w/v) BSA. Determine the concentration of each fluorescent antibody in the mixture (from 0.7 to 2 µg/mL) by performing titration experiments of each antigen/antibody pair[22].

7.4.2) Incubate for 1 h at ambient temperature, as described in step 7.2.5, or for 12 h at 4 °C.

7.5) Washing out the fluorescent antibodies
7.5.1) Remove the fluorescent unbound antibodies, as in step 7.3.

7.5.2) Disassemble the cassette and immerse the slide into 0.1X PBS (*e.g.,* in a 50-mL tube) for a quick rinse.

7.5.3) Dry the slide as in step 7.1.3.

8) **Scanning for fluorescence**

8.1) Scan the slide for fluorescence at the maximum emission fluorescence peak for far-red fluorescent dye in a scanner for fluorescence. Take several images of the microarrays at different scanning parameters, generally by lowering the laser gain value.

NOTE: Avoid saturated spots (>65,000 fluorescence counts)—they are out of scale and may introduce quantification errors.

9. Image processing and data analysis

9.1) Use a commercial software for image analysis and quantification; the software provides measurements of the fluorescence intensity (FI; median or mean of all pixels from a single spot) for each spot of the whole microarray. Subtract the local background around the spots:
$FI = FI_{spot} - FI_{local\ background}$.

9.2) Save the FI data and open them using a spreadsheet program.

9.3) Use the values obtained for the blank arrays as the negative control to identify and discard the false positives. Apply the following equation to calculate the FI for each antibody spot:
$FI = (FI_{sample} - FI_{blank})$, where $FI_{sample} = FI_{spot} - FI_{local\ background}$ in the microarrays run with sample extracts and $FI_{blank} = FI_{spot} - FI_{local\ background}$ in the blank microarrays.

9.4) Apply an additional cut-off threshold of 2- to 3-fold the average of the FI of the whole microarray to minimize the probability of false positives; this is especially relevant for poor-quality microarray images and low signal-to-noise ratios.

9.5) Create plots and/or perform further analysis as necessary.

REPRESENTATIVE RESULTS:

This work describes a multiplex immunoassay test for the simultaneous identification of the most relevant freshwater cyanobacterial species (Table 1) using the CYANOCHIP antibody microarray. The microarray can be a 3 x 8 microarray format printed onto microscope slides. Each microarray is made up of a set of 17 antibodies printed in a triplicate spot pattern, their corresponding pre-immune antibodies and BSA as negative controls. The microarrays also include a fluorescent frame, using a fluorescently-labeled pre-immune antibody to easily localize the microarray pattern[22] (Figure 1).

The microarray was tested in the field for the *in situ* analysis of water samples collected at the shore of the freshwater Lozoya Reservoir, which supplies the city of Madrid. Samples were processed as described above, and the main positive immunoreactions corresponded to antibodies to planktonic cyanobacteria, such as *Microcystis* spp. (K4 and K5), and to benthic Oscillatoriales, such as *Leptolyngbya* spp. (K10 and K15). Lower fluorescence signals were obtained for Nostocales (K6 and K12, two planktonic *Aphanizomenon* spp.), benthic *Rivularia* sp. (K8), *Anabaena* sp. (K1), and planktonic *Microcystis flos-aquae* sp. (K3). Optical microscope

observations (not shown) showed a diverse cyanobacterial community within abundant terrigenous debris from the shore. Several species of *Anabaena* dominated the community, but *Microcystis* spp. and *Pseudanabaena* spp. were also present.

Additionally, the microarray was also validated by assaying a dry, nearly 1,000-year-old microbial mat collected in the McMurdo Ice Shelf in Antarctica to test for the presence of cyanobacterial markers. Figure 1 shows highly-fluorescent, positive reactions in antibodies produced to benthic cyanobacteria isolated from other Antarctic mats, including *Anabaena* sp. and *Leptolyngbya* spp. (K14 and K15, respectively). Additional positive immunoreactions were detected with antibodies to planktonic cyanobacteria, such as *Microcystis* spp. (K4 and K5), *Aphanizomenon* spp. (K6 and K12), and *Planktothrix rubescens* sp. (K17). Low signals were obtained from antibodies to other benthic species isolated in an Antarctic mat, including *Tolypothrix* sp. (K16) and *Anabaena* sp. (K1). Fluorescent optical microscopy revealed the presence of cells structurally similar to cyanobacteria and green algae (not shown). No filamentous cyanobacteria were identified. Nevertheless, multiple amorphous fluorescent structures of about 1 μm in size and copious diffuse fluorescence were detected, which could be attributed to the presence of cell remains (broken and dead cells) and extracellular polymeric substances (EPS), respectively. Accordingly, the biochemical analysis showed that the mat consisted of profuse amounts of biopolymers and cell remains (complex biological matter) that could be the targets for the antibodies in the microarray.

Figure 1: Quick and reliable multiplex microarray immunoassay for detecting *Cyanobacteria*. A) Scheme showing the main steps of the FSMI for the analysis of multi-target samples with a CYANOCHIP. B) Schematic of a printing pattern layout (by triplicate) of the anti-cyanobacteria antibody collection: (0) BSA, bovine serum albumin; (X) only printing buffer; (1-17) each of the antibodies as in Table 1 in Blanco *et al.* 2015 (K1 to K17). From p1 to p17, the corresponding pre-immune antibodies act as controls. The yellow rectangles correspond to a fluorescent spot gradient as a frame reference. C and D) Picture showing the top part of a 1,000-year-old dry microbial mat from the McMurdo Ice Shelf (Antarctica) and the CYANOCHIP image detecting cyanobacteria in this mat, respectively. E) Panoramic view of the Lozoya Reservoir showing transparent water with no visible green particles at the time of sampling. F) microarray image after the *in situ* analysis of the water sample.

Table 1. List of the antibodies (Abs) and the cyanobacterial strains used to produce the CYANOCHIP[22]

DISCUSSION:

Here, a multiplex fluorescent sandwich immunoassay using the CYANOCHIP, a 17-antibody microarray for the detection and identification of a wide range of cyanobacterial genera, is described[22]. These cyanobacteria represent the most frequent benthic and planktonic genera in freshwater habitats, some of them being toxin producers. Recently, the fluorescent sandwich immunoassay format has been used to identify microorganisms and/or bioanalytes in environmental applications[26-28]. The protocol is primarily based on two steps: (i) immobilizing or capturing antibodies to specifically bind analytes from a test sample and (ii) detecting analyte-

antibody pairs by using fluorescently-labeled antibodies (tracer or detector antibodies). Because the sandwich assay requires at least two accessible antibody binding sites (epitopes) in the analyte for the reaction to take place, any positive fluorescent signal indicates the presence of relatively large and complex oligo- or multimeric analytes that are identical or highly similar to the ones used to produce the capturing antibodies.

Even though this method requires small volumes and basic sample preparation, without the need for special expertise or knowledge, several drawbacks could ruin the assay. Low fluorescent signals or a complete lack thereof may be due to poor antibody purification or poor labeling efficiency. For the isolation of rabbit IgG, protein A is the best choice, because it specifically binds with high efficiency to the Fc region of immunoglobulins. As indicated in section 4, fluorescent antibodies with a labeling range between 3-7 mol of dye per mole of antibody must be used. Furthermore, a lack of fluorescent spots can be explained by the concentration of analytes lying under the limits of detection. In this case, the sample can be concentrated before incubating it with the microarray, or greater amounts can be used for a new extraction. High fluorescent backgrounds are the result of inefficient chip blocking or/and washing steps and of extracts from samples composed of minerals and complex organic matter that stick onto the chip. When complex samples are used as analytes, it is desirable to increase the salt and/or the detergent concentration to favor the specific interaction between analyte-antibody pairs.

In recent years, antibody microarray technology has been developed for environmental applications. Nonetheless, the use of this technique does involve some limitations. Polyclonal antibodies are faster and cheaper to produce and, more importantly, the possibility to bind any target epitope in a complex environmental sample are theoretically higher than with monoclonal antibodies. However, the fact that they can recognize different epitopes increases the number of cross-reactions in FSMI. To gain high specificity, the use of methods to disentangle these cross-reactivity events from the true cognate antigen-antibody reactions by using deconvolution methods[26,27], for example, is highly desirable. Basically, the microarray is considered a qualitative biosensor for the multiple detection and classification of cyanobacteria. In this regard, it is essential to determine the limit of detection for each antibody one-by-one to use their optimal working concentrations in the assay. Although this microarray, together with FSMI, is not conceived as a quantitative method, the biosensor implies high sensitivity, because the lower detection limit of most of the antibodies contained in the CYANOCHIP[22] is from 10^2 to 10^3 cells/mL.

Despite these limitations, this methodology has several advantages against other techniques used in environmental monitoring. Antibody-biosensors allow the possibility of recognizing different molecules simultaneously in a single analysis, the possibility of detecting low concentration of analytes, and the possibility of producing antibodies against almost any substance. Furthermore, the microarray can achieve up to 24 analyses in a single assay, with limits of detection from 10^2 cells/mL. In comparison with other methods, antibodies can detect living or dead cells, extracellular material, and cellular debris. Although it takes at least 4 h to complete the whole assay, it is faster than other analytical methods applied to environmental

monitoring. The CYANOCHIP was originally conceived for the identification of cyanobacterial strains. This biosensor was not formally designed for that purpose, so it can identify potential toxin producers and could be improved in the future by adding antibodies against a wide range of new strains. Biochemical assays, such as ELISA, PPIA, and neurochemical tests in mice, allow the identification of cyanotoxins, but they are restricted to a few known toxins and can give false positives. In addition, using the CYANOCHIP does not require special training, while optical microscopy and mouse bioassays require trained personnel or labor-intensive work with live animals, and they do not give information about the type of toxins present in a sample. Cyanotoxins can also be identified and quantified by other analytical methods, such as HPLC, GC-MS, or MALDI-TOF. In these methods, the sample must be purified, and the lack of reference standards limits the identification of cyanotoxins. Furthermore, analytical methods require costly equipment and supplies and specialized training. Molecular methods are based on DNA extraction from the samples, while FSMI only requires an environmental extract prepared in a couple of very simple steps, without cyanobacteria purification.

The high performance of the CYANOCHIP for the *in situ* detection of cyanobacteria in freshwater reservoirs makes it a new tool for the early warning of water administrators. In addition, the microarray is also interesting for the field of astrobiology, particularly for searching for microbial markers as evidence of life. The study of extremophiles can help us to understand the origin of life on Earth and how life could survive in the extreme environments present in our solar system and beyond. As several environments on Earth are very similar to places on other planets, such as Mars, it might be possible to find remains of photosynthetic prokaryotes as evidences of extinct life. The microarray was able to identify cyanobacterial markers from living or nonliving cells; from population remains and/or extracellular material in old microbial mats (Figure 1); and from water, soil, and rocks collected in extreme environments, such as the Antarctica, Atacama, the Andean lakes, the High Arctic, or the Rio Tinto area in Spain (not shown). Considering that cyanobacteria are the primeval microorganisms on Earth, there is reason to believe that they might once have lived on other planets.

In conclusion, the fact that CYANOCHIP-FSMI can identify *in situ* cyanobacterial markers and can even associate them to different phylotypes or groups, and that the microarray covers a broad range of habitats, including those of plankton, benthos, and endoliths, demonstrates that this technique could a tool for environmental monitoring. Future improvements to the microarray could be to increase the number of antibodies to new strains so that relevant phylogenetic groups still pending are included. Additionally, the chip can be implemented with antibodies to specific cyanobacterial compounds, such as toxins or cyanophicin polymer. This is especially relevant for monitoring fresh water reservoirs, pipes, and installations in human facilities. The current microarray and future versions will be very useful in the field of astrobiology, either for life detection or for monitoring human space facilities (*e.g.,* monitoring water reservoirs or life support systems). In fact, we routinely use the microarray in field campaigns to extreme environments as a method for the “on-site” detection of life remains. The microarray is part of the so-called Life Detector Chip (LDChip), a microarray with more than 300 antibodies for the search for life in planetary exploration missions[29,30]. The microarray alone or as part of the

LDChip will be implemented in the Signs of Life Detector (SOLID) instrument[29] to validate the SOLID-LDChip concept for planetary exploration in multiple field campaigns to terrestrial analogues. The microarray will provide useful information by identifying cyanobacteria and/or their toxins. By determining the strains, it will give information about the environments and the habitats in which they developed.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure1

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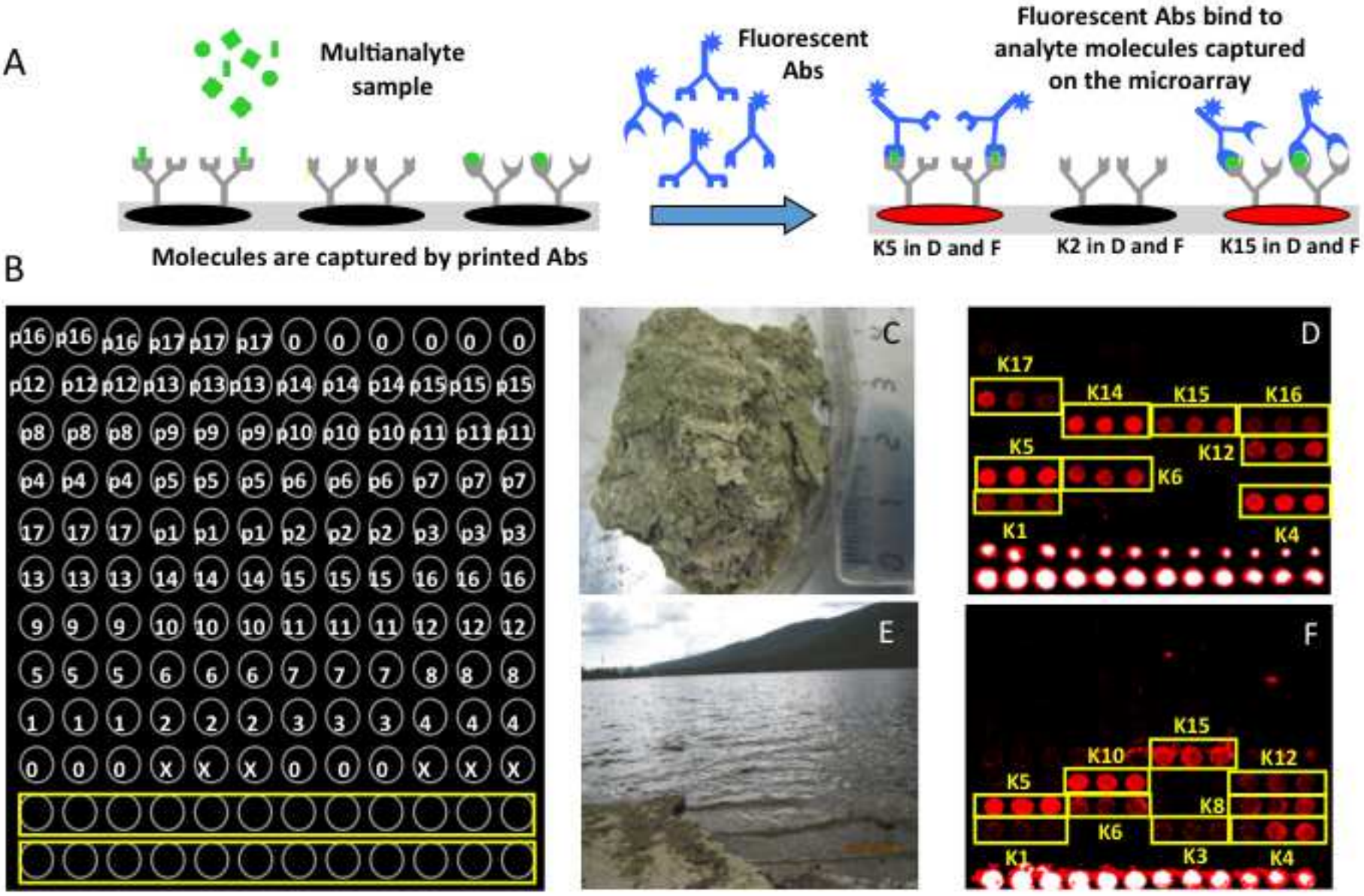

Ab code	Immunogen (strain)	Order	Habitat
K1	*Anabaena* sp.	Nostocales	unknown
K2	*Anabaena* sp.	Nostocales	unknown
K3	*Microcystis flos-aquae*	Chroococcales	planktonic
K4	*Microcystis novacekii*	Chroococcales	planktonic
K5	*Microcystis aeruginosa*	Chroococcales	planktonic
K6	*Aphanizomenon ovalisporum*	Nostocales	planktonic
K7	*Phormidium* sp.	Oscillatoriales	benthic
K8	*Rivularia* sp.	Nostocales	benthic
K9	*Chamaesiphon* sp.	Chroococcales	benthic
K10	*Leptolyngbya boryana*	Oscillatoriales	benthic
K11	*Tolypothrix distorta*	Nostocales	benthic
K12	*Aphanizomenon aphanizomenoides*	Nostocales	planktonic
K13	*Nostoc sp. (Antarctica)*	Nostocales	benthic
K14	*Anabaena* sp.	Nostocales	benthic
K15	*Leptolyngbya* sp.	Oscillatoriales	benthic
K16	*Tolypothrix* sp.	Nostocales	benthic
K17	*Planktothrix rubescens*	Oscillatoriales	planktonic

Medium	Culture conditions
BG11 and nitrate	30 ºC, continuous light
BG11o	30 ºC, continuous light
BG11	28 ºC, continuous light
BG11	28 ºC, continuous light
BG11	28 ºC, continuous light
BG11o	28 ºC, continuous light
BG11	18 ºC, 16-8 photoperiod
CHU-D	18 ºC, 16-8 photoperiod
BG11	18 ºC, 16-8 photoperiod
BG11	18 ºC, 16-8 photoperiod
BG11o	18 ºC, 16-8 photoperiod
BG11o	28 ºC, continuous light
BG11o	13 ºC, 16-8 photoperiod
BG11o	13 ºC, 16-8 photoperiod
BG11	13 ºC, 16-8 photoperiod
BG11	13 ºC, 16-8 photoperiod
none	none

Name of Reagent/ Equipment	Company	Catalog Number
0.22 μm pore diameter filters	Millipore	GSWP04700
Eppendorf 5424R microcentrifuge	Fisher Scientific	
Phosphate buffer saline (PBS) pH 7.4 (10X)	Thermofisher Scientific	70011036
Ultrasonic processor UP50H	Hielscher	
Complete Freund's adjuvant	Sigma-Aldrich	F5881
Incomplete Freud's adjuvant	Sigma-Aldrich	F5506
Protein A antibody purification kit	Sigma-Aldrich	PURE1A
Centrifugal filter devices MWCO<100 KDa	Millipore	UFC510096-96K
Dialysis tubings, benzoylated	Sigma-Aldrich	D7884-10FT
Illustra Microspin G-50 columns	GE-HealthCare	GE27-5330-02
Bradford reagent	Sigma-Aldrich	B6916-500 mL
MicroBCA protein assay kit	Thermo Scientific	23235
Protein arraying buffer 2X	Whatman (Sigma Aldrich)	S00537
Tween 20	Sigma-Aldrich	P9416
Bovine serum albumin (BSA)	Sigma-Aldrich	A9418
384-wells microplate	Genetix	X6004
Robot arrayer for multiple slides	MicroGrid II TAS arrayer from Digilab	
Epoxy substrate glass slides	Arrayit corporation	VEPO25C
Alexa Fluor-647 Succinimidyl-ester	Molecular probes	A20006
DMSO	Sigma-Aldrich	D8418
Heidolph Titramax vibrating platform shaker	Fisher Scientific	
Illustra Microspin G-50 columns	Healthcare	27-5330-01
Safe seal brown 0,5 ml tubes	Sarstedt	72,704,001
Nanodrop 1000 spectrophotometer	Thermo Scientific	

3 µm pore size polycarbonate 47 mm diameter filter	Millipore	TMTP04700
1M Trizma hydrochloride solution pH 8	Sigma-Aldrich	T3038
Sodium chloride	Sigma-Aldrich	S7653
20 µm nylon filters	Millipore	NY2004700
10-12 mm filter holders	Millipore	SX0001300
Protease inhibitor cocktail	Sigma-Aldrich	P8340
1M Trizma hydrochloride solution pH 9	Sigma-Aldrich	T2819
Heidolph Duomax 1030 rocking platform shaker	VWR	
VWR Galaxy miniarray microcentrifuge	VWR	C1403-VWR
Multi-Well microarray hybridization cassette	Arrayit corporation	AHC1X24
GenePix 4100A microarray scanner	Molecular Devices	
GenePix Pro Software	Molecular Devices	

Comments/Description

For preparation of immunogens

For preparation of immunogens

50 mM potassium phosphate, 150 mM NaCl, pH 7.4

For preparation of immunogens

Immunopotentiator

For boost injections

For isolation of IgG

For isolation of IgG

For isolation of IgG

For isolation of IgG

To quantify the antibody concentration

To quantify the antibody concentration

Printing buffer; 30-40% glycerol in 1X PBS with 0.01% Tween 20

Non-ionic detergent

Control for printing; blocking reagent

For antibody printing

For antibody printing

Solid support for antibody printing

Fluorochrome

Fluorochrome dissolvent

For antibody labeling

For purification of labeled antibodies

For labeled antibodies storage

To quantify antibody concentration and labeling efficiency

To concentrate cells

For TBSTRR preparation; to block slides

For TBSTRR preparation

For environmental extract preparation

For environmental extract preparation

For environmental extract storage

To block slides

To block slides; for incubation processes

To dry slides

Cassette for 24 assays per slide

Scanner for fluorescence

Software for image analysis and quantification

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Dear Editor,

Please find attached the second-round report from the manuscript JoVE54994 entitled "Experimental protocol for detecting cyanobacteria in liquid and solid samples with an antibody microarray chip", previously submitted to be considered for the JoVE publication.

We have thoroughly reviewed the manuscript to address editorial and reviewers' comments. We acknowledge the criticism and advice from the reviewers, and we have done the changes to carefully correct and improve all the grammatical errors, typos and oversimplification/generalizations to address his/her main criticisms in order to fully integrate these feedbacks. In addition, we have rewritten several paragraphs of the discussion according to the editorial and reviewer 1's recommendations. Also, we have reduced the highlighted material following the editorial's suggestion.

We honestly think that this video may be of interest for researchers using immunological techniques for environmental or biomedical applications. It contains a complete description of the fluorescent sandwich microarray immunoassay (FSMI) from the microarray printing process to the analysis.

Sincerely

Victor Parro.

Answers to the editorial's comments: Thanks for the exhaustive reading.

1. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Done.

2. Please revise the highlighting of protocol to be 2.75 pages or less. Done.

3. Formatting:
-"in field" is not latin, and does not need to be italicized. Changed.
-Please refer to "section" not "step" when referring to sections separated by bold headings (see 3.1, 4.1). Done.
-Line 480 – Please do not use "excellent"; please use a more objective term. Changed for "high".

4. Length exceeds 2.75 pg of highlighted material and should be reduced accordingly. We suggest eliminating highlighting of sections 1-3 for continuity. We accepted your suggestion. Thank you for your advice.

5. Grammar:
-Please copyedit the manuscript for numerous grammatical errors. Such editing is required prior to acceptance, and some errors are indicated below. This editing should be performed by a native English speaker.
-Line 35 – "it is described an experimental protocol". Changed for "To evaluate the risk of water contamination, this article describes an experimental protocol for in field detection of cyanobacterial strains in liquid and solid samples by an antibody microarray chip".

-Line 39 – “make some aquatic ecosystem to behave”. Changed for “make some aquatic ecosystems behave as true bioreactors”.
-Line 42 - “damages”. Changed for “Many cyanobacterial strains are toxin producers and few cells are necessary to induce irreparable damages to the environment”.
-Line 57 – “of 1 h incubation each one”. Changed for “The whole process takes around 3 h, most of it corresponding to 2 1-hr periods of incubation”.
-Line 67 – “it has been developed a multiplex fluorescent sandwich…”
-Line 70 – “to proportionate tools” Both grammatical errors changed for “a multiplex fluorescent sandwich microarray immunoassay (FSMI) has been developed as a tool to water managers for helping them in…”
-Line 80 - “that frequently makes difficult to distinguish”. Changed for “they require a certain level of expertise considering that it is often very difficult to distinguish between closely related species[2]”.
-Line 87 – “it has been described false positives”. Changed for “false positives have been described”.
-Line 100 – “quantified”. Changed for “quantify”.
-Line 112 – “a higher research”. Changed for “further studies”.
-Line 117 – “hard-working” – not correct use of term. Changed for “very laborious”.
-Line 120 “ssandwich”. Changed for “sandwich”.
-Line 140 – “leads to identify”. Changed for “This microarray allows for the identification of potential…”.
-4.2 – “continuous agitating”. Changed for “continuous agitation”.
-Line 239 – “homogenates” – wrong word. Changed for “homogenizes”.
-6.2.1 – “Weight”. Changed for “Weigh”.
-6.2.2 – “If use a hand-held sonicator”. Changed for “Give at least 5 x 30 sec cycles at 30 kHz stopping 30 sec on ice”.
-7.1.2 – “avoiding touching”. Changed for “trying to avoid”.
-7.5.2 – “Disassembly the cassette”. Changed for “Disassemble the cassette”.
-Line 374 – “proportionates measurements”. Changed for “provides measurements”.
-Please remove all instances of you or your. Done.
-Please rewrite the second paragraph of the discussion so that complete sentences are used rather than a long list of sentence fragments. Done.
-Line 473 – “it is recommended the use of methods”. Changed for “the use of methods to disentangle these cross-reactivity events from the true cognate antigen-antibody reactions by, for example, using deconvolution methods[26,27], is highly desirable”.
-Line 489 – “considering…demonstrate” – incorrect subject/verb pair. Changed for “the fact that CYANOCHIP-FSMI can identify *in situ* cyanobacterial markers, even to associate to different phylotypes or groups, and that CYANOCHIP covers a broad habitats from plankton, benthos and endoliths, demonstrates…”.

6. Additional detail is required: 5.1.1 – What is the composition of the protein printing buffer? Protein printing buffer is a commercial mixture composed of solvents, viscosity enhancers, stabilizers and buffering components to improve sample surface properties during printing. The exact composition is unknown.

7. Branding: 4.4 note, 7.4.1, 8.1 – Alexa 647.
4.1: “Alexa-647” changed for a far-red-fluorescent dye”.
4.3: “Sephadex G-50” changed for “a gel with a fractionation range between 1.5 and 30

kDa trapped into a column".
4.4 note: "Alexa-647" changed for "the fluorescent dye".
7.4.1: The sentence with Alexa-647 changed for "Add 50 µl of an antibody mixture containing the 17 anti-cyanobacterial strain antibodies labeled each of them with the fluorochrome in TBSTRR, 1 % (w/v) BSA".
8.1: The sentence with Alexa-647 changed for "Scan the slide for fluorescence at the maximum emission fluorescence peak for the far-red-fluorescent dye in a scanner for fluorescence".

8. Discussion: Please discuss the significance with respect to alternative methods; please also discuss the limitations of the protocol.

Answers to the reviewer 1's comments: Thanks for the exhaustive reading.

Reviewer #1:

Major Concerns:
Many details in the protocol are missing and should be explained clearly. Otherwise, nobody would be able to repeat successfuly the experiment.
Following information are missing in the protocol:
1. Preparation of immunogens:
The authors have to explain the key point that grown bacteria cells have to be inactivated before immunization of animals. Done. We have added a note at the end of the section 1 to explain the reason why we inactivate cells before animal immunization.
1.1. Information about growth medium is missing. Done. Culture medium and growth conditions for each cyanobacteria are listed in Table 1.
Quantification method for determination of cell concentrations is missing. Done.
1.2 The Quantification method for determination of cell concentrations is missing. Done. These two steps rewritten.

2. Production of polyclonal antibodies: the authors have to explain the screening method to find rabbits that produce the polyclonal antibodies of interest. Which titer should be found in serum? The screening method is normally performed by a specialized company. They supply results of ELISA experiments carried out with each rabbit serum, providing the serum titer. This titer give us information if there is a positive humoral response in the animal. A serum titer between 1:2000 and 1:10000 is recommended by providers to work with.
Each antibody have to be characterized (sensitivity and selectivity) before usage in microarray experiments!. We do not consider that one of the objectives of the present paper is to describe how the sensitivity and selectivity of antibodies is performed. These protocols are highly described in the literature (e.g. Blanco et al., 2015) and they are easy to follow for a new user.

3. Antibody purification: Besides protein concentration the activity of antibodies should be tested. Wrong treatment could reduce the activity of antibodies. Please give an advice. Yes, it is very important to test the activity of antibodies before labeling. We always perform it by ELISA, but we considered that extending current manuscript in the description of the standard immunological methods was out of the scope. Anyhow, we mention it in the new version.

4. ok

5.1.1)The term protein printing buffer have to be explained in detail because this is the main point for microarray production! We use a commercial buffer. See the answer to editorial comments.

5.1.2. ditto. OK.

Note: please give the advice that low binding plates should be used!. Done.

Discussion: It is not the case that the analysis can be performed rapidly and simply in the field. The total analysis from sample incubation to data processing takes at least 4 h and needs a large set of equipment and many manual process steps. Please, write your statements in a more realistic way. Ok. We are agree with your comment. We include this observation in the discussion.
Please explain, why cyanobacteria with the same immunogen pattern should be found on other planets. My opinion is that antibodies are very selective and only the set of antigens used for immunization can be found in a sample. We are agree with you up to a point because we can only study life in other planets as we know it on Earth. Then, we must based our research on terrestrial life. Also, we only need a signal in the CYANOCHIP to detect something related with cyanobacteria markers. We understand a pattern as several signals in the CYANOCHIP that are consistent in different places. However, we only look for single positive signals in space exploration.
Please explain in detail the process for quantitative analysis of cyanobacteria by the CYANOCHIP used for early warning systems. For such tasks, the CYANOCHIP has to be calibrated with standard concentration of cyanobacteria. Working ranges, sensitivity, selectivity and precision has to be determined.
We consider CYANOCHIP a qualitative biosensor for multiple detection and classification of cyanobacteria. We determine the working dilution for each antibody in a titration curve by FSMI. And we also test the sensitivity and specificity for each antibody as described in Blanco *et al*.[22].

The authors have to explain in detail which experiments can be done in future using the CYANOCHIP and which scientific conclusion can be made from such experiments.

The conclusion paragraph has been modified and extended so that it contains the reviewer request.

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Answers to the reviewer 2:

Reviewer #2:
Manuscript Summary:
A microarray for the detection of cyanobacteria was presented. A multiplexed system for the monitoring of at least 17 cyanobaterial strains was developed. A protocol

including sample preparation is given. The total process needs about 3 hours.

Major Concerns:
It is not clear, how the cyanobacteria should be designated unambiguously.
These cyanobacteria are from the most common genera found in European water bodies.
Are the strains obtained from a collection or from natural samples?. All the strains, with the exception of K17, belong to the Antonio Quesada's group from Autonoma University (Madrid, Spain). Antibody against *Planktothrix rubescens* was generated from a natural sample of this cyanobacteria monospecific bloom from Vilasouto reservoir (northern Spain).

Minor Concerns:
Some typos should be removed by careful revision, eg. Fig. "Multitanalyte". Thanks. Done.

Additional Comments to Authors:
N/A