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A new fluorescent oligonucleotide probe for in-situ identification of *Microcystis aeruginosa* in freshwater

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ABSTRACT

Cyanobacteria colonize different environments and blooms can occur in both contaminated and non-contaminated water bodies (freshwater, brackish and marine areas). Among 150 known cyanobacteria genera, > 40 species are able to produce toxins, which are natural compounds that differ from both a chemical and toxicological point of view and are responsible for acute and chronic poisoning in animals and humans. Among the main classes of cyanotoxins, microcystins are frequently found in the environment. Fast and accurate methods for unequivocally identifying microcystin-producing cyanobacteria, such as *Microcystis aeruginosa* in water bodies, are necessary to distinguish them from other non-toxic cyanobacteria and to manage and monitor algal blooms. For this purpose, we designed, developed and validated an oligonucleotide probe for FISH (Fluorescence In Situ Hybridization) analysis to detect *Microcystis aeruginosa* at the species level even at relatively low concentrations in freshwater. The FISH probe, MicAerD03, was designed using the ARB software with the Silva database within the framework of the MicroCoKit project, also with the intention of adding it to the microarray from the EU project, µAQUA, for freshwater pathogens, which had only genus level probes for *Microcystis*.

We tested various fixative methods to minimize the natural autofluorescence from chlorophyll-*a* and certain accessory pigments (viz., phycobilins and carotenoids). The FISH probe was tested on pure cultures of *Microcystis aeruginosa*, and then successfully applied to water samples collected from different sampling points of the Tiber River (Italy), using a laser confocal microscope. Subsequently, the probe was also conjugated at the 5′ end with horse-radish peroxidase (HRP-MicAerD03) to apply the CAtalysed Reported Deposition-FISH (CARD-FISH) for increasing the fluorescence signal of the mono-fluorescently labelled probe and make it possible to detect *M. aeruginosa* using an epifluorescence microscope. Samples taken within the EU MicroCokit project indicated that microarray signals for *Microcystis* were coming from single cells and not colonial cells. We confirmed this with the CARD-FISH protocol used here to validate the microarray signals for *Microcystis* detected at the genus level in MicroCokit.

This paper provides a new early warning tool for investigating *M. aeruginosa* at the species level even at low cell concentrations in surface water, which can be added to the µAqua microarray for all freshwater pathogens to complete the probe hierarchy for *Microcystis aeruginosa*.

1. Introduction

In recent years, the frequency, intensity and geographical distribution of algal blooms in freshwater have been growing worldwide, with the major causes generally recognized as water eutrophication and climate warming [1–3]. A small fraction of overall planktonic species has the capability to produce potent toxins, potentially causing the so-called Harmful Algal Blooms (HABs). These blooms of toxin-producing

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algae can impact environmental and human health. The risk of human exposure comes from contaminated recreational surface waters and from the consumption of unsuitably treated drinking water or ingestion of contaminated food, (e.g. shellfish poisoning) [4,5].

Cyanobacteria are Gram-negative prokaryotic photosynthetic bacteria that can live in a wide variety of terrestrial and aquatic environments [6]. Some cyanobacteria are capable of producing highly toxic secondary metabolites, named cyanotoxins [7]. Containment of cyanobacterial blooms represents one of the major challenges in water safety. Many cyanotoxins are not commonly monitored in water bodies and are thus considered emerging environmental contaminants [8]. Cyanobacterial blooms and cyanotoxin concentrations are known to be regulated by different combinations of environmental parameters, such as light exposure, water temperature and the trophic status of the aquatic system [9]. Being a highly variable biological phenomenon, it is difficult to monitor, prevent and control them and to predict which factor contributes to biomass concentration vs cyanotoxin accumulation. Few attempts have successfully managed to relate these phenomena with specific environmental causes and with toxin production [10,11].

Microcystins are among the most toxic and frequently detected cyanotoxins in water bodies and are mainly produced by *Microcystis*, *Anabaena*, *Planktothrix*, *Anabaenopsis*, and *Aphanizomenon* species [12]. The World Health Organization published a first Drinking-water Guideline [13] where a threshold value of 1 μg/L was fixed for the microcystin-LR because of its high toxicity [14] and several countries use this as a reference value. The most accurate techniques currently applied in environmental analysis for determining trace-level concentrations of microcystins in water samples use liquid chromatography-mass spectrometry (LC-MS) [12]. A common approach for detecting and monitoring the presence of cyanobacteria is to measure the chlorophyll-*a* and/or specific cyanobacterial pigments, such as phycocyanins [7]. Other traditional methods used are the enumeration and identification of cells by microscopy, based exclusively on the determination of morphological features [15]. The latter method not only requires high experience in cyanobacterial identification, but also is not reliable in the case of different species, with similar phenotypic characteristics. Some colonial species, e.g., *Microcystis*, can also revert to single cells making their identification difficult. The use of Fluorescent In-Situ Hybridization (FISH) and in particular of the catalyzed reporter deposition (CARD)-FISH method has been successfully applied for labelling akinetes of the *Nostocales* species (cyanobacteria), such as *Aphanizomenon ovalisporum*, *Anabaena planctonica* and *Cylindrospermopsis raciborskii* [16].

Except in the case of an algal bloom, in which the cyanobacteria are visible, it is very difficult to detect them in water samples because they occur in low concentrations in freshwaters compared to other bacterial species. Knowledge of cyanobacterial ecology is still quite scarce, as well as is the availability of robust and accurate cyanotoxin and cyanobacteria monitoring procedures. Because there is currently no ideal way of eradicating algal blooms, the use of preventive measures becomes essential, including an early detection of cyanotoxin-producing species even at low concentrations, using rapid and accurate methods, such as molecular methods. Because it is difficult to detect toxic from non-toxic strains of cyanobacteria, any early warning system can point to the toxic potential of any water body, until sufficient biomass is accumulated to allow toxin detection.

Microcystis aeruginosa is a freshwater cyanobacterium occurring worldwide except in Antarctica [17] and its blooms can occur in both natural (lakes and rivers) and artificial water (drinking water reservoirs) under specific conditions and when water temperatures exceed 15 °C [18]. *Microcystis* are coccoid-shaped cells with a diameter ranging between 1 and 9 μm, which can exhibit a variety of colonial morphologies consisting of dense aggregations of cells under specific environmental light, temperature and nutrient loading conditions [10,19–21]. When grown under laboratory conditions, they often revert to single cells (G. Codd, pers. comm.). They are unable to fix nitrogen and for their growth they require an external nitrogen source. The availability of the latter plays a role in bloom regulation [7,9,22].

The combination of high nutrient loads and a high temperature can promote the formation of toxic cyanobacterial blooms in freshwater bodies [21,23]. The strong increase in algal bloom phenomena in the last few years can be attributed to anthropogenic pollution and climate change [24].

Cyanotoxin concentrations are largely dependent on cyanobacterial concentrations and, consequently, knowing the abundance of potentially toxic species, such as *M. aeruginosa*, is a starting point for establishing alert levels and performing risk assessments before, or even without, any toxin analysis [25]. For this reason, we report the design and validation with pure cultures of a16S rRNA-targeted oligonucleotide fluorescent probe that can detect and be used to quantify *M. aeruginosa* at the species level. The ARB software and its corresponding rRNA databases were used for the probe design. The probe was applied using both the FISH and CARD-FISH methods, in both pure cultures and environmental samples. FISH combines the precision of molecular genetics with direct visual information from microscopy, allowing simultaneous visualization, identification, enumeration and localization of individual microbial cells within their natural microhabitat. Because whole cells are hybridized, errors arising from biases in DNA/RNA extraction, PCR amplification and cloning are avoided [26,27].

The optimized FISH and CARD-FISH protocols developed in the EU MicroCokit project were applied for detecting *M. aeruginosa* in both pure cultures and surface waters, as an indicator for the toxin potential presence in any water body for early warning systems. Cell counts from this probe were used to validate the genus probe signals from the μAqua microarray that was used in the EU MicroCokit project for the detection of freshwater pathogens in the Tiber River, Italy. The protocols for CARD-FISH optimized here enabled this probe to be used alone for monitoring purposes where needed.

2. Materials and methods

2.1. Oligonucletide probe design

Probe design for fluorescent in situ hybridisation was performed for the selected cyanobacterium *M. aeruginosa*. Based on the structure and the alignment of 16S and 23S rDNA gene sequences from different cyanobacterial species belonging to the genus *Microcystis* (EMBL database), we used the ARB software (http://www.arb-home.de) [28] with Silva (from Latin *silva*, forest) database (http://www.arb-silva.de) to design RNA-targeted probes at genus and at species level (Table 1). High quality and comprehensive rRNA databases comprising sequences for *Bacteria*, *Archaea* and *Eukarya* have been compiled from GenBank (https://www.ncbi.nlm.nih.gov/genbank) and released to form the public alignment, SILVA database, since 2007.

A genus probe, henceforth called “GNMICSO5” (5′-GCGTGAGGGA GGAAGGTCTTT-3′) was covalently labelled at the 5′ end with the isothiocyanate derivative (Cy3) for FISH identification of species belonging to *Microcystis*. The probe was shortened from 28 nt for the best performing genus level probe on the μAqua microarray to 22 nt to facilitate FISH hybridization with the newly designed species level probe. The species level probe “MicAerD03” (5′-CTTGATCAACGCCACTTCC

Table 1
RNA oligonucleotide probes designed for *Microcystis* (genus level) and *Microcystis aeruginosa* (species level).

Probe name	Species	Gene	Sequences 5′-3′-fluorochrome
GNMICSO5	*Microcystis*	16S rRNA	GCGTGAGGGAGGAAGGTCTTT-Cy3
MicAerD03	*Microcystis aeruginosa*	23S rRNA	CTTGATCAACGCCACTTCCCTC-FITC

CTC-3′) was covalently labelled at the 5′ end with the FITC (FAM) for FISH identification of *M. aeruginosa*. Probes were synthesized by Metabion International AG (Planneg, Germany).

2.2. Pure cultures cultivation

Experiments were carried out with selected strains of *M. aeruginosa* obtained from the algal culture collection of the Veterinary College at Complutense University (Madrid, Spain). Details on the isolation procedures and culture methods are described by Carrillo et al. [29]. Cells were grown axenically in cell-culture flasks with 20 mL of BG-11 medium (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), at 20 °C and a photon irradiance of 60 μmol/m^2/s over the wavelengths 400–700 nm, in a 16:8 L:D photoperiod. Cells were maintained in mid-log exponential growth by serial cell transfers to fresh medium. Prior to the experiments, the cultured cells were re-cloned (by isolating a single cell) to assure genetic homogeneity in all the cultures.

2.3. Optimization of hybridization conditions and probe specificity in FISH

The FISH method was adjusted to label *M. aeruginosa* and, during the fixation step, bleaching chlorophyll that interferes with probe fluorescence. The method/protocol for the probe application comprised five sequential steps: (1) sample filtration, (2) fixation, (3) bleaching chlorophyll, (4) hybridization and (5) visualization under a laser confocal microscopy.

(1) Filtration: 150 μL of laboratory culture strain of *M. aeruginosa* was mixed with 800 μL PBS (1×10^6 cell/mL), vortexed and filtered onto white polycarbonate membrane filters (pore size 0.2 μm) applying a gentle vacuum.

(2) Fixation and (3) bleaching of pigments: different fixation methods were tested in order to minimize the autofluorescence caused by the presence of chlorophyll in the cyanobacterial cells. We used a Saline-EtOH fixative solution, prepared fresh for every experiment, composed of 100% ethanol: bi-distilled water: 25× SET buffer (25:2:3) (v/v), (25× SET buffer: 3.75 M NaCl, 25 mM EDTA, 0.5 M Tris–HCl pH 7.8) [30]. Saline-EtOH fixation was applied for two different times (1 h and overnight) with and without 1 h of 50% dimethylformamide (DMF) treatment [31] to remove the chlorophyll autofluorescence. The optimal fixation method was the simultaneous use of the one-hour Saline-EtOH fixative solution and one-hour of 50% of DMF [32]. 10 mL of Saline-EtOH fixative solution was aliquoted directly into the filter funnel of our filtration equipment under sterile cabinet at room temperature for 1 h; afterwards we applied a gentle vacuum. When all of the Saline-EtOH solution was removed, 10 mL of Washing Buffer (5× SET buffer +0.1% v/v Nonidet) were added directly into the filter funnel of the filtration equipment for 5 min; afterwards we applied a gentle vacuum. When all the Washing Buffer was removed, we added 5 mL of 50% DMF directly into the filter funnel for 1 h; also applying a gentle vacuum. Finally, we added 10 mL of Washing Buffer (5× SET buffer + 0.1% v/v Nonidet) directly into the filter funnel for 5 min under a gentle vacuum. The filters were stored at −20 °C in the dark.

(4) Hybridization for FISH: Each filter was cut into about 16 sections and two filter sections were hybridized with the same probe. No probe controls were also hybridized. The hybridization parameters considered were temperature and stringency (formamide concentration). Two formamide concentrations (15% and 20%, respectively) with increasing temperatures (45 °C, 47 °C, 49 °C and 51 °C) were tested to optimise the FISH method. The best hybridisation conditions were 20% formamide and a temperature of 49 °C.

M. aeruginosa pure cultures were hybridized with each probe. Aliquots of hybridization buffer (5× SET, 0.1% (v/v) Nonidet-P40, 20% (v/v) formamide) with a 50 ng/μL FITC labelled probe, and DAPI (4′,6-diamidino-2-phenylindole) solution (10% v/v) were aliquoted onto each filter piece, previously placed on a slide. After hybridization, the filter sectors were washed twice in buffer solution (1× SET) at 49 °C for 10 min and air-dried.

(5) The filter sections were mounted with ProLong® Gold Antifade Mountant (Thermo Fisher, liquid mounting applied directly to fluorescently labelled cell) on a glass for the examination and (5) counting under a laser confocal microscope (LEICA SP-2 AOBS Laser Confocal Microscope) at the Complutense University Microscopy Service, Madrid. The theory for using a genus level probe with one fluorescent signal and a species level probe with a different label is that the two probes could be used in a hierarchical fashion. The membrane could be scanned with the Cy3 filter for microscope searching for signals from the genus level probe and when positives were encountered switch to the FITC filter to confirm the identification of the species or vice versa. In this manner both generic and species level counts could be made. *E. coli* OP50 was the negative control and pure cultures of *Microcystis aeruginosa*, the positive controls.

The Cy3-labelled probes were excited using an Ar laser in a range of 340–380 nm through a band-pass (BP) filter and the fluorescence recovered through 425 LP. The FITC-labelled probes were excited using a He—Ne laser emitting at 590 nm and the fluorescence was recovered using a 515–560 BP filter. When the FITC- and Cy3-labelled probe was used in the same sample, sequential image acquisition was used. Lasersharp and Laserpix softwares (Bio-Rad) were used to analyze the images. Cells binding cyanobacterial species probes were reported as percentage of the total DAPI-positive cells. The hybridization efficiency of the oligonucleotide probes was estimated by monitoring the fluorescence intensity of pure cultures of the target cyanobacteria.

2.4. River water sampling

Water samples were collected from the Tiber River, the main river in central and peninsular Italy. The four selected sampling points were: T1: pristine area, (Monte Fumaiolo, 43°47′11″N, 12°04′50″E) at the source of the river. T2: agriculturally polluted area, (Attigliano, 42°30′30″N, 12°16′59″E). T3: industrial and urban polluted area, where Aniene River flows into the Tiber River in Rome city (41°56′22″N, 12°30′26″E) and in proximity of the Rome East wastewater treatment plant (WWTP). T4: urban polluted area, (Fiumicino 41°48′15″N, 12°14′50″E), downstream the Southern WWTP of Rome and close to the river mouth (Fig. 1). These sites were selected in the EU MicroCokit project so that the entire river could be sampled from a variety of pollution sites to analyze the total microbial community using various molecular tools. We applied two different microbiological methods: the μAQUA microarray (designed in a previous work [32]) and the FISH method for identifying *M. aeruginosa*.

Water samples were collected at each of the four sampling points at the water surface in two seasons (Spring and Autumn) for two years (2014 and 2015) for the microarray analysis. For the microarray, 50 L of water were concentrated into a one litre sample using a hollow fibre and aliquots of the concentrate were used for RNA extraction [32]. Total RNA was analysed using the μAQUA microarray and these results were presented elsewhere [32]. The samplings for the analysis of *Microcystis aeruginosa* here reported were performed in whole water samples, collected during the second year (Spring 2015 and Autumn 2015) and pre-filtered in situ through a 0.5 mm steel sieve to remove any coarse particles. The FISH probes were applied only to 2015 samplings, because of the time required for designing and testing them in lab. Water samples (3 bottles of 1 L each for each sampling point) for direct epifluorescence microscope methods (total abundance, cell viability and *M. aeruginosa* by FISH and CARD-FISH) were collected using sterile flasks from each sampling point and were kept at 4 °C during transport to the lab and processed immediately.

For inorganic and ion metals, samples were collected in polyethylene bottles (3 replicates) previously washed with HNO_3 (pH < 2) for at least 24 h and then washed with MilliQ water until a neutral pH was reached.

The physico-chemical parameters such as temperature, pH, redox

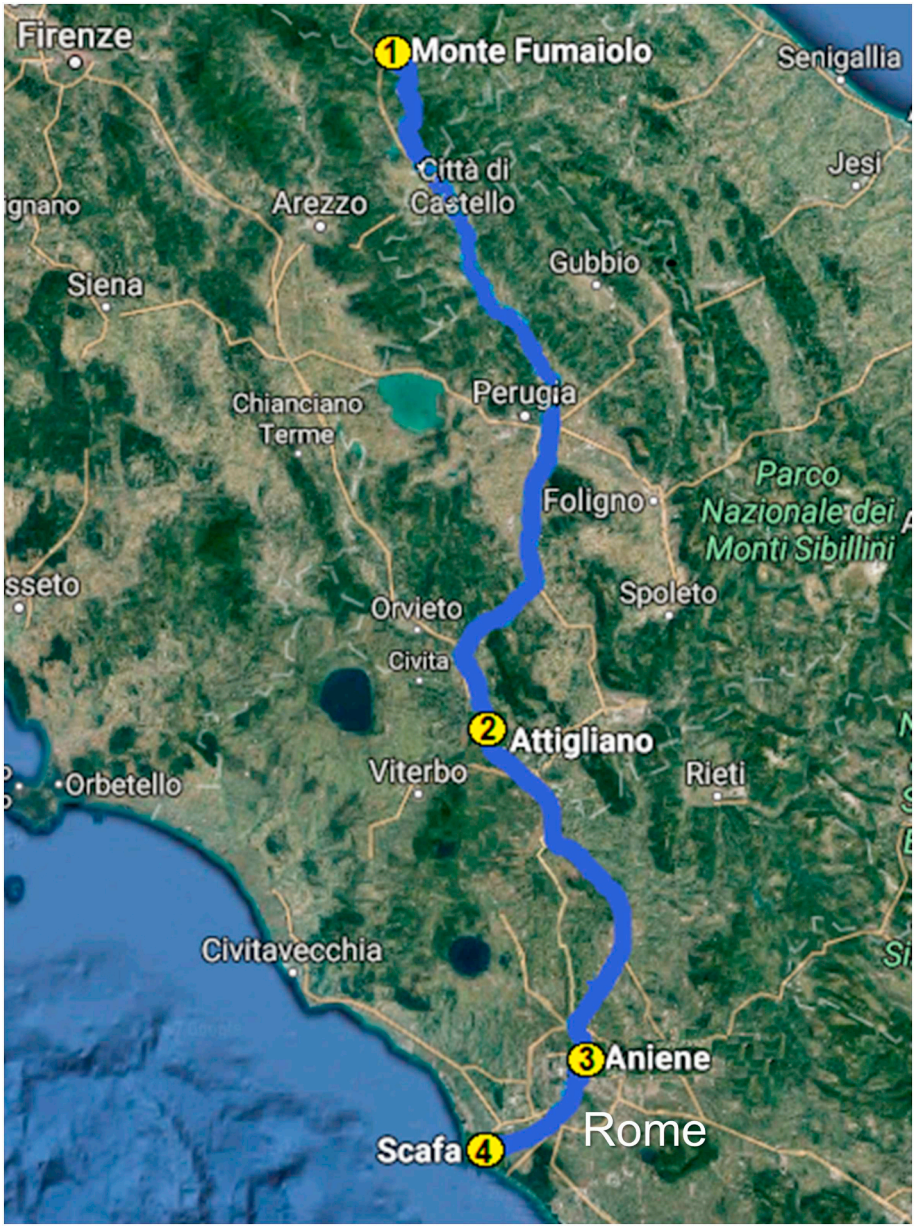


Fig. 1. Map of the Tiber River showing the water sampling sites. T1: river source; T2: Attigliano, agricultural area; T3: urban area where the Aniene River joins the Tiber River; T4: Scafa, river mouth.

potential (mV), conductivity (μS/cm), and dissolved oxygen (mg/L) were measured during sampling (on-site) using a Hydrolab DSS multi-parameter water quality probe (Ott Hydromet; Kempten, Germany). Water samples for inorganic ions and metal analysis were collected using acid-washed polyethylene bottles.

2.5. Application of the FISH method to freshwater samples

For each sampling point (T1, T2, T3, T4), water aliquots of 50 or 100 mL each (3 replicates) were filtered onto polycarbonate 0.2 μm GTTP Millipore filters and fixed for 1 h with Saline-ETOH solution and subsequently treated for one-hour with 50% dimethylformamide. Each filter was cut into about 16 sections and two filter sections were hybridized with the same FITC- or Cy3- labelled probes for *Microcystis* and then analysed using a Confocal laser microscopy. Negative controls were used to assess potential non-specific binding to non-target reference bacteria. Cell autofluorescence was also determined using no probe controls. The results are reported as the percentage of DAPI positive cells that hybridized with the fluorescent probe multiplied for the total microbial abundance.

2.6. Total microbial abundance and cell viability

Water samples were analysed by epifluorescence direct methods for assessing microbiological parameters, such as total microbial abundance and cell viability. Water samples for microbial abundance were fixed with formaldehyde (2% final concentration) and volumes ranging from 0.5 to 3 mL were filtered through a 0.2 μm polycarbonate membrane (Merck Millipore, 25 mm diameter) with a gentle vacuum (< 0.2 bar). The volume of filtered water was adjusted for each sample in order to obtain enough cells for a representative enumeration. Filters for evaluating microbial abundance by the total direct count (cells/mL) were treated using DAPI as the nucleic acid intercalant [33]. DAPI binds principally to dsDNA, therefore all microbial cells were stained, regardless their physiological state and metabolic activity. Cells were visualized and enumerated with a fluorescence microscope (Leica DM

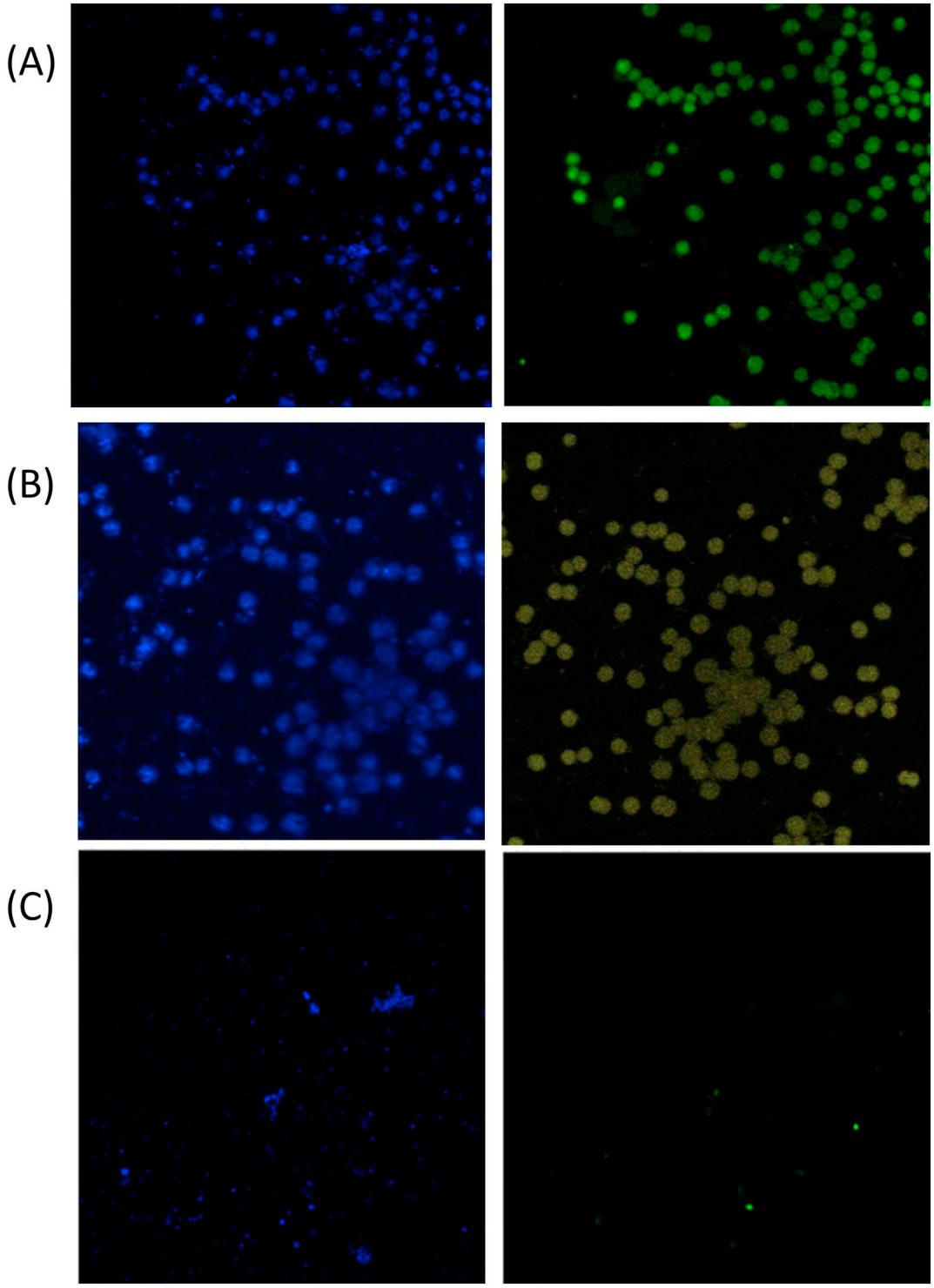


Fig. 2. Images of FISH assays under the laser confocal microscope using the designed FITC (FAM) MicAerD03 probe for identifying *Microcystis aeruginosa*. A. Pure culture: DAPI-stained cells (blue cells on the left), *M. aeruginosa* cells hybridized with the probe (on the right, green); B. DAPI-stained cells (blue), *M. aeruginosa* without probe (yellow autofluorescence); C. application of the probe to an environmental sample of the Tiber River (Autumn): DAPI-stained cells (on the left), *Microcystis aeruginosa* cells found in the T1 Tiber River sampling point (on the right).

4000B, Leica Microsystems GmbH, Wetzlar, Germany). A minimum of 300 cells were counted for each replicate in at least 30 random fields selected on each filter.

The percentage of live cells (% live cells/live + dead) was measured in non-fixed samples filtered through a 0.2 μm polycarbonate membrane. Filtered volumes were adjusted as described above. Two fluorescent dyes, SYBR Green II and propidium iodide (Sigma-Aldrich, Germany), were used to distinguish between viable (green) and dead (red) cells [34]. In a similar way to the DAPI counts, a minimum of 300 cells were counted for each replicate in at least 30 random fields selected on each filter. Each microbiological datum was the average of six values for each sampling point (3 samples, each in duplicate).

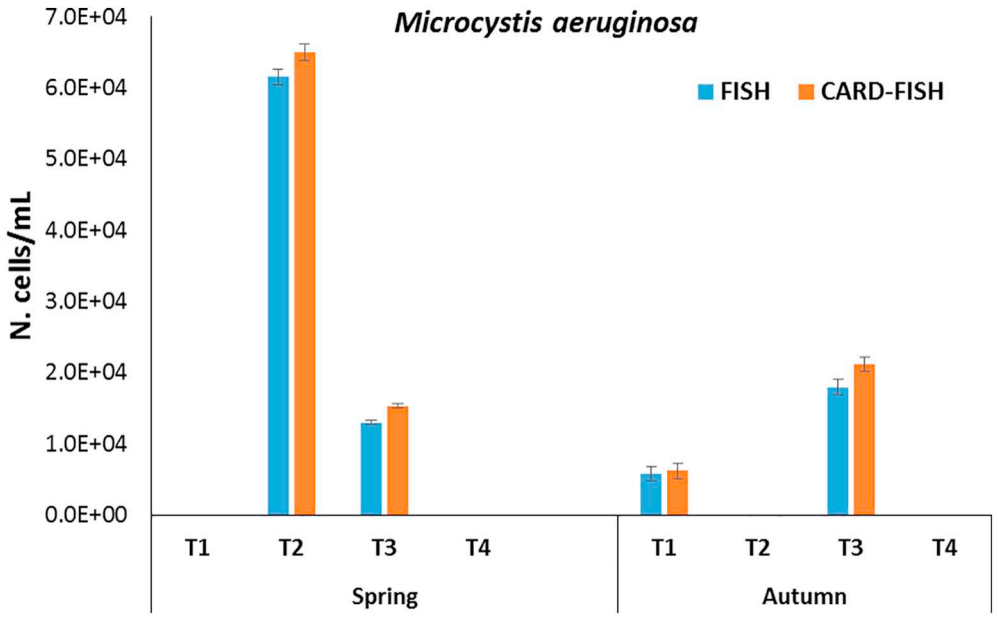


Fig. 3. Number of *M. aeruginosa* cells detected by the MicAerD03 probe in river sampling in Spring and Autumn using both regular FISH and CARD-FISH. The values are means of six analyses. The vertical bars are the standard errors.

2.7. Inorganic ions and metals

Analyses of inorganic ions and metals were performed in accordance with the Italian Official Guideline and APHA, AWWA, and WEF methods [35,36].

To ensure that the chemicals to analyze remain stable and unaltered during the period between sampling and analysis, samples were filtered immediately by gentle vacuum through 0.45 μm polycarbonate filters. Inorganic anions (fluoride, chloride, nitrite, nitrate and sulphate) were determined by ion chromatography using a Dionex DX-120 Ion Chromatograph.

A pre-acidification step (HNO_3 1%) was performed for metal and cation analyses. Metals (barium, antimony, arsenic, cadmium, total chromium, copper, lead, mercury, nickel, selenium, vanadium, iron, zinc, manganese, aluminium, lithium, caesium, uranium, cobalt, strontium) were analysed by a Inductively coupled plasma-mass spectrometry (ICP-MS), (Agilent technologies 7500c) with Octopole Reaction System (ORS). Major cations (calcium, magnesium, sodium, potassium) were determined by an Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) using a Perkin Elmer P400 spectrometer.

These parameters were selected to provide a comprehensive water quality characterisation at each site to compare with the microbial community analysis.

2.8. Application of the HRP-MicAerD03 designed probe for CARD-FISH

The probe MicAerD03, designed for *M. aeruginosa*, was also conjugated at the 5′ end with horse-radish peroxidase (HRP-MicAerD03) to apply the CARD-FISH method (also known as tyramide signal amplification or TSA) to both pure cultures and environmental samples. CARD-FISH increases the signal of mono-fluorescently labelled probes > 10-fold and for this reason can be very useful to highlight better a specific hybridization signal and identify bacteria independent of their rRNA content [37]. The CARD-FISH method was applied to the same filters obtained as described in the 2.3 paragraph (steps 1, 2 and 3) following published methods [38]. Pre-warmed 0.2% low melting point ultrapure agarose was pipetted on each filter section (previously cut) for avoiding cell loss. The sections were incubated in a lysozyme solution (20 mg/mL lysozyme) for 60 min at 37 °C and then in a Proteinase K solution (1 mg/mL) for 30 min at 37 °C. Finally, all sections were put (10 min, room temperature) in a 0.01 M HCl solution to inactivate endogenous peroxidases.

The hybridization step was achieved by immersing the sections on a glass slide overnight (46 °C) in a hybridization buffer, containing 20% formamide and the MicAerD03 HRP-labelled probe (50 ng/μL) in humidified 50 mL tubes. Subsequently, a washing step was performed by dipping the entire slide into 50 ml of a washing solution (NaCl 5 M, Tris/HCl 1 M, EDTA 0.25 M, SDS 10%). Finally, the signal amplification using the fluorescein-labelled tyramide (1 mg/mL) was performed in accordance with Lupini et al. [38].

2.9. Statistical analysis

Between groups, Principal Component Analysis (PCA) was performed on the correlation matrix using Log (x + 1) transformed data of *M. aeruginosa* presence, physicochemical parameters (pH, O_2, T°C, NO_2, NO_3, SO_4, F, Ca, Mg, K, B) and metal concentrations, using the program Past version 3.11 [39].

3. Results

The oligonucleotide probes designed for FISH were first tested with a pure culture of *M. aeruginosa*; other cyanobacterial species were tested at the University of Madrid [30]. A positive signal was obtained from the MicAerD03 species probe with the FITC fluorochrome. The GNMICSO5 genus probe labelled with Cy3 showed a signal indistinguishable from the red autofluorescence of the cells seen under the Cy3 filter and for that reason it was never used as originally planned in contrast to the green signal from the FITC species level probe that was easily distinguished from yellow autofluorescence of the cells. The application of the MicAerD03 species probe with a FITC label to a pure culture (Fig. 2A) showed a positive hybridization (100%) with a well-defined signal, distinguishable from cyanobacterial autofluorescence (Fig. 2B).

Cells positive to the MicAerD03 probe were also found in the 2015

Table 2
Total microbial abundance, cell viability, pH, Oxygen and temperature found at T1, T2, T3 and T4 points in the Spring and Autumn samplings of Tiber River.

	Live cells/live + dead	Total microbial abundance	pH	O_2	T
	(%)	No. cells/mL		mg/L	°C
Spring					
T1	77	$1.70 \cdot 10^5$	8	10	7
T2	69	$7.33 \cdot 10^5$	8	11	13
T3	91	$6.21 \cdot 10^5$	7	7	15
T4	58	$4.80 \cdot 10^5$	7	8	14
Autumn					
T1	78	$1.43 \cdot 10^5$	7	8.5	9.9
T2	88	$8.20 \cdot 10^5$	7	8.2	20.4
T3	76	$2.76 \cdot 10^6$	7	4.6	18.4
T4	81	$2.23 \cdot 10^6$	7	4.3	18.9

river water samples at both Spring and Autumn samplings considered in this study. Single cells of *Microcystis aeruginosa* were found at T2 (8.4% vs DAPI) and T3 (2.09%) in Spring and at T1 (4.05%) and T3 (0.65%) in Autumn. The percentages of positive probes multiplied by the total microbial number (obtained by DAPI counts) at each sampling point are reported in Fig. 3. Cells positive to the MicAerD03 probe were never found at T4. An image of the positive hybridization at the T3 sampling site (Autumn) is reported in Fig. 2C. The values of total microbial abundance obtained by DAPI counts are shown in Table 2. Although the overall abundance of the microbial community was lower in Spring than in Autumn, cell viability was comparable and in some cases (e.g. T3) higher in the Spring than in the Autumn sampling (Table 2). The physico-chemical parameters measured during samplings and the inorganic ions and metals analysed in the lab are reported in Tables 2, 3 and 4.

The PCA analysis performed with the physico-chemical data (Tables 3 and 4) and with presence/absence data of *M. aeruginosa* (Fig. 4) was able to represent successfully the variability in the data. The first two components captured 91.9% of the total variance. The first component representing most of the inorganic ions and metal concentrations accounted for 80.7% of the variation in the samples, whereas the second one, representing *M. aeruginosa*, Na and Cl accounted for 11.2%.

The four sampling sites (T1, T2, T3, T4) were displayed separately in the four quadrants of the PCA biplot. In the upper quadrants, *M. aeruginosa* vector was associated with T1 Autumn, T2 Spring and T3 Spring and Autumn. Most of the physico-chemical parameters measured (T°C, NO_2, NO_3, SO_4, F, Ca, Mg, K, B) and the metal concentration vectors were associated with sites T3 and T4 in the right quadrants of the biplot. In the lower right quadrant, Na^+ and Cl^- ions were associated with site T4, in the opposite direction than *M. aeruginosa*.

The HRP-MicAerD03 probe designed for CARD-FISH was tested on the same pure culture of *M. aeruginosa* and on river samples to verify if the probe signal intensity could substantially increase. This can be particularly useful for visualising environmental samples using an epifluorescence microscope instead of a laser confocal one. The images of the pure cultures and of some environmental samples obtained by the CARD-FISH method and using the epifluorescence microscope (Leica Digital Microscope 4000 B) are reported in Fig. 5(A, B and C). As expected, CARD-FISH made it possible to obtain comparable values (Fig. 3) and with a higher fluorescent signal than that obtained using FISH (Fig. 5).

4. Discussion

The FISH MicAerD03 probe was successfully applied to both pure cultures of *M. aeruginosa* and river water samples using a laser confocal microscope and is ready to be added to the μAqua microarray to complete its coverage of freshwater pathogens. The overall data obtained from the water samples suggest that *M. aeruginosa* is a naturally occurring species in the Tiber River, even at a low temperature of 10 °C. *M. aeruginosa* was found in relatively low concentrations (ranging from $5.78 \cdot 10^3$ cells/mL at T1 in Autumn to $6.16 \cdot 10^4$ cells/mL at T2 in Spring) if compared to the total river microbial abundance (T1: $1.43 \cdot 10^5$ cells/mL and T2: $7.33 \cdot 10^5$ cells/mL). The presence of this cyanobacterium in this river has been shown in previous studies. In fact, microarray signals were obtained at the genus level for *Microcystis* at all three polluted sites (2014 sampling) with higher values in Spring and lower in Autumn ([36], Supplemental Fig. 1). In 2015, lower signals were obtained but still at all three sites, with highest values in the Autumn. Because the microarray detects RNA, this suggests that in Autumn, cyanobacteria were less active than those in the Spring in 2014. The genus level signals at T4 from the microarray analysis were likely from a different species because no signals were observed with the CARD-FISH probe.

A comparison of FISH and microarray signals revealed that in no case there was a FISH signal obtained where a microarray signal was not [32]. However, in few sites, a microarray signal was obtained and a FISH signal was not. This can likely be attributed to the great difference in water collected for the two analyses (50 L vs. 50–100 mL).

The fact that algal blooms of *Microcystis* have never been reported in this river may be ascribable to the relatively low nutrient level found (Table 3) and to the fact that the cells could be flushed from the ecosystem with high rainfall, which usually occurs in Spring and Autumn. Seasonal *Microcystis* algal blooms in rivers can occur with a high nutrient level (in particular of N) and with cell abundance several-fold higher than 10^5 cells/mL [22]. In rivers, cells can float downstream and become trapped in pools and along channel margins where they can accumulate biomass as mats and cause toxic events. Nutrient availability is reported to be the major factor controlling the proliferation of *Microcystis* [10,40], but temperature and light can also influence population dynamics [21]. The highest abundance of *M. aeruginosa* found

Table 3
Inorganic ions (mg/L) found at T1, T2, T3 and T4 points in the Spring and Autumn samplings of Tiber River.

	NO_2	NO_3	Cl	SO_4	F	Ca	Mg	Na	K	B
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Spring										
T1	0	2.1	16.8	7.9	0.1	52.3	2.7	4.9	0.4	0.018
T2	0.02	5.2	28.1	71.2	0.4	77.1	17.2	24.1	11.7	0.107
T3	0.06	8.8	104	245	0.6	183.8	42.8	51.2	13.3	0.654
T4	0.08	9.1	352	181	0.6	123.9	49.4	282.3	15.4	0.285
Autumn										
T1	0.01	2.7	16.1	7.1	0.1	48.6	2.5	4.8	0.6	0.012
T2	0.03	3.5	31.3	62.9	0.4	71.3	17.5	27.2	9.8	0.095
T3	0.05	10.4	52.6	165	0.6	178.3	35.9	48.3	13.9	0.542
T4	0.09	12.0	514	331	0.8	167.6	110.6	772.5	38.6	0.506

Table 4
Main metals (μg/L) found at T1, T2, T3 and T4 points in the Spring and Autumn samplings of Tiber River.

Spring	Ba μg/L	Sb μg/L	As μg/L	Cd μg/L	Cr μg/L	Cu μg/L	Pb μg/L	Hg μg/L	Ni μg/L	Se μg/L	V μg/L	Fe μg/L	Zn μg/L	Mn μg/L	Al μg/L	Autumn	Ba μg/L	Sb μg/L	As μg/L	Cd μg/L
T1	23.4	< 0.1	< 0.1	< 0.1	0.8	0.2	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	6.1	0.4	0.2	5.1	1.2	< 0.1	< 0.1	< 0.1	338
T2	92.5	0.16	2.9	< 0.1	0.3	0.5	0.2	< 0.1	1.3	0.3	2.7	7.5	5.5	0.3	1.6	10.3	0.2	1.9	0.3	861
T3	42.3	0.21	62.6	< 0.1	0.4	0.4	0.2	< 0.1	1.2	0.3	5.1	13.2	2.4	0.2	1.4	31.2	6.6	1.9	0.9	2352
T4	59.2	0.24	8.1	< 0.1	0.4	1.7	0.3	< 0.1	1.3	0.3	2.7	7.8	2.1	0.4	1.5	19.8	0.6	1.5	0.6	1323

Autumn	Ba μg/L	Sb μg/L	As μg/L	Cd μg/L	Cr μg/L	Cu μg/L	Pb μg/L	Hg μg/L	Ni μg/L	Se μg/L	V μg/L	Fe μg/L	Zn μg/L	Mn μg/L	Al μg/L	Li μg/L	Cs μg/L	U μg/L	Co μg/L	Sr μg/L
T1	21.2	< 0.1	< 0.1	< 0.1	1.1	0.3	< 0.1	< 0.1	< 0.1	< 0.1	0	7.8	0.3	0	3	1.1	< 0.1	< 0.1	< 0.1	286
T2	98.4	< 0.1	2.7	< 0.1	0.3	0.9	0.3	< 0.1	1.2	0.2	3	8.1	0.6	0	2	10	0.1	1.8	0.3	805
T3	43.5	< 0.1	56.2	< 0.1	0.4	1.5	0.2	< 0.1	1.6	0.3	6	13	29	1	2	31	4.3	2.4	0.9	1885
T4	65.4	< 0.1	8.7	< 0.1	0.7	0.3	0.3	< 0.1	0.6	0.3	4	22	0.7	0	1	38	0.5	1.7	0.3	2096

in T2 in the Spring may be associated with a higher day length during this season; at the same time, its absence in T1 may be caused by a very low temperature (7 °C) or low levels of nutrients. Although high abundances for cyanobacteria are usually found in the summer samples [41], these are usually reported from lakes and ponds where biomass accumulates as opposed to rivers where biomass is continually flushed downstream. Thus, a significant signal as shown here in the Spring samples is likely related to nutrient availability because high rainfall occurs at this time and underscores the ability of this cyanobacterial species to reach potential bloom proportions in a river. Because cells are rapidly transported downstream, the impact of *Microcystis* can be transported to the entire watershed, even impacting marine organisms. *Microcystis* has also been reported from several estuaries along the Iberian Peninsula (see references in [42] and microcystins have also been reported in marine organisms.

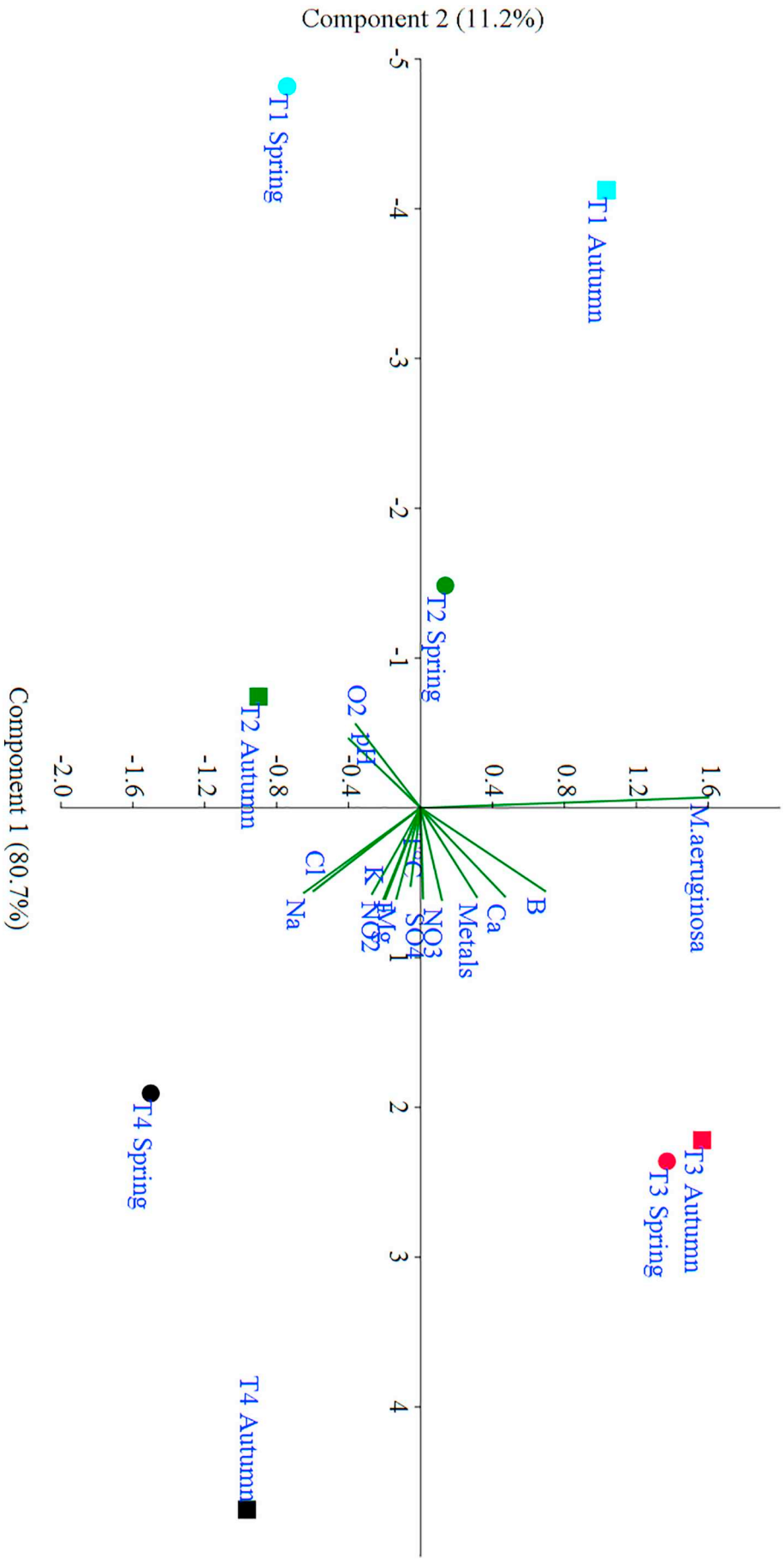
M. aeruginosa was not found at T4, at the river mouth. This may be because of the higher NaCl concentration at this point caused by saline intrusion from the sea. This was confirmed by the PCA analysis showing that Na and Cl were associated with T4, the opposite to the abundance of *Microcystis* at T2 and T3. Although many cyanobacterial species can survive salinities up to 16‰ [43], *Microcystis* is usually not one of them [44]. Genus level probes for *Microcystis* were detected at T4 [32], but this may be another species.

The HRP-MicAerD03 probe was tested on pure cultures of *M. aeruginosa* and some river water samples. The CARD FISH method had comparable results in terms of cell numbers obtained in all sampling points, but the hybridization positive signal was significantly stronger than that of classic FISH using the same epifluorescence microscope. Because the latter is an instrument commonly present in laboratory facilities, the use of the HRP-MicAerD03 probe for identifying *M. aeruginosa* with CARD-FISH can be proposed as a useful routine identification method for a single species. The probe is also ready for incorporation into the μAqua microarray for a total pathogenic community analysis [32]. A combination of the μAqua microarray and CYANO RT-array developed in the same EU project to detect cyanobacterial toxins [43] will offer the best molecular monitoring for cyanobacterial blooms. The species array will detect the species long before any visible signs of a bloom are recorded by monitoring agencies and the toxin array will detect the expression of toxin genes before they are produced in sufficient quantity to be detected by chemical analyses. If only a single species needs to be monitored, then the CARD-FISH protocol tested here offers the cheapest and faster solution for detection of a single species.

5. Conclusions

It is of critical importance to water monitoring authorities to detect toxic cyanobacterial bloom rapidly. This study makes available new fluorescent oligonucleotide probes (MicAerD03 and HRP-MicAerD03), to be applied using molecular and direct methods, such as FISH and CARD-FISH for detecting one of the most commonly occurring harmful cyanobacteria, *M. aeruginosa*. Moreover, this probe can be added to the μAqua microarray for a total pathogenic community analysis. This paper both contributes to the ecological knowledge of this species especially in riverine ecosystems and provides a new early warning tool for investigating *M. aeruginosa* even at low cell concentrations in surface water before substantial biomass is accumulated to facilitate toxin detection. It therefore has practical implications for the proper management of fresh water bodies. This probe can now be added to the suite of probes in the μAqua microarray, which was missing a species level probe for *Microcystis aeruginosa* to analyze the total freshwater pathogenic community, including cyanobacteria, bacteria and protozoa.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2019.05.017.



(caption on next page)

Fig. 4. Principal Component Analysis biplot of Log + 1 transformed data of *Microcystis aeruginosa* presence, physicochemical parameters and concentrations of metals in the four points of the Tiber River, in the Autumn (squares) and Spring (dots) campaigns.

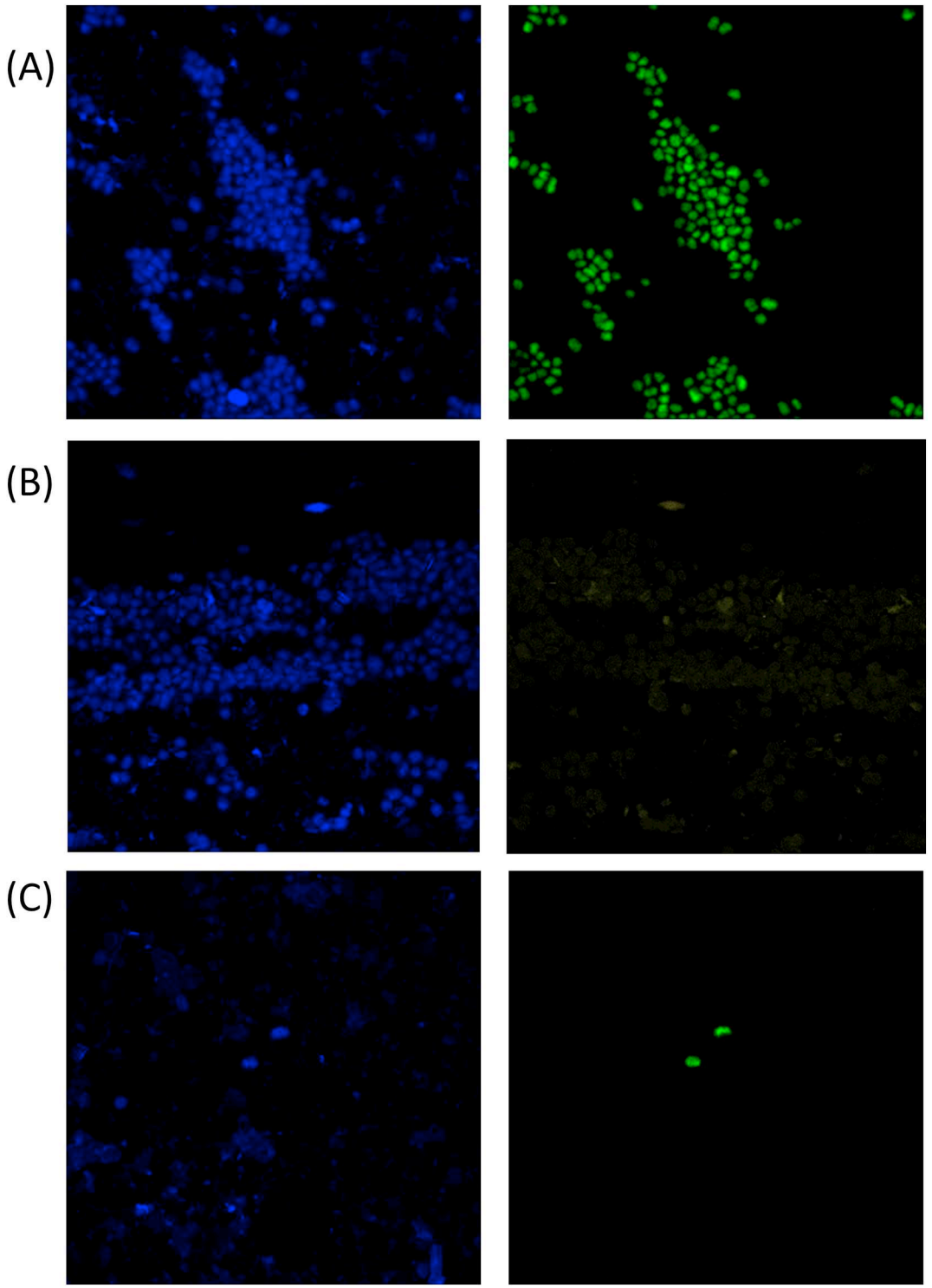


Fig. 5. Images of CARD-FISH assays under a epifluorescence microscope using the designed HRP-MicAerD03 probe for identifying *Microcystis aeruginosa*. A. Pure culture: DAPI-stained cells (blue, on the left); *M. aeruginosa* cells hybridized with the probe (green, on the right); B. DAPI-stained cells (blue, on the left), cells without probe (yellow, on the right); C. Application of the probe to an environmental sample of the Tiber River (Autumn): DAPI-stained cells (blue, on the left), *Microcystis aeruginosa* found in the T1 Tiber River sampling point (green, on the right).

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