THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE

Coulibaly Kalpy J et al, The *Experiment, 2014, Vol.26 (1)1775-1787*

DETECTION OF POTENTIALLY TOXIC MICROCYSTISAND CYANOBACTERIA BY MOLECULAR METHOD IN CÔTE D'IVOIRE

ABSTRACT

The cyanobacteria or blue-green algae are natural inhabitants of water bodies. The eutrophication due to anthropogenic pressure favors the massive development of strains of cyanobacteria, some of which are potentially toxic. The main goal of this work was to demonstrate for the first time in Ivory Coast the presence of toxicity gene of cyanobacteria from cyanobacteria culture. Water used by the population for their different needed were sampled in different cities of the country. Several genera known for their ability to produce toxic were highlighted including Microcystis, anabeana, oscillatoria, plancktothrix.

Oscillatoria was the most isolated genus with 93% of presence in the samples. The pcr has allowed us to confirm the existence of the gene mcy encoding microcystin in Microcystis.

PCR identified the mcygène of microcystis in all the sample (100%) while microcystis was found only 86% by microscopy. The contribution of chromatographic and ELISA techniques will ultimately confirm their toxin production or not.

Key words Cyanobacteria, microcystin, mcy; microcystis

INTRODUCTION

Cyanobacteria are cosmopolitan and former inhabitants of surface waters, freshwater and marine but also terrestrial environment [Whitton and Potts, 2000]. The quality of surface water is a major issue for the future of our planet. So, access to drinking water for the population was identified to be one important part of the Millennium Development Goals. However, the eutrophication of water bodies could compromise this objective. These phenomena are mainly related to human activities (urban, industrial and agricultural development) and favor the frequent occurrence of cyanobacteria blooms, which is a problem known worldwide. Today, more than 40% of lakes and surface waters are considered eutrophic and prone to the proliferation of algae and / or cyanobacteria [Briand et al, 2003].Africa is not to be outdone of this natural phenomenon disturbing [Okelloet al, 2010; Krienitzet al, 2012]. This problem is even more serious than the species responsible for these blooms can produce water-soluble substances that are potentially toxic [Carmichael et al, 1994] [Oudraet al, 2002] [Teet al, 2011]. Toxic cyanobacteria blooms occur at different places in the world at an average frequency of 59% [Sivonen and Jones, 1999], and those related to Microcystisaeruginosa are the most commonly reported [Carmichael et al, 1994]. Cyanobacteria produce different types of toxins including microcytins. These are hepatotoxins whose production is mediated by a cluster of 10 genes mcy different, from mcyA to mcyJ [Tillettet al, 2001; Teet al, 2010].

By inhibition of the phosphatase PP2A and PPA, hepatotoxins "microcystins" are currently considered as promoters of cancerous tumors in the liver by ingestion and chronic exposure to sublethal doses [Tilletet al, 2001]. Thus, their presence in water bodies used for the production of drinking water, livestock watering and recreation, is a potential hazard and a serious threat to the aquatic biota, human and animal health [Carmichael and Falconer, 1993; Falconer et al, 1999; Vasconceloset al, 2001;Bittencourt-Oliveira et al, 2011]. In addition, they are not removed effectively by conventional processing techniques drinking water [Rositano and Nicholson, 1994].
Accidental poisoning of livestock due to contamination of surface waters by toxins synthesized by cyanobacteria is known for over a century. It is the same for dogs and other animals [Merveet al, 2012; Lurlinget al, 2013; Backer et al, 2013]. In 1996, in Brazil, 76 patients in a dialysis unit died due to direct exposure to high concentrations of microcystins, due to the use of contaminated water by cyanotoxin[Pouriaet al, 2002; Bittencourt-Oliveira et al, 2011].

Therefore, the presence of toxin producing cyanobacteria in water bodies intended for use by men is considered an emerging public health problem [Manigliaet al, 2010, Chorus I and Bartram J].

Cyanobacteria are morphologically very different bacteria. They are typically differentiated on phenotypic base (morphology and physiology). This phenotypic differentiation does not differentiate between toxic and non-toxic strains of the same species. Also, the

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limitations of this approach have led it to develop molecular methods for the identification and monitoring of cyanobacterial strains especially those capable of producing toxins [Glowackaet al, 2011]. Indeed, the molecular approach has facilitated the identification and detection of environmental microorganisms and or those difficult to grow. In addition, the molecular method as cyanobacteria identification tool no requires axenic culture [Glowackaet al, 2011]).

Microcystin production depends on mcy gene possession or not by the bacterium. Any cyanobacterium harboring this gene is supposed to produce this toxin. In Côte d'Ivoire, few studies have been conducted on cyanobacteria and those relating to toxic cyanobacteria are almost nonexistent. The presence of potentially toxic cyanobacteria was also reported on various bodies of water [Ouattaraet al, 2001; Ngohesseet al, 2007; Komoéet al, 2010; Humbertet al, 2012; Grogaet al, 2012; Adonet al , 2011].

Some of these waterbodies are used for production of drinking water or likely to be. Given the health risks that could represent the presence of cyanobacteria toxins, particularly microcystin in those waters for the consumer, this study was undertaken with the objectives of highlighting the presence of potentially toxic cyanobacteria, by detecting the presence of cyanobacteria andmcygene in cultures using molecular method.

MATERIALS AND METHODS

Measurement and sampling campaigns

Our various water samples were collected in the lagoon Aghien,Kan's water pond in Bouaké, the Comoé River, and the BandamaRiver (Figure 1). The lagoon has an area of 19.5 km ² and is located about 5 km north of Bingerville in Comoé River watershed. This hypertrophic water plan [Ngohesseet al, 2007] is the venue for the development of various kinds of cyanobacteria such as Microcystissp, Anabeanasp, Oscillatoriasp, Cylindrospermopsissp, and many other species [Ngohesseet al, 2007; Humbertet al, 2011].

Water pond of Kan is located at the southern entrance of the city of Bouaké, the second largest city in Côte d'Ivoire in terms of population. This restraint and Loka's one are used for the production of drinking water. Restraint has a catchment area of 18.5 km^2 with an average annual contribution 2,900 10^6 m^3 of water. It is located in the watershed of the River Bandama.

Rivers Bandama and Comoé are two of the four largest rivers in Côte d'Ivoire. They cross towns within 150 km to the east and west of the city of Abidjan.

A sampling plan was prepared according the selected points from the expert report on the phytoplankton of the lagoon Aghien [Humbert et al, 2011]; the expert report on the microbiological quality of rivers Bandama and Comoé [IPCI a, 2012], and the expert report on the phytoplankton population of the retaining Kan [IPCI b, 2012]. The simplest access points with a high number of genera were selected (Figures 2 and 3). Bandama's shore at the village M'Brimbo (banana plantation) and the bridge over the Comoé to Alépé were identified.

Sampling techniques

All those waters were sampled without any efflorescence. Cyanobacteria and phytoplankton in general were collected by plankton nets of 20 µm in mesh size. Using a bucket of 5 liter capacity, 20 liters of water was taken and filtered through the plankton net. The pellet retained at the collector was transferred to two tubes Falcon 50 ml each corresponding to different sampling points. A tube was attached to lugol 1% v / v for microscopic identification and other non-fixed tube was used for cultivation of cyanobacteria for molecular analyzes.

Identification andinventory of the types of cyanobacteriain presence

All samples were fixed and analyzed to identify the types of cyanobacteria present. The identification was made by observation between slide and cover slip at light microscope (x400) according to the morphological keys of cyanobacteria described by Komarek and Anagnostidis (1998, 2005) and John et al (2003). Quality control of the media was achieved by sterile distilled water as a control medium seeding. The quality control of Media used have made by the seeding of distilled sterile water.

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Culture of cyanobacteria

A quantity of 10 ml of unfixed pellet was cultured in glass flask of 250 ml capacity containing 100 ml of BG11 medium. Incubation took place under controlled temperature (25 ° C + / - 1), at continuous light. The incubator is constituted by a polyethylene enclosure (55 cm x 40 cm x 37 cm). It is surmounted by a ramp with two bulbs of 18 watts producing 1350 lumens of light and measuring 60 cm each. The incubator had a total light intensity of 50,766 lux.

After 21 days growth, 2 mL of each sample were taken and used for molecular analysis [Tillettet al, 2001].

Molecular analysis

The DNA extraction was performed according to Boom's method modified from the centrifugation pellet of Cyanobacteria culture. To do this, an isolation kit (Mobio Power water® DNA isolation Kit (#14900-50-NF, Mobio laboratories)) was used.

PCR (Polymerase Chain Reaction) was used for the diagnosis of cyanobacteria including the identification of the presence of cyanobacteria (16S rRNA) [Neilanet al, 1999], phycocyanin [Neilanet al, 1995] and research mcyA gene [Tillettet al, 2001] and mcyB gene [Nonnemanet al, 2002] of microcystin, a cyanotoxin.

The different amplification reactions by conventional PCR targeting the 16S rRNA and the genes encoding microcystin and phycocyanin have generate respectively PCR products of 1.1 Kb, 1.3 Kb and 685 bp. The reaction were performed in a final volume of 50 µl containing 50 mMKCl, 10 mMTris-HCl pH 8.3; 1.5 mM MgCl2; 0.2 mM of each deoxynucleotide triphosphate(dNTPs); 0.4 µM of each oligonucleotide (sense primer and antisense), 1 U of Taq DNA polymerase (Promega) and DNA (<0.5 µg / 50 µl)

The amplification was carried out in a the rmocyclerGeneAmp 9700 (Applied Biosystems) with hybridization temperatures ranging between 56 ° C and 62 ° C (Table 1).

For research of mcyB gene size 320 bp coding for microcystin, preparing the reaction mixture contained: a Roche isolation kit (water, buffer 10X (5µl per sample), MgCl2 (25 mM) 3 µl per sample, the enzyme TaqDNA 0.3µl samples by polymerase), dNTPs (10 mM) 0.5 µl per sample, the primers MCY-R1 and MCY -F1 (10µM) 1µl of each primer per sample, and 5µl of the DNA extract.

The DNA on Synechococcus strain (PCC 7502) of the Pasteur Institute of Paris cyanobacteria collection was used as positive control.

The amplification reaction was conducted according to the following program: initial denaturation at 94 ° C for 1 min, full denaturation at 94 ° C for 30 seconds, annealing at 57 ° C for 45 seconds, extension at 72 ° C for 1 min, 35 cycles in total, and finally the final elongation at 72°C for 5 min. The revelation of the amplified products was performed after electrophoresis of 1.5% agarose gel containing ethidium bromide. A molecular weight marker (100 bpSmartLadderEurogentec) was used to assess the size of the amplified products.

RESULTS AND DISCUSSION

Four waterbodies were sampled (Aghien Lagoon, Pond of Kan, Bandamariver and Comoé river). A total of 15 samples were collected at the rate of six (6) samples in lagoon Aghien, seven (07) in pond of Kan, one (01) for the Bandamariver and one (01) in the Comoé river.
Inventory of cyanobacteria isolated

Based on microscopic analyzes, a total of 15 genera of cyanobacteria has been identified (Table 2). Were isolated from 15 genera of cyanobacteria present, nine potentially toxic genera in which six (06) capable of producing microcystins [Coudertet al, 2014; Mbukwaet al, 2012]. There were mainly the genera Oscillatoria present in 93% samples, Microcystis (86%), Anabeana (66%) and Planktothrix (60%). The genus Oscillatoria was the only kind common to all waterbodies sampled.

In the lagoon Aghien, it was identified 8 genera of cyanobacteria including 7 potentially toxic and 5 capable of secreting microcystins.

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According to the sampling stations, the isolated genera vary between 3 and 8. The genera Microcystis, Anabeana, Planktothrix were absent in sampling stations 1, 2 and 3. In pond of Kan at Bouaké, 6 genera at least, and 11 differents cyanobacteria genera maximum were observed according to sampling stations. Among them, 8 potentially toxic genera were isolated which 6 can secrete microcystins in which Anabeana, Microcystis, Planktothrix.

The river Comoé contained only two genera of cyanobacteria. All of them were potentially toxic and may produce microcystin. There were not any major microcystin producing genera of cyanobacteria in the river comoe. However Bandamariver was the area of isolation of seven different genera of cyanobacteria including 5 potentially toxic. This river did not contain bacteria of the genus Anabeana.

Molecular analysis:

Several genes have been investigated: the gene targeting 16S RNA and PC-IGS gene of phycocyanin to confirm the presence of cyanobacteria in samples mcyA and mcyB genes to search potential toxic Microcystis.

All samples examined showed the presence of cyanobacteria. PCR products of a size range between 1100 and 1500 bp corresponding to the 16S RNA from cyanobacteria were observed in all samples analyzed. The PC IGS gene of phycocyanin with amplification products size between 680 bp and 710 was also detected in the sample except from that of Comoériver (Figure 3).
They were also found in Samples the genes mcyA and mcyB coding for microcystin (Figure 4 and 5).

DISCUSSION

This study was undertaken with the aim of making an inventory of the genera of potentially toxic cyanobacteria in water bodies of Côte d'Ivoire using microscopic techniques for phenotypic identification and the search of mcy toxicity genes by molecular technique.

After microscopic analysis, all samples contained cyanobacteria. 15 different genera of cyanobacteria have been identified. The genera identified have also been reported in previous studies on Ivorian waterbodies [Grogaet al (2012); Sallaet al (2012)]. However, no study on a single waterbody has not shown a great diversity of genera. Indeed, Grogaet al (2012), who worked on the site of Lake Taabo and Sallaet al (2011), who worked on two rivers, the Boubo and Mé rivers did not found many bacterials genera.

The studies conducted on one site always showed a smaller number of cyanobacteria's genera, such as the study of Adonet al (2011), which identified only eight (8) different genera of cyanobacteria at Adzopé.

The presence of the genera Microcystis and Anabeana one hand and on the other hand Planktothrix in the lagoon Aghien confirmed the respective previous results of Ngohesseet al (2007) and Humbertet al, (2012).

However, it is noted that if the genera of isolated cyanobacteria are qualitatively different depending on the studies and sites, some are consistently found in all studies including Aphanocapsa, Microcystis, Anabeana. The number of genera of potentially toxic cyanobacteria varies according to the authors. He was found nine genera of potentially toxic cyanobacteria in this study in which six potentially secreting microscystines. This number is higher than the toxic genera identified in previous studies in Côte d'Ivoire. Thus Grogaet al (2012) found five potentially toxic genera when Adonet al (2011), and Sallaet al (2012) identified respectively 4 and 7 genera. These genera were virtually identical and dominated by Microcystis, Anabeana that are found in all studies [Sallaet al, Ouattaraet al, Grogaet al, Adonet al .....].

The genus Planktothrix highlighted in this study was not found in previous studies as well as the genus Oscillatoria found in over 90% of the samples of this study. So it would appear that the toxic genera like Microcystis and Anabeana would be found in most waterbodies studied in Côte d'Ivoire unlike to Oscillatoria and Planktothrix less found.

The simultaneous presence of Microcystis and Anabeana in waterbodies has been found in Asia including Singapore [TE et al, 2011] and Australia [Baketet al, 2001].

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In this study, molecular analyzes confirmed the presence of cyanobacteria in all samples. Indeed, the DNA 16S and PC-IGS gene were amplified. However, as noted Manigliaet al, (2010) amplification of the PC-IGS gene allowed certainly to note the possible presence of cyanobacterial strains in polybactérien product but do not differentiate between toxic and non-toxic cyanobacteria. This gene, however, could help the molecular identification of many types of cyanobacteria [Manigliaet al, 2010]. The absence of amplification of the PC-IGS gene in the Comoé river sample while the 16S rDNA was amplified could be allocated according to Baker et al, (2001), to a problem of the detection limit.

Indeed, the author believes that the PCR remains negative for a lower number of cells to a ratio of 1: 10 in the solution [Baker et al, 2001]. The PC-IGS gene is adapted to the molecular characterization of cyanobacteria because it allows the use of molecular tools such as sequencing or RFLP to identify cyanobacteria. This is due to the highly constant of subunits α and β of the phycocyaninintergenic spaces when variables subunits allow differentiation of genera and species of cyanobacteria especially the samples non monospecific [Manigliaet al, 2010; Baker et al, 2001; Neilanet al, 1995].

The Microscopic techniques do not allow to differentiate between toxic and non-toxic cyanobacterial strains [Teet al, 2011], the present study investigated the presence of genes encoding the secretion of microcystin. All the PCR analyzes have identified the gene mcyA and mcyB coding for microcystin secretion. This result suggests a high probability of the presence of microcystin in Ivorian waters. There is a strong correlation between the presence of mcyA and mcyB and secretion of microcystin by cyanobacterial strains harboring these genes. [Ouahidet al, 2005].

The primers used to detect genes mcyA and mcyB are oriented to detect strains toxic of Microcystis [Tillettet al, 2001; Manigliaet al, 2010]. This study chose to investigate firstly the genus Microcystis because it is the most common microcystin producing cyanobacterium in the world [Rantalaet al, 2006; Okelloet al, 2010; Mbukwaet al, 2012].

Indeed, several types of cyanobacteria are potentially secreting microcystins including Microcystis, Anabeana, Planktothrix. Previous studies have led to the development of the primer pairs for the direct detection of microcystin producing cyanobacteria but also highlight the genera that are responsible of that. [Manigliaet al, 2010; Glowakaet al, 2011]. For the detection of Anabeana, Planktothrix and Microcystis strains, potentially microcystin-producing, Hisbergueet al (2003) have developed a pair of primers seeking a fragment of mcyA different of those of Tillettet al (2001) and Rantalaet al (2004), a pair of primers seeking mcyE. However, the presence of mcy genes is a good indicator of the presence of potentially toxic cyanobacteria of the genus Microcystis. Neilanet al., 1999; Baker et al, 2001, 2002; Tillettet al, 2001; Kurmayeret al, 2002; Pan et al, 2002; Bittencourt-Oliveira et al, 2003; Hisbergueset al, 2003; Mikalsenet al, 2003; Vaitomaaet al, 2003).

The results of the research of gene toxicity in selected water bodies are not always stackable to microscopics observations. Molecular biology has indeed shown that all samples have amplified specific mcyA segment of Microcystis and also mcyB, while 24% of the samples did not contain Microcystis by microscopy. This fact already mentioned by Glowakaet al, (2011) indicates the possibility of detecting microorganisms by molecular techniques when microscopy remains negative.

Baker et al, (2001) think that the positivity of a molecular test when microscopy analysis did not give satisfactory response to the research of toxic Microcystis strains is due to the low concentration of these strains in the medium (Table 3).

This work presents a number of limits. Indeed, the identification of toxicity genes mcyA and mcyB does not certify toxin production but the ability to express it. The presence of gene mcy in strains not secreting toxin by modification of the latter has been reported in previous studies [Bittencourtet al, 2009; Glowackaet al, 2011]. A dosage of microcystins by ELISA or by chromatography is necessary to confirm the production of microcystin in Ivorian waters as pointed Bittencourtet al, (2009).

The using of specific primers for microcystin producing Microcystis, cannot discriminate the case of the genera Anabeana, Planktothrix and other cyanobacteria observed in this study. However, it has the merit of highlighting a possible risk to people because in most studies, the presence of mcy gene is synonymous with production of microcystins [Kurmayer and Kutzenberger, 2003 Via-Ordorikaet al, 2004; Dittmann and Börner, 2005]. To this risk, it must be incorporated the cases where the amount of toxin is below the detection limit, makes them undetectable. [Bittencourtet al, 2009]

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CONCLUSION

This study highlights the presence of potentially toxic cyanobacteria in large bodies of water in Côte d'Ivoire. The molecular technique, including PCR, revealed the presence of genes mcyA and mcyB specific of Microcystis. It must be complemented by the determination of microcystin, but also by the study and research of other kinds of microcystin-producing cyanobacteria and possibly other toxins. This importan knowledge could help sanitariesauthorities to build cyanobacteria monitoring programme for the water bodies used by the population in Cote d'Ivoire

ACKNOWLEDGEMENTS

- Pasteur Institut of Côte d'Ivoire for the financial assistance and the provision of its equipment.
- The National Office of Potable Water (ONEP), which permitted access to his sites particularly to Mrs KONE and Dr CLAON
- Thanks Also to SODECI for its frank and sincere cooperation
- This work may not be possible without the members of Unit of Chemistry and Environmental Microbiology of Pasteur Institute of Côte d'Ivoire, all my thanks to them.

Gene region and primer	Sequence 5'-3'	Tm (°C)
mcyANMT		
MSF	ATCCAGCAGTTGAGCAAGC	59
MSR	TGCAGATAACTCCGCAGTTG	60
Phycocyanin		
PCβF	GGCTGCTTGTTTACGCGACA	62
PCαR	CCAGTACCACCAGCAACTAA	60
16S rDNA		
27F1	AGAGTTTGATCCTGGCTCAG	57
1494Rc	TACGGCTACCTTGTTACGAC	56
mcyB		
MCY F1	TGGGAAGATGTTCTTCAGGTATCCA	57
MCY R1	AGAGTGGAAACAATATGATAAGCTAC	57

Table 1: Primers used in this study

	AghienLagoon						**Pond of Kan Bouaké**							**Bandama**	**Comoé**
	Ag1	Ag2	Ag3	Ag4	Ag5	Ag6	K1	K2	K3	K4	K5	K6	K7	Ban	Co
Aphanocapsa	+	+	+	+			+		+	+	+	+	+		+
Microcystis	+		+	+	+	+	+	+	+	+	+	+	+	+	

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Anabeana	+		+	+		+	+	+		+	+	+	+		
Gleocapsa							+		+			+		+	
Oscillatoria	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Planktothrix	+	+		+			+	+	+		+	+		+	
Pseudoanabeana	+	+	+	+	+		+	+	+		+		+		
Merismopedia									+	+		+		+	
Lyngbya							+	+	+	+	+	+	+		
Synecocystis														+	
Woronichinia														+	
Aphanizomenon	+			+											
Limnothrix	+	+													
Chroococcus									+	+	+	+	+		
Anabeanopsis										+					

+: presence: -: Absence

Table 2: List of Cyanobacteria by sampling station

	AghienLagoon						Pond of Kan Bouaké							Bandama	Comoé
	Ag1	Ag2	Ag3	Ag4	Ag5	Ag6	K1	K2	K3	K4	K5	K6	K7	Ban	Co
Cyanobacteria	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Microcystis	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
mcyA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
mcyB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ADNr 16S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CPC-IGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

+: presence: -: Absence

Table 3: Correlation between Microscopic and molecular result

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Figure 1: Sampling sites

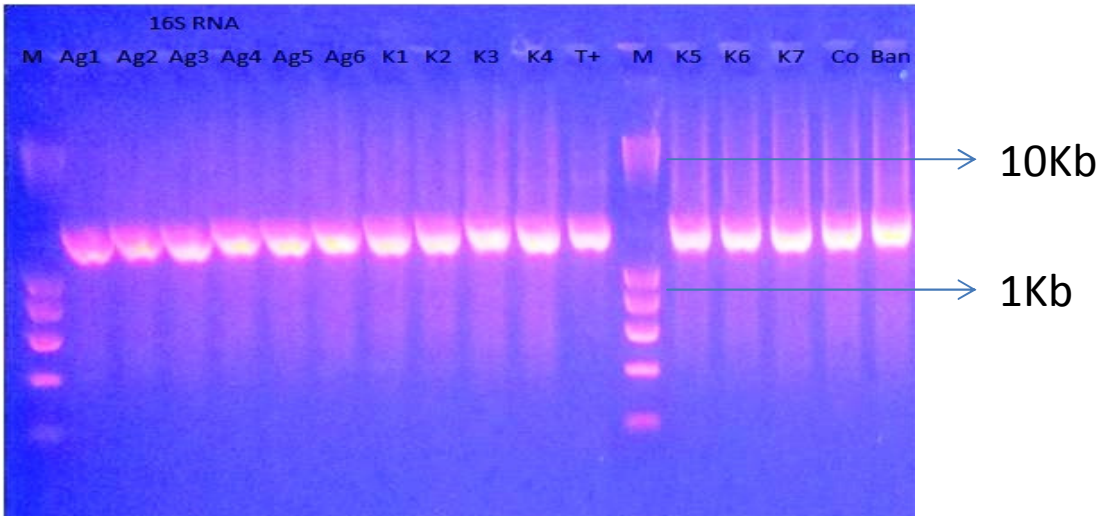

Figure 2: Revelation of the amplification products of the 16S RNA gene of cyanobacteria in agarose gel 1.5%; The expected band is between of 1100 to 1500 bp; lane 12: positive control (strain PCC7502), lane 1 and 13: molecular weight marker: SmartLadder 10000pb (Eurogentec), lane 2-7: Aghien lagoon samples, lane 8 to11 and 14 to 16: pond of Kan samples, Track 17: River Comoé sample, track 18: Rivers Bandama samples

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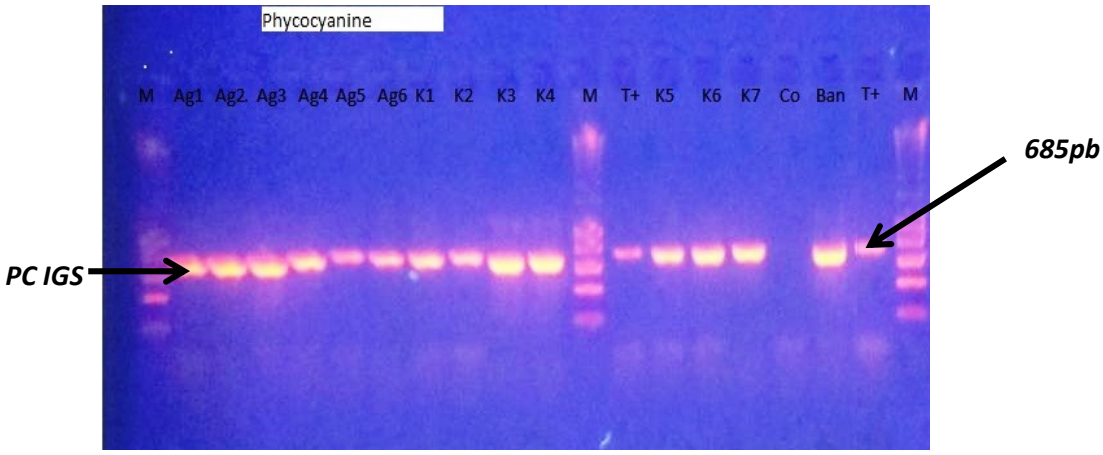

Figure 3: Revelation of the amplification products of the PC-IGS gene for the phycoyanines in agarose gel at 1.5%; The expected band is between 680 and 710 bp. Lane 13 and 19: positive control (strain PCC7502); Lane 1, 12 and 20: Molecular weight marker: SmartLadder 10000 bp (Eurogentec); Lane 2-7 samples Lagoon Aghien, Lane 8-11 and 14-16 samples of pond Kan, Lane 17: River Comoé sample, Lane 18 River Bandama sample

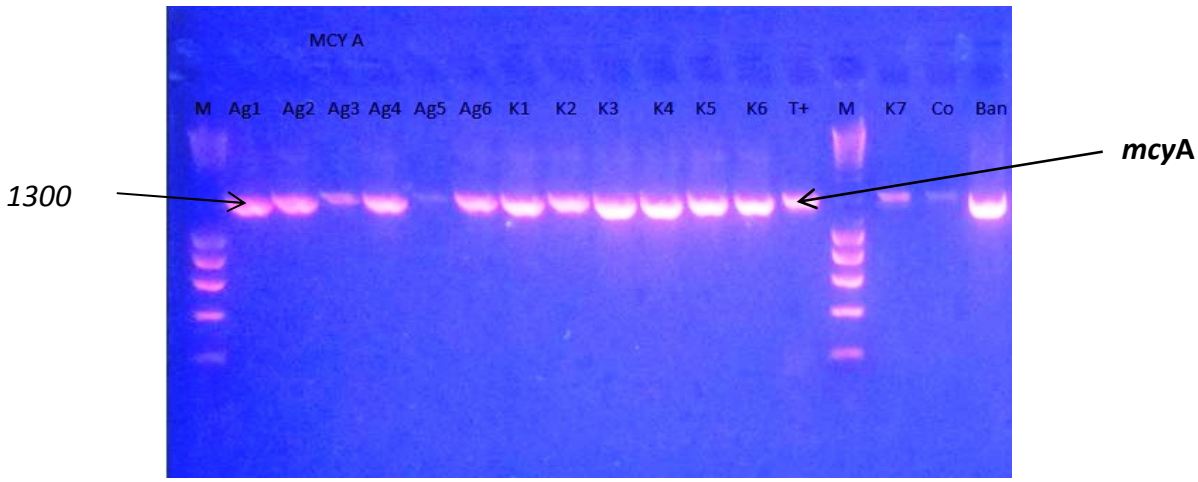

Figure 4: Revelation the amplification products mcyA gene on agarose gel 1.5%; the expected band is 1300 bp. Lane 14: Positive control (strain PCC7502), Lanes 1 and 15: Molecular weight marker: SmartLadder 10000 bp (Eurogentec); Lanes 2-7: samples Aghien Lagoon; Lane 8-13 and 16: samples pond Kan; Lane 17: River Comoé sample; Lane 18: samples River Bandama

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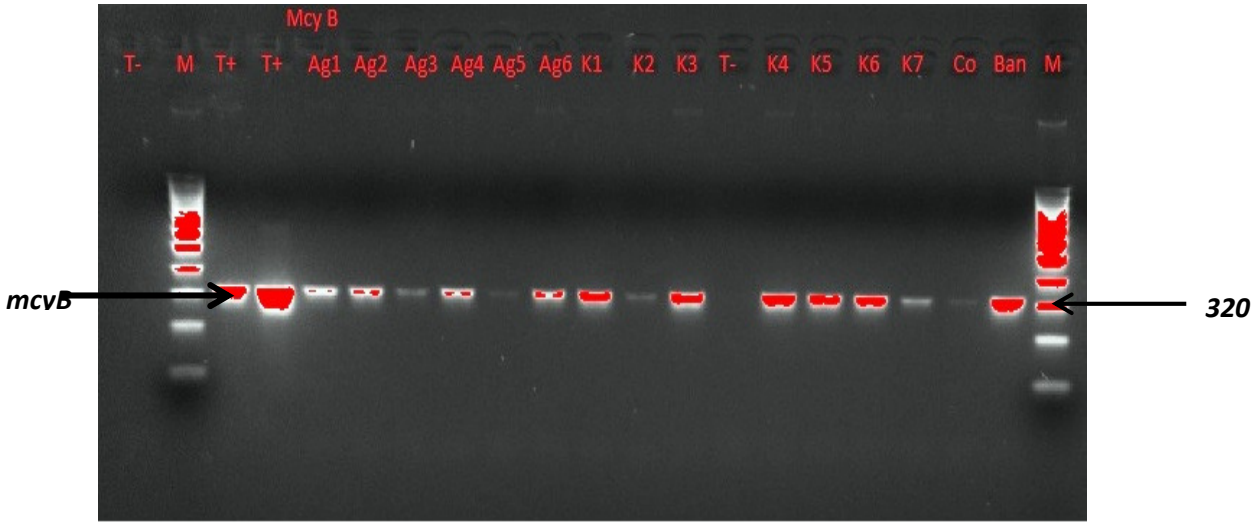

Figure 5: Revelation of the amplification products mcyB gene on agarose gel 1.5%, the expected band of 320 bp; Lanes 1 and 13: Negative control; Lanes 3 and 4: positive control (strain PCC7502); Lane 2 and 21: Molecular weight marker: SmartLadder 1000 bp (Eurogentec); Lane 5 to 10: samples Lagoon Aghien, Lane 11, 12 and 14 to 18: sample pond of Kan track 19: sample Comoé River, Track 20 sample Bandama River

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THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE

Coulibaly Kalpy J et al, The *Experiment, 2014, Vol.26 (1)1775-1787*

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