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Evaluating methods for purifying cyanobacterial cultures by qPCR and high-throughput Illumina sequencing

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ABSTRACT

Cyanobacteria are commonly found in association with other microorganisms, which constitutes a great challenge during the isolation of cyanobacterial strains. Although several methods have been published for obtaining axenic cyanobacterial cultures, their efficiency is usually evaluated by observing the growth of non-cyanobacteria in culture media. In order to verify whether uncultured bacteria should be a concern during cyanobacterial isolation, this work aimed to detect by molecular methods sequences from cyanobacteria and other bacteria present before and after a technique for obtaining axenic cultures from plating and exposure of *Fischerella* sp. CENA161 akinetes to the Extran detergent and sodium hypochlorite. Solutions containing 0.5, 1, and 2% sodium hypochlorite were able to remove contaminant bacterial CFUs from the culture. However, qPCR pointed that the quantity of sequences amplified with universal bacteria primers was higher than the number of cyanobacteria-specific sequences before and after treatments. The presence of uncultured bacteria in post-hypochlorite cultures was confirmed by high-throughput Illumina sequencing. These results suggest that culturing may overlook the presence of uncultured bacteria associated to cyanobacterial strains and is not sufficient for monitoring the success of cyanobacterial isolation by itself. Molecular methods such as qPCR could be employed as an additional measure for evaluating axenity in cyanobacterial strains.

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1. Introduction

Cyanobacteria is a bacterial phylum comprising the only organisms known to carry out both oxygenic photosynthesis and nitrogen fixation. As a consequence of this capacity, these microbes play important ecological roles and are commonly found in association with other organisms. These ecological associations provide a considerable challenge during the isolation of cyanobacterial strains, as several heterotrophic bacteria develop strong attachments to their cells (Rippka 1988; Eiler and Bertilsson 2004). In addition, some molecules synthesized by cyanobacteria, such as toxins and other compounds, can be used as carbon sources by other bacteria (Ishii et al. 2004). Thus, achieving axenity in a cyanobacterial culture is usually a very slow and difficult process when feasible.

In spite of this difficulty, a few methodologies for obtaining axenic cyanobacterial cultures have been developed. Most of these methods include techniques such as using broad spectrum antimicrobial drugs (Ferris and Hirsch 1991); sodium hypochlorite (Fogg 1942; Tassigny et al. 1969; Vaz et al. 2014); membrane filtration (Heaney and Jaworski 1977; Azma et al. 2010); or micromanipulation and successive streaking (Bowyer and Skerman 1968). All these techniques demand time and careful monitoring, but the use of chemicals is perhaps the most suitable treatment for obtaining pure cyanobacterial cultures in shorter time spans (Vaz et al. 2014). Washing cyanobacterial cells with detergent solutions (McDanile et al. 1962) is a supplementary treatment that is usually helpful in the removal of heterotrophic bacteria suspended in cultures or adhered to mucilaginous sheaths.

Success in obtaining axenic cyanobacterial cultures is traditionally evaluated by plating samples in culture media specific for heterotrophic bacteria and verifying growth. Alternatively, spreading samples in media for culturing cyanobacteria may be used after the addition of 0.2% glucose and 0.2% casamino acids and subsequent incubation in dark for three days (Rippka et al. 1979). Nevertheless, uncultured bacteria make up the majority in samples obtained from the environment, with only an estimated 1% of bacterial species being possible to culture by current methods (Torsvik et al. 1990, 1996; Rappé and Giovannoni 2003), and co-culturing is capable of helping previously uncultured bacteria to thrive under laboratory conditions (Vartoukian et al. 2010; Stewart 2012).

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Under this light, evaluations of axenity in cyanobacterial cultures based on culturing heterotrophic bacteria might be misleading as they do not consider the possibility of uncultured bacteria still being present and feeding on cyanobacterial metabolites or decaying cells. In order to evaluate whether uncultured bacteria should be a concern during procedures for obtaining axenic cyanobacterial cultures, this work aimed to verify by quantitative PCR and Illumina sequencing the presence of bacteria in samples from a cyanobacterial culture after purifying procedures.

2. Material and methods

2.1. Strain origin and culture conditions

Fischerella is a cyanobacterial genus capable of differentiating heterocytes, cells in which nitrogen fixation is carried out, and akinetes, resistant cells produced under unfavorable environmental conditions (Moore 1977; Singh and Kashyap 1988; Komárek 2013). *Fischerella* sp. strain CENA161 (Fig. 1) is maintained at the Laboratory of Cellular and Molecular Biology, located in the Center for Nuclear Energy in Agriculture, University of São Paulo, in Piracicaba, São Paulo, Brazil. This strain was obtained in 2004 from a small concrete dam in Piracicaba, São Paulo, after successive streaking in solid media (Fiore et al. 2009). However, this culture is non-axenic, and as such the cyanobacterium is co-cultured with other bacteria. Culturing was performed in liquid BG-11_0 medium (Allen 1968) lacking nitrogen sources, under a temperature of 25 ± 1 °C, 40 μmol photons$\cdot m^{-2} \cdot s^{-1}$ fluorescent light irradiance and a photoperiod of 14 h of light and 10 h of darkness.

2.2. Cells washing

Initially, cyanobacterial cells were washed with detergents in order to reduce the number of contaminant bacteria. For this purpose, liquid cultures were concentrated by centrifuging 1 mL aliquots in microtubes at 7690 $\times g$ for 10 min, and the pellet was washed three times with 2 mL of sterile ultrapure water at 7690 $\times g$ for 10 min. After discarding the supernatant, 2 mL of 0.05% Extran® (Merck, Darmstadt, Germany) were added to the pellet, which was mixed and centrifuged at 2370 $\times g$ for 10 min. Afterwards, cells were washed three times by centrifuging at 4650 $\times g$ for 10 min with 2 mL of a solution consisting of 50 mM NaCl, 10 mM pH 7.5 Tris-HCl, 2.5 mM pH 8 EDTA, and 50% ethanol. Finally, 2 mL of 0.85% NaCl were added to the pellet, which was mixed and centrifuged to 7690 $\times g$ for 10 min.

2.3. Exposure to sodium hypochlorite

Since cyanobacterial akinetes are more resistant to chemicals than vegetative cells, an akinetes-containing 90 days old culture was used for sodium hypochlorite treatment as described by Vaz et al. (2014). After 20 mL samples were homogenized with a syringe in order to fragment filaments and expose akinetes and vegetative cells to the chemical, 500 μL from this suspension was deposited on the surface of a vacuum-driven filtration system through an 8 μm pore membrane (Merck Millipore, Darmstadt, Germany). Cells were washed for 20 s with 3 mL solutions of sodium hypochlorite in 0.25, 0.5, 1, and 2% concentrations, followed by washing with 3 mL of ultrapure sterile water for 20 s. After this process, cells were collected from the surface of the membrane and placed into a 125 mL Erlenmeyer flask containing 20 mL of BG-11 medium lacking inorganic nitrogen. The flask was incubated during 60 days at 25 ± 1 °C under 40 μmol photons$\cdot m^{-2} \cdot s^{-1}$ irradiance with a photoperiod of 14 h light and 10 h dark.

2.4. Growth in solid culture media

After incubation of treated cells, a 100 μL culture aliquot from each experimental Erlenmeyer was collected and spread on the surface of Petri dishes containing the Tryptic Soybean Agar medium (HiMedia, Mumbai, India) supplemented with 1% glucose or the BG-11 medium lacking inorganic nitrogen and supplemented with 0.2% glucose and 0.02% casamino acids. Petri dishes were incubated at 25 °C for 96 h and checked constantly for the growth of heterotrophic bacteria.

2.5. DNA extraction

DNA extraction was performed on 1 mL culture aliquots using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences,

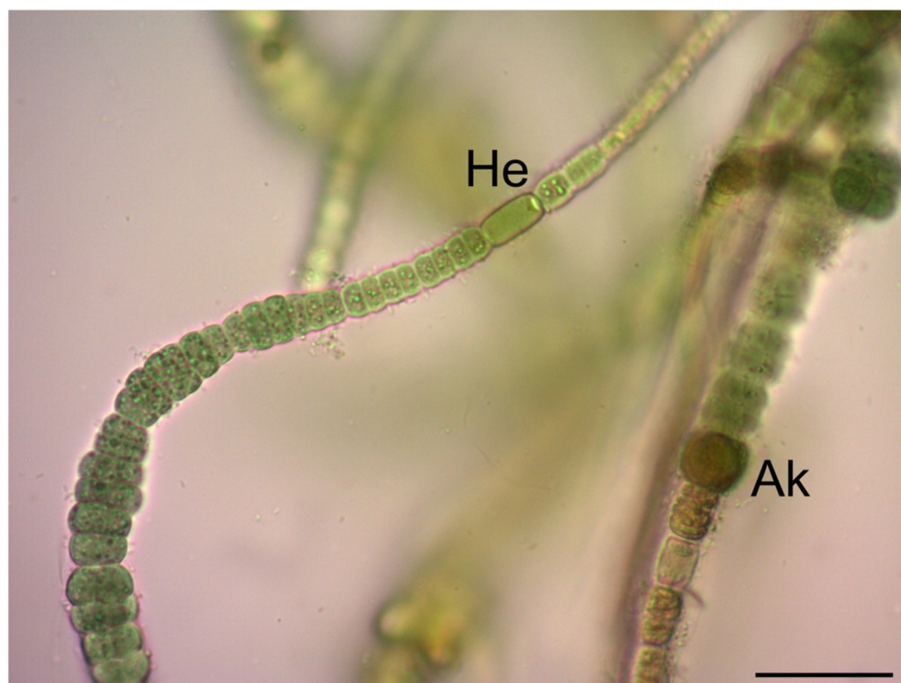

Fig. 1. Microphotograph of *Fischerella* sp. strain CENA161 showing the structure of its filaments. He: heterocyte. Ak: akinete. Scale: 10 μm. A colored version is available online.

Union City, CA, USA) according to manufacturer's instructions. Integrity of the total genomic DNA extracted was verified by 1% agarose gel electrophoresis. The extracted DNA was purified using the AxyPrep™ PCR Clean-Up Kit (Axygen) and quantified in the QuBit™ fluorometer with the Quant-iT™ dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. DNA extraction was performed on cells from three different treatments in replicate: 0.5% sodium hypochlorite and washing with the Extran detergent (SHW); 0.5% sodium hypochlorite without washing (SH); and a control treatment without sodium hypochlorite or washing (C).

2.6. Real-time PCR quantification

In addition to growth in solid media, the presence of bacteria persisting in the culture was verified by Real-time quantitative PCR (qPCR) using the samples extracted DNA. Primers CYA359F and CYA781R-b were used for amplifying a cyanobacteria-specific region of the 16S rDNA, and primers P1-F and P2-R were used as universal primers for total bacterial DNA in the sample, including the Cyanobacterial phylum (Table 1). Reactions were carried out in three replicates on the StepOnePlus Real Time PCR System (Life Technologies) using the SYBR Green I system of detection (Qiagen, Valencia, CA, USA). In all reactions, a final denaturing curve was performed with temperatures ranging from 72 to 94 °C (Bustin et al. 2009). Standard curves were obtained with serial dilutions of a plasmid standard containing the target region generated for each primer set. Threshold values obtained from sample amplification were interpolated in the standard curve determining the number of 16S rDNA copies found per mL of samples. Analysis of variance and mean comparisons were performed using Tukey test, $P < 0.05$, by the program Sisvar (Ferreira 2011).

2.7. High-throughput DNA sequencing

Illumina high-throughput DNA sequencing was also used as an additional method for verifying the presence of non-cyanobacteria in the SHW and C treatments. Paired-end libraries were constructed with the Nextera XT DNA Sample Prep Kit (Illumina, San Diego, CA, USA) and sequenced at the MiSeq platform with the MiSeq Sequencing Kit v.3 (Illumina). Overlapping read pairs were merged with PEAR 0.9.6 (Zhang et al. 2014a). Bases with quality lower than Phred 20 and sequences shorter than 50 bp were discarded with Seqyclean 1.3.12 (http://cores.ibest.uidaho.edu/software/seqyclean). Abundance and taxonomic composition in the processed sequences were determined with FOCUS 0.26 (Silva et al. 2014).

3. Results and discussion

Treatments with 2% sodium hypochlorite did not allow recovery of *Fischerella* sp. strain CENA161 akinetes to vegetative cells after a 60 days incubation period. Nevertheless, the cyanobacterium proved to be able to recover vegetative cells from akinetes 60 days after 0.25, 0.5, and 1% sodium hypochlorite treatments, turning the whitish coloration caused by the hypochlorite oxidant effect into their characteristic green coloration after photosynthetic pigments were resynthesized. Except for 0.25% solutions, which resulted in a 58 $CFU \cdot mL^{-1}$ count after 60 days, all hypochlorite concentrations were successful in eliminating heterotrophic bacterial CFU in supplemented Tryptic Soy Agar and nitrogen-free BG-11 media after 96 h incubation.

Success of these methods depends mainly on the capacity of solutions in dissolving lipids from plasma membranes, denaturing structural proteins, disrupting the trichome-involving exopolysaccharides layer, and the oxidation of cell constituents in vulnerable vegetative cells while relying on akinetes resistance. Fogg (1942) used this method for achieving axenic cultures for *Anabaena* spp. strains after exposing cells to sodium hypochlorite 0.0025% for 2 min. Tassigny et al. (1969) achieved axenic cultures for filamentous cyanobacterial strains after treatment with sodium hypochlorite 2% for 5 min. Vaz et al. (2014) showed it was possible to achieve axenic cultures from three *Nostoc* spp. strains by exposing their akinetes to sodium hypochlorite at 1, 2, or 3% concentrations for just 10 s.

At first sight, the lack of colony forming units from recovering akinetes after exposure to sodium hypochlorite is a strong indication that this method was capable of producing an axenic culture of the *Fischerella* sp. CENA161 strain. However, the abundance of 16S rRNA gene sequences in the 0.5% sodium hypochlorite and Extran washing treatment (SHW) as estimated by qPCR using primers specific to cyanobacteria and for total bacteria accused an amount of non-cyanobacterial DNA remained in the culture, suggesting the persistence of uncultured bacteria in treated cultures (Fig. 2).

A high number of heterotrophic bacteria are capable of degrading and uptaking molecules synthesized by cyanobacteria, including carbohydrates, peptides, toxins, and other compounds, and uses them as carbon or nitrogen sources (Ishii et al. 2004; Tsuji et al. 2006). A common shelter for contaminants is found in mucilaginous sheaths surrounding the cells of some cyanobacterial species, which may harbor heterotrophic bacteria nourished by its exopolysaccharide layer (Hoiczyk 1998; Vázquez-Martínez et al. 2004). Consequently, even in culture media lacking carbon and nitrogen sources, bacteria may be able to obtain nutrients from cyanobacterial cells or metabolites.

Despite being unable to completely remove uncultured bacteria in addition to cultured bacteria, cells that received sodium hypochlorite and detergent washing before DNA extraction (SHW) showed a decrease in the abundance of total bacteria sequences ($3.16 \cdot 10^{10}$ 16S rDNA copies) in comparison to cells exposed to sodium hypochlorite alone (SH) ($7.94 \cdot 10^{10}$) and the untreated control cells (C) ($3.16 \cdot 10^{12}$). In addition, the SHW treatment presented the highest abundance of cyanobacterial sequences ($2.51 \cdot 10^{9}$ 16S rDNA gene copies), and as a consequence the cyanobacteria/total bacteria ratio for this treatment was superior to other treatments.

Results of qPCR suggest that exposing akinetes to detergents and chemicals may be efficient for the elimination of cultured contaminants, but also indicate this exposure is unable to completely remove uncultured bacteria. Taxonomic identification of sequences obtained after

Table 1
Primers and conditions used in qPCR amplifications.

Primer	Sequence	Cycling	Reference
CYA359F CYA781R-b	5′-GGGGAATYTTCCGCAATGGG-3′ 5′-GACTACAGGGGTATCTAATCCCTTT-3′	95 °C for 2 min 94 °C for 1 min 47 °C for 1 min 72 °C for 1 min (35 cycles)	Nübel et al. (1997)
P1-F P2-R	5′-CCTACGGGAGGCAGCAG-3′ 5′-ATTACCGCGGCTGCTGG-3′	95 °C for 3 min 94 °C for 30 s 55 °C for 30 s 72 °C for 30 s (35 cycles)	Muyzer et al. (1993)

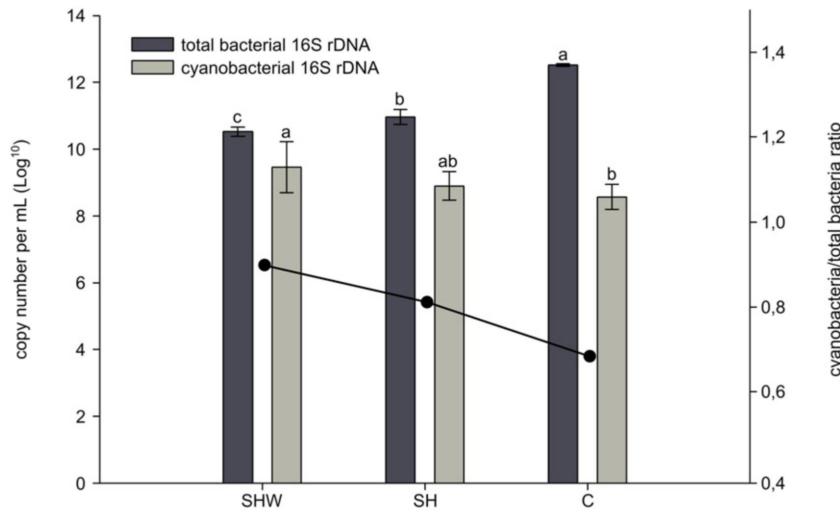

Fig. 2. Quantification of 16S rRNA gene copies from cyanobacteria as opposed to sequences from total bacteria among different treatments. SHW: sodium hypochlorite and detergent washing. SH: sodium hypochlorite without washing. C: control.

high-throughput DNA sequencing confirmed these observations, accusing approximately 74% of sequences after treatment could be identified as coming from non-cyanobacterial organisms. Nevertheless, these methods also showed the methods employed are capable of reducing the number of contaminant bacteria and increasing the cyanobacteria/total bacteria ratio, thus enabling better results for subsequent DNA-based methods.

DNA sequencing results also showed reduction on the number of contaminant bacteria after hypochlorite exposure. These results suggested even though the BG-11 culture medium is turned to the necessities of cyanobacterial physiology, there was an overwhelming relative abundance of non-cyanobacterial sequences on the control treatment (approximately 99%). A large increase in the relative abundance of cyanobacterial sequences was observed after the SHW treatment (26%), which led *Fischerella* sp. CENA161 to become one of the dominant bacteria in the culture (Fig. 3).

A sharp decrease in the abundance of alphaproteobacteria observed after the culture was exposed to hypochlorite suggests a general susceptibility of this class to the chemical employed. This probably opened ground for thriving bacteria to proliferate by exploring niches under lowered competition, allowing betaproteobacteria, gammaproteobacteria, and the cyanobacterium to occupy dominant positions previously held by alphaproteobacteria in this culture. Consequently, the taxonomic composition of the cyanobacterium-associated

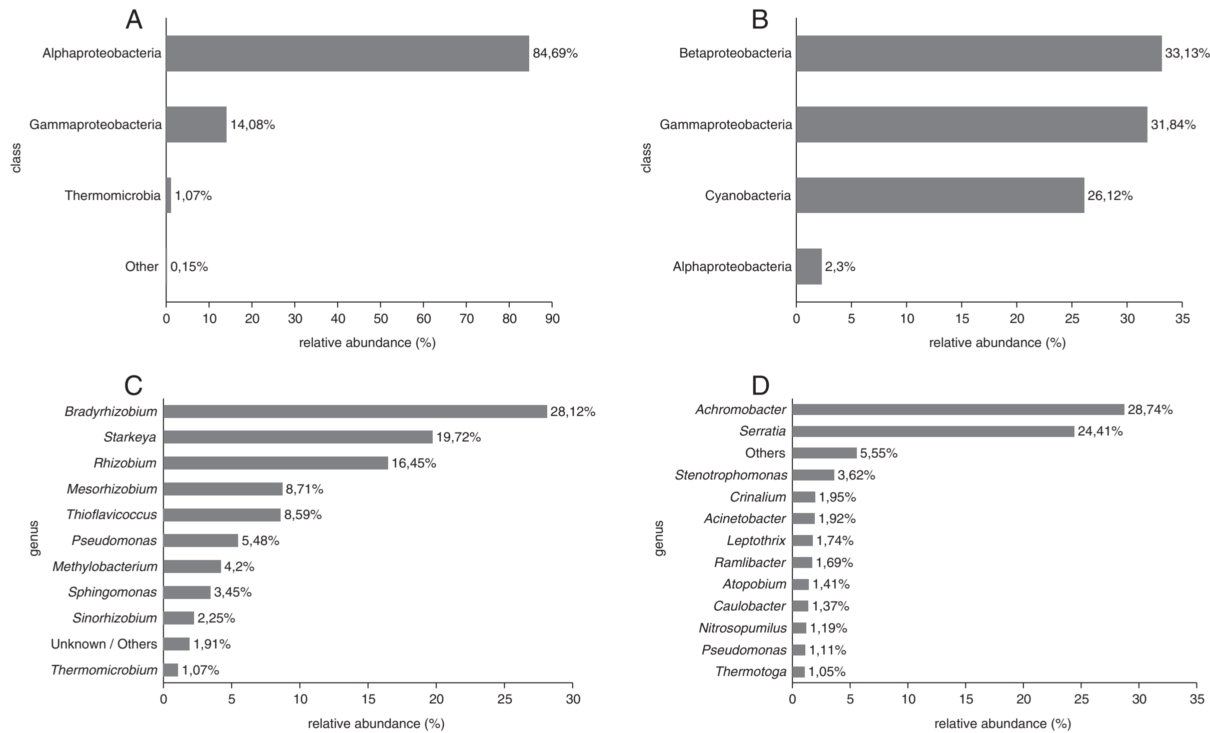

Fig. 3. Relative abundances of bacterial taxa identified on cultures before and after chemical treatments as estimated by high-throughput sequencing. A: abundance of bacterial classes before exposure to sodium hypochlorite. B: most abundant bacterial classes after the culture was subjected to sodium hypochlorite and detergent washing. C: bacterial genera present on the culture before chemical treatment. D: non-cyanobacterial genera detected after chemical exposure.

bacterial community was deeply restructured from being dominated by the genera *Bradyrhizobium*, *Starkeya*, and *Rhizobium* to a predominance of *Achromobacter* and *Serratia*.

According to Fogg (1942), success in eliminating heterotrophic bacteria from cyanobacterial cultures during hypochlorite treatments depends on the absence of spore-producing bacteria in the community. Cells most susceptible to the chemical agents should be eliminated during treatment, while some recalcitrant uncultured bacteria may resist due to being highly specialized and strongly attached to the target cyanobacterium. These recalcitrant bacteria are likely part of an ecological network interacting intimately with cyanobacterial cells after surviving the selection pressure brought by the aggressive treatment.

An increasing number of cyanobacterial genomes has become available by next generation sequencing technologies, prompting evolutionary, ecological, and bioprospection studies based on whole genomes (Shih et al. 2013). Although it is possible to perform the assembly of cyanobacterial genomes from non-axenic cultures (Nederbragt et al. 2010; Albertsen et al. 2013) and to separate contigs belonging to cyanobacteria and contigs from other bacteria coexisting in the culture (Wood and Salzberg 2014), genome coverage of the target strains could remain low, leading to the necessity of additional sequencing to contemplate genome regions not properly covered.

For better chances of success in genome assemblies, DNA extraction from axenic cultures is recommended for higher genomic coverage and reduced contamination. However, not only achieving but also keeping cyanobacterial cultures axenic after isolation is difficult because of a high contamination risk in all phases. Therefore, it is crucial that cyanobacterial cultures are once again evaluated before sequencing. Preferentially, cultures should be sequenced right after observation of axenity, because bacterial cells not eliminated along the isolation process could recover themselves and reproduce until dominance.

In comparison to other methods for obtaining axenity in cyanobacteria such as using antimicrobials or mechanically isolating cells by streaking, akinetes isolation promises results in shorter times. Recovering *Fischerella* sp. CENA161 akinetes after hypochlorite exposure has taken only 60 days, but on the other hand was unable to completely remove uncultured bacteria. Although the same remains to be observed in other methodologies, it is reasonable to assume other methods for achieving axenity might present similar results regarding uncultured bacteria. In that case, we have performed the Petri plate exhaustion for only one time, after the cleaning procedure, for achieving a previous short axenic filament.

Although the number of 16S rRNA gene copies differs between bacterial genomes and that difference can occasionally distort abundance profiles, the 16S rRNA gene remains as an important molecular marker for taxonomic and phylogenetic inferences since it is present in all bacteria, its mutation taxa is low, and it presents conserved regions that facilitate representations of bacterial diversity (Zhang et al. 2014b). Under this light, qPCR may not necessarily quantify contaminants with precision, but it could still be considered a method for positive detection of contaminants in cyanobacterial cultures.

Molecular tools used for detecting uncultured bacteria provide a more thorough monitoring of the cleaning and purification processes during cyanobacterial isolation and maintenance of isolated cultures, allowing to detect and even identify bacteria not properly uncovered by other methodologies. In addition to the qPCR technique, restriction fragment length polymorphism (RFLP) and denaturing gradient gel electrophoresis (DGGE), commonly used for separating and sorting PCR-amplified DNA fragments, may also have potential for verifying the persistence of non-cyanobacterial organisms during isolation, as these techniques have been implemented in the evaluation of bacterial communities associated to cyanobacteria in natural environments (Kolmonen et al. 2004; Giovannoni and Stingl 2005).

4. Conclusions

Techniques for evaluating axenity in cyanobacterial cultures by relying on culturing heterotrophic bacteria are not entirely sufficient for this purpose as they may overlook uncultured bacteria. Molecular techniques provide a better framework for the evaluation of purifying efficiency since they also allow uncovering uncultured bacteria. qPCR allows for a quick detection of non-cyanobacteria in culturing samples and constitutes a relatively cheaper and faster alternative to sequencing that could be implemented as supplementary to other techniques commonly used for this purpose.

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