

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL



**IMPACT OF MICROCYSTINS ON THE GROWTH
AND ANTIOXIDANT SYSTEM OF AQUATIC
BACTERIA**

Master thesis

Diana Marta Luís Miguéns

MESTRADO EM MICROBIOLOGIA APLICADA

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Dissertação orientada pela Doutora Elisabete Valério (INSA)

e Prof. Doutora Ana Reis (FCUL)

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Prof. Doctor Ana Reis was the internal designated supervisor in the scope of the Master in Applied Microbiology of the Faculty of Sciences of the University of Lisbon, Portugal.

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ABSTRACT

Microcystins (MC) are the type of hepatotoxins more abundantly produced by cyanobacteria. Studies have shown that these toxins affect many multicellular organisms that inhabit aquatic ecosystems, however their impact on bacteria that cohabit with freshwater cyanobacteria is still unclear.

In this work the impact of three variants of the MC (-LR,-RR,-YR) was evaluated on growth and antioxidant system of heterotrophic bacteria isolated from three Portuguese reservoirs where blooms of cyanobacteria are often observed, some having microcystin-producing strains, and also in bacteria isolated from a reservoir where these phenomena do not occur. To this end, morphological and molecular characterization of the bacterial isolates was proceeded and these bacteria were exposed to three different concentrations of each variant of the MC, and the effect on the bacterial growth curves was evaluated. The enzymatic activity of catalase (CAT) and SuperOxide Dismutase (SOD1 and SOD2) was determined spectrophotometrically at 240 nm and 550 nm, respectively, in cells exposed to the microcystin variants.

It was found that MC can reduce the growth of most bacteria tested (62.5%), and some bacterial cultures grown with no effect (37.5%), while others reacted differently depending on the variant and concentration used on the same isolate. However, in two isolated bacteria a slight stimulation of growth was observed, although with no statistical significance.

The results of the determination of CAT and SOD activities showed that the bacterial isolates were susceptible to 10 nM of each variant MC. In all strains tested there was an increase in CAT activity and, in relation to the SOD1 and SOD2 activities it was observed that, most bacteria had an increase of the each SOD activity when exposed to MC. However, not all isolates showed effects on SOD1 or SOD2 activities in the three variants of the MC used.

This study showed that MCs can induce a reduction on the growth of most bacteria isolated from freshwater. In respect to the antioxidant system enzymes, all results point out that microcystins can induce oxidative stress in the bacteria tested and that CAT and SOD activities were activated as a defense mechanism to scavenge reactive oxygen species (ROS) increment.

RESUMO

As microcistinas (MC) são o tipo de hepatotoxinas mais abundantemente produzido pelas cianobactérias. Estudos revelaram que estas toxinas afetam diversos organismos multicelulares que habitam ecossistemas aquáticos, no entanto o seu impacto em bactérias, que coabitam com cianobactérias de água doce encontra-se ainda por esclarecer.

Neste trabalho avaliou-se o impacto de três variantes da MC (-LR, -RR, -YR) no crescimento e sistema antioxidante de bactérias heterotróficas isoladas de três albufeiras portuguesas, onde frequentemente se observam "blooms" de cianobactérias, sendo algumas estirpes produtoras de MC, assim como em bactérias isoladas de uma albufeira onde estes fenómenos não acontecem. Para tal, procedeu-se à caracterização morfológica e molecular das bactérias isoladas e estas foram expostas a três concentrações diferentes de cada variante da MC, e os efeitos nas curvas de crescimento bacteriano foram avaliados. A atividade enzimática da catalase (CAT) e da Superóxido Dismutase (SOD1 e SOD2) foram determinadas, espectrofotometricamente a 240 nm e 550 nm, respetivamente, nas células expostas às variantes da microcistina.

Verificou-se que as MC podem reduzir o crescimento da maioria das bactérias testadas (62,5%), sendo que algumas bactérias cresceram sem efeito algum induzido (37,5%), enquanto outras reagiram de forma diferente consoante a variante e a concentração usada no mesmo isolado. No entanto, em dois isolados observou-se uma ligeira estimulação do crescimento, embora sem significado estatístico.

Os resultados da determinação das atividades da CAT e SOD revelaram que os isolados bacterianos são na sua maioria suscetíveis à exposição de 10 nM de cada variante da MC. Em todos os isolados testados observou-se um aumento da atividade da CAT e, em relação à SOD1 e SOD2 verificou-se que, na maioria das bactérias testadas, ocorreu um aumento da atividade de cada SOD quando expostas à MC. Contudo, nem todos os isolados apresentaram efeitos na atividade SOD1 ou SOD2 nas três variantes da MC usada.

Este estudo demonstra que as MCs podem reduzir o crescimento da maioria das bactérias isoladas das albufeiras. Em relação às enzimas do sistema antioxidante, todos os resultados indicam que as microcistinas podem induzir stress oxidativo nas bactérias testadas e que as atividades da CAT e da SOD foram ativadas como um mecanismo de defesa para eliminar o aumento de espécies reativas de oxigénio (ROS).

TABLE OF CONTENTS

1. INTRODUCTION	2
1.1. Aquatic cyanobacteria and heterotrophic bacteria	2
1.2. Cyanobacterial blooms and microcystins.....	3
1.3. Bacterial cell growth	5
1.4. Bacterial antioxidant system and oxidative stress	6
2. MATERIALS AND METHODS.....	8
2.1. Sampling reservoirs	8
2.2. Isolation of bacteria.....	9
2.3. Characterization of the isolated bacteria.....	10
2.4. Molecular identification of the isolates	11
2.5. Bacterial cell growth	12
2.6. Determination of the activity of the antioxidant system enzymes	13
3. RESULTS.....	15
3.1. Characterization of the heterotrophic bacteria isolated	15
3.2. Effects of microcystins on the bacterial growth	23
3.3. Effects of microcystins on the bacterial antioxidant system.....	35
4. DISCUSSION	41
4.1. Characteristics of the isolated bacteria.....	41
4.2. Effects of microcystins on the bacterial growth	42
4.3. Effects of microcystins on the bacterial antioxidant system	45
5. CONCLUSION.....	48
6. REFERENCES	49

1. INTRODUCTION

Cyanobacteria are phototrophic microorganisms that can produce a variety of toxins including microcystins (Best *et al.*, 2002). Also known as blue-green *algae*, these cyanobacteria are ubiquitous unicellular organisms which mainly inhabit aquatic ecosystems. In aquatic reservoirs these bacteria live in community with others organisms, such as heterotrophic bacteria.

The increase of nutrients concentration, mostly nitrogen and phosphorous in water bodies contributes to the cyanobacterial proliferation which, in some cases, could lead to the proliferation of toxic cyanobacterial species (Kuriama *et al.*, 2012). These toxins are secondary metabolites such as heptapeptide microcystins, of which over 70 structural variants are recorded and they inhibit protein phosphatases causing changes in membrane integrity (Codd *et al.*, 2005).

Most studies about the toxicity of microcystins are focused on animals and on higher plants. However, few studies have been made on the possible effects of these cyanotoxins on heterotrophic bacteria (Dixon *et al.*, 2004 and Yang *et al.*, 2008), which are important as other organism in the trophic web in aquatic ecosystems. Thus, the aim of this study was to examine the effects of three variants of microcystin (Microcystin-LR, Microcystin-RR and Microcystin-YR) exposure on aquatic heterotrophic bacteria that live in the ecosystem as cyanobacteria, and observe their impact on the bacterial growth and on enzymes of the antioxidant system (Catalase and Superoxide Dismutase) of these bacteria, to increase the knowledge about microcystin effects on microbial cells.

1.1. Aquatic cyanobacteria and heterotrophic bacteria

The phytoplankton in aquatic ecosystems is constituted by several eukaryotic microscopic species, as well as, prokaryotic species such as cyanobacteria, which are photosynthetic organisms with a worldwide distribution (Saker *et al.*, 2009). These photosynthetic bacteria, with the certain amount of nutrients and light, can rapidly grow in high density populations called cyanobacterial blooms. Thus, some blooming-forming cyanobacteria cause ecological, economic and health problems (Paulino *et al.*, 2009), due to these overgrown in a short time period, they may break the natural balance of the aquatic system.

Regarding heterotrophic bacteria, they are prokaryotes that are involved in many geochemical cycles in freshwater reservoirs, and their subsistence on aquatic

ecosystems can be due to natural or anthropogenic factors (Figueiredo *et al.*, 2007) that include biological processes. As a result of their role in those biogeochemical processes, bacteria are, therefore, essential to the management of the aquatic ecosystem, as they are the unit base of the trophic web. Furthermore, it has already been hypothesized that the presence of heterotrophic bacteria in water may have an important role in the natural cleansing of the chemically stable hepatotoxins (Berg *et al.*, 2009).

Some studies showed that many blooming cyanobacterial species prefer to grow in the presence of other bacteria (Berg *et al.*, 2009). Nevertheless, some bacteria are able to degrade cyanobacterial hepatotoxins, such as microcystins (Berg *et al.*, 2009).

Giaramida *et al.*, (2012) reported that exposure of microcystins significantly contributed to the bacterial communities shape and microbial physiology of the water bodies under study. That fact could explain the role of toxic cyanobacteria in the control of phytoplankton diversity and species abundance, causing ecological unbalances and contamination of the environment (Campos *et al.*, 2013). However, the role of cyanobacteria and their interactions with heterotrophic bacteria is still barely known.

Cyanobacteria and heterotrophic bacteria are an important part of aquatic ecosystems (Berg, 2009), so studies that combine the effects of both organisms on each other provide new evidence towards the kind of relationships that occur in aquatic ecosystems. Evidences showed that cell concentrations of heterotrophic bacteria can be substantially higher during and immediately after cyanobacterial water blooms than in their absence (Bouvy *et al.*, 2001; Eiler and Bertilsson 2004; Berg, 2009).

1.2. Cyanobacterial blooms and microcystins

Cyanobacterial blooms are not axenic and typically have many heterotrophic bacteria associated with them as shown, for example by Islam *et al.* (1994) who found *Vibrio cholerae* within the mucilaginous sheath of *Anabaena* sp. filaments. These cyanobacteria capable to produce a range of secondary metabolites (Bártová *et al.*, 2010) and their mass occurrences (blooms) cause problems to humans and animals. The problems caused by cyanobacteria are often associated with the toxins that they produce and with the endotoxic lipopolysaccharide (LPS) structures of their cells (Berg *et al.*, 2009) and although cyanobacteria are not listed among waterborne pathogens,

their cyanobacterial cells and toxins that develop present waterborne hazards to health, ranging from mild to fatal, on humans and animals (Codd *et al.*, 2005).

Blooms of cyanobacteria have occurred in many regions all over the world and produce a number of toxins, including hepatotoxins such as microcystins (Yang *et al.*, 2008). Several factors contribute to the prevalence of algae blooms, for instance, nutrient inputs, climate changes and the construction of water barriers which often lead to water eutrophication (Churro *et al.*, 2010). The cyanotoxin contamination of water occurs mainly when the cyanobacteria die, the cell walls burst, releasing the toxin thus resulting in the liberation of high amounts of toxins into the water (Blom *et al.*, 2001), and one of the toxins most commonly found are microcystins (Best *et al.*, 2002).

As mentioned before, microcystins are one of the main cyanotoxins. These are cyclic peptides produced by species of freshwater cyanobacteria, primarily *Microcystis aeruginosa* (Jos *et al.*, 2005; Dawson, 1998), that are capable of specifically inhibit the protein phosphatases 1 and 2A (PP1 and PP2A) of both mammals and higher plants (Mackintosh *et al.*, 1990; Hu *et al.*, 2005). Microcystins being hepatotoxins, their main target is the liver by specific binding to the organic anion transport system in hepatocyte cell membranes, inhibiting type 1 and type 2A eukaryotic serine/threonine protein phosphatases (Valério *et al.*, 2009). The toxin is extremely stable and resists to hydrolysis or oxidation under conditions found in most natural water bodies (Butler *et al.*, 2009). These toxins can break down slowly at high temperature (40°C) and at either very low (<1) or high (>9) pH (Harada *et al.*, 1996).

Microcystins comprise over 80 analogs and they have a particular chemical structure (Hawkins *et al.*, 2006). They are cyclic peptides containing seven amino acids, sharing the common structure of Adda_D-Glu-Mdha_D-Ala_L-X_D-MeAsp_L-Z (Valério *et al.*, 2009). The general structure of the cyanotoxin with variable portions shown as X, Z is illustrated in Fig. 1.

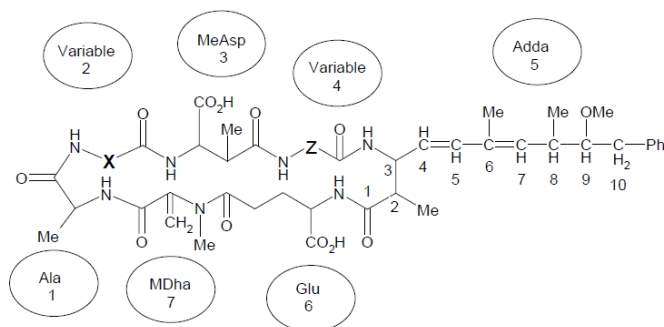


Figure 1 - General structure of microcystin consisting of D-alanine (Ala); two variable amino acids (position X and Z); D-β-methylaspartic acid (MeAsp); (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda); isolinked D-glutamic acid (Glu) and N-methyl dehydroalanine (MDha) (from Hawkins *et al.*, 2006).

The major isoforms of microcystin and most studied ones are microcystin-LR (MCLR), microcystin-RR (MCRR) and microcystin-YR (MCYR) (Li *et al.*, 2009). MCLR has a leucine (L) and an arginine (R) in the X-position and Z-position amino acids, respectively. Microcystin-RR (MCRR) with an arginine (R) in the X-position and in the Z-position amino acids; and the third variant has microcystin-YR (MCYR) with a tyrosine (Y) and an arginine (R) in the X-position and Z-position amino acids, respectively (Butler *et al.*, 2009).

The three microcystin variants are naturally occurring cyclic heptapeptide produced by some strains of cyanobacteria (Guzman and Solter, 1999) and MCLR is the most studied variant of microcystin and it is the most representative variant of all (Campos *et al.*, 2013). MCLR was the first microcystin chemically identified and has been associated with most of the incidents of toxicity involving microcystins in most countries (Fawell *et al.*, 1993), consequently, its toxicity is well known in animals (Honkanen *et al.*, 1990; Guzman and Solter 1999; Jos *et al.*, 2005; Dias *et al.*, 2009; Sabatini *et al.*, 2011; Huguet *et al.*, 2013). However, in microorganisms such as other bacteria, the studies are few (Dixon *et al.*, 2004 and Yang *et al.*, 2008). In some studies, MCLR revealed to be less cytotoxic than MCRR (Huguet *et al.*, 2013) and MCLR and MCYR showed a similar effect on microbial growth (Valdor and Aboal, 2007). The LD₅₀ for MCLR in mice is 50 µg/kg (Dittmann and Wiegand, 2006). The acute lethality of MCYR is slightly lower than MCLR (Gupta *et al.*, 2003; Stotts *et al.*, 1993). LD₅₀ estimates for MCYR is 70 µg/kg in mice (Dittmann and Wiegand, 2006). The LD₅₀ for MCRR is about 10 times higher than the other two variants, with an estimate value of 600 µg/kg in mice (Dittmann and Wiegand, 2006).

1.3. Bacterial cell growth

Bacterial cell growth defines duplication of its cells (Madigan *et al.*, 2012) and in microbial growth usually growth parameters, as lag phase and growth rate are obtained by measuring turbidity as optical density (OD).

Turbidity is measured with a spectrophotometer at a certain wavelength and the presence of more cells in the cell suspension results in a turbidity increase (Madigan *et al.*, 2012).

Bacterial growth is defined with four different phases: lag, exponential, stationary and a death phase. The exponential phase is where the cell duplication occurs and this

period is dependent on several factors such as temperature, pH, water availability and oxygen (Madigan *et al.*, 2012).

There are few studies about the microcystins effects on bacterial growth, however, Yang *et al.*, (2008) observed that *E. coli* had a growth inhibition at initial growth phase when cells were treated with MCRR. A similar effect was observed by Hu *et al.*, (2005) in cyanobacterium *Synechococcus elongates* when exposed to the same microcystin variant.

1.4. Bacterial antioxidant system and oxidative stress

Microcystins are capable to elicit oxidative stress in aquatic organisms (Jos *et al.*, 2005) and induce formation of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$) as a result of oxidative metabolism (Jos *et al.*, 2005). These ROS might cause serious cellular damage (Ding *et al.*, 2008) such as peroxidation of lipid membranes, genotoxicity, or modulation of apoptosis (Ding and Ong, 2003). The presence of ROS triggered secondary reactions of defense based on enzymatic mechanisms (Hu *et al.*, 2005). Under stress conditions, the balance between oxidative impact and the antioxidant defense system could be disturbed leading to oxidative stress. Studies made in aquatic macrophytes, as well as in other higher plants showed that the exposure to cyanotoxins have promoted oxidative stress (Pflugmacher 2004; Pflugmacher *et al.*, 2006).

Oxidative stress is imposed on cells in one of three ways: (1) an increase of the oxidants generation, (2) a decrease in the antioxidant protection, or (3) a failure to repair oxidative damage (Vassilakaki and Pflugmacher, 2008). Oxidative stress may be caused by overproduction of ROS or to the depletion of cellular antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) (Sabatini *et al.*, 2011) that are synthesized for scavenging ROS. Elevated levels of ROS, such as superoxide ($O_2^{\bullet-}$) may also lead to DNA damage and mutations (Carmel-Harel and Storz, 2000).

SOD and CAT were found in almost all organisms and are known as important antioxidant enzymes (Yang *et al.*, 2008). SOD converts superoxide radicals to H_2O_2 and molecular oxygen thereby; the level of cellular damage is decreased (Rahda, 2010). SOD is widely distributed to protect such cells against the toxic effects of superoxide anion ($O_2^{\bullet-}$) and protects cells against ROS by lowering the steady state level of $O_2^{\bullet-}$ (Rahda, 2010). There are three types of SOD containing Mn, Fe or Cu and Zn as

prosthetic metals (Rahda, 2010) and they are SOD1 (cytosolic Cu/Zn-SOD), SOD2 (mitochondrial Mn-SOD), and SOD3 (extracellular Cu/Zn-SOD) (Trevigen manufacturer's instructions). The Fe SOD and Mn SOD types occur together in many eubacteria and plants. The Cu-Zn and Mn/Fe types of SOD have quite different mechanisms of action and contain different types and numbers of metal ions (Smith and Doolittle, 1992).

When H_2O_2 is high, catalase acts catalytically and removes it by forming H_2O and O_2 (Radha, 2010). However, at a low concentration of H_2O_2 and in the presence of a suitable hydrogen donor such as ethanol and others, CAT acts peroxidically, removing H_2O_2 , but oxidizing its substrate (Turkseven *et al.*, 2005). CAT decomposes H_2O_2 and protects the bacterial cell from highly reactive OH^{\bullet} (Rahda, 2010). Most of the catalases characterized until now can be classified in two types: typical catalases and bifunctional catalase-peroxidases and have been shown to be present in bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumonia* and *Streptococcus coelicolor* (Kim *et al.*, 1994). Bifunctional catalase-peroxidases are pH-dependent with a pH optimum at 6 - 6.5, and are more sensitive to temperature, chloroform/ethanol and H_2O_2 than typical catalases (Kim *et al.*, 1994).

There are some studies on microcystin effects on antioxidant system and the majority of them concluded that the cyanotoxins induces oxidative stress in eukaryotic (Pflugmacher, 2004) and prokaryotic cells (Yang *et al.*, 2008). Li *et al.*, (2009) demonstrated that MCRR could induce the oxidative stress in *Synechocystis* sp. PCC6803 and the increase gene expressions of antioxidant enzymes might protect the algae from the oxidative damage.

Ding *et al.*, (2008) found out that microcystins induced stress on aquatic plants *Lemna minor* and *Myriophyllum spicatum* and that stress induced SOD activity increase which may contribute to the microcystin tolerance. However, CAT activity had little benefits to the tolerance in these aquatic plants (Ding *et al.*, 2008).

As cited before, there are few reports about the effects of microcystin on SOD and CAT activity on bacterial cells that co-inhabit in the same ecosystem as cyanobacteria and for the first time the present study assessed the impact of the three microcystin variants on the antioxidant system enzymes of the isolated bacteria in study.

2. MATERIALS AND METHODS

2.1. Sampling reservoirs

Sampling was performed on the 29th October 2012 and 29th April 2013 using 1 l sterile bottles. The first sampling occurred at Albufeira de Magos, Albufeira de Monte da Barca and Albufeira de Patudos, where cyanobacterial blooms are frequently observed. The second sampling was made at Albufeira de Castelo de Bode, a reservoir where these mass occurrences do not occur.

Albufeira de Magos is located in Ribeira de Magos and it belongs to Rio Tejo basin river system (Fig. 2(A)). This reservoir was a swim area that is currently forbidden for bathing due to bacterial contamination and the regular presence of cyanobacteria in water (Decreto Regulamentar N^o 2/88). Albufeira de Monte da Barca (Fig. 2(B)), Albufeira de Patudos (Fig. 2(C)) and Albufeira de Castelo de Bode (Fig. 2(D)) which also belong to the Rio Tejo basin river system, but these reservoirs are located near Coruche, Alpiarça and Tomar, respectively.

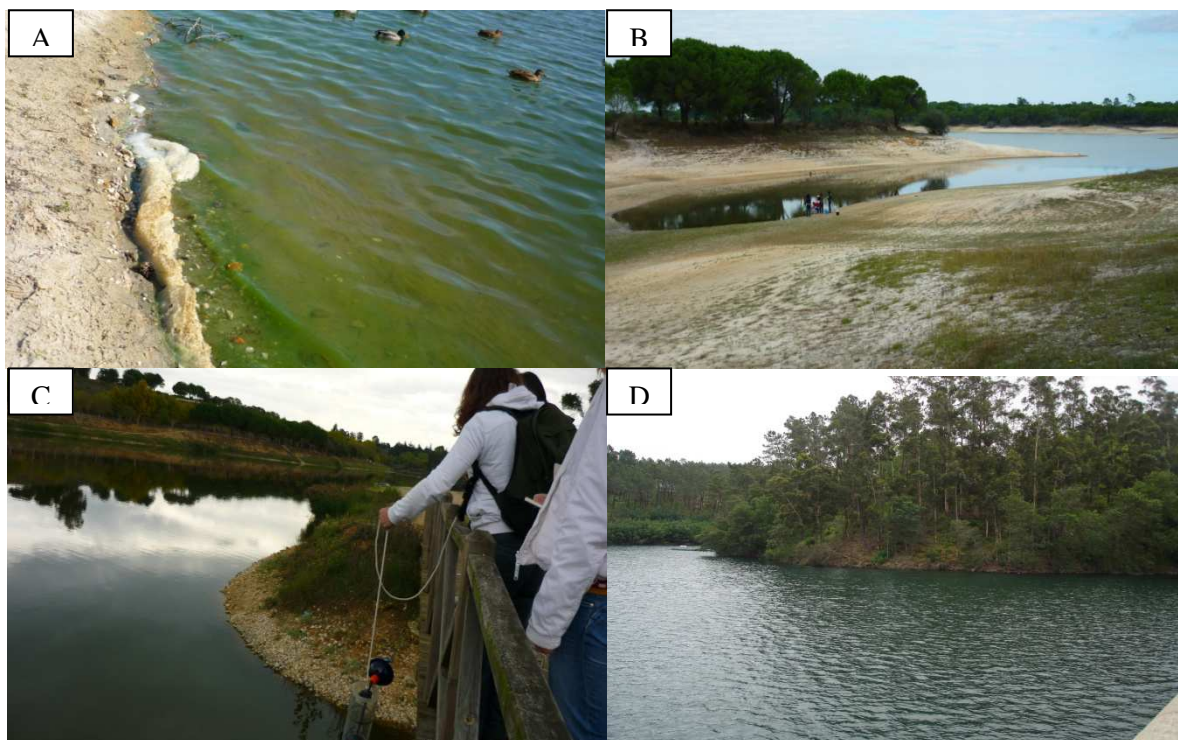


Figure 2 - Sampling reservoirs. (A) Albufeira de Magos; (B) Albufeira de Monte da Barca; (C) Albufeira de Patudos; (D) Albufeira de Castelo de Bode (Taken by Diana Miguéns).

Water samples were transported in a cooler bag in the dark to prevent cyanobacterial growth and the increase of the water temperature.

2.2. Isolation of bacteria

Bacteria were isolated from water samples from each reservoir by two methods, water filtration method and plating beads method.

The filtration system was assembled with a filtering ramp and a cellulose membrane (pore diameter = 0.45 μm). A portion of 20 ml of water was filtered from each reservoir and the membranes were placed directly on the surface of Petri dishes containing three different culture media.

The same water samples were also inoculated by viable counting method, where 100 μl of the samples were spread using sterile glass beads. The plates were then incubated until colonies appear (Madigan *et al.*, 2012).

The same media were used in both methods, one was the non-selective Reasoner'2A medium (R2A) which was originally made for counting heterotrophic bacteria in drinking water samples (Reasoner and Geldreich, 1985), but currently is used for heterotrophic bacterial growth from water samples (Massa *et al.*, 1998; Zinder and Salyers, 2001); the Lysogeny Broth medium (LB) that is usually used for bacterial growth (Bertani, 2004) and the Z8 medium was also inoculated as is it a rich medium appropriate for cyanobacterial growth (Skulberg and Skulberg, 1990). The last one intended to verify if the heterotrophic bacteria that live in the same ecosystem as cyanobacteria could also grow with the same nutrient medium that cyanobacteria.

All of the plates inoculated were incubated at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in the dark, to prevent cyanobacterial growth, during four days. After the incubation period, four different bacterial colonies were selected from each sample incubated at 20°C from the R2A and from the LB medium. Since no bacterial growth on the Z8 medium was observed, and there were no macroscopic differences between colonies from the plates incubated at 20°C and at 30°C , the bacterial colonies were selected from plates incubated at 20°C because this temperature is more similar to the water temperature from the reservoirs where sampling occurred. In the end, 28 colonies were picked and further cultured in Nutrient Agar (NA), an enrichment culture medium, until pure cultures were obtained. After confirming the purity of the isolated bacteria, cryopreservation was performed to maintain the primary features of the isolates, due to the lost of certain features by genetic variation of the isolated bacteria that usually adapts to culture medium conditions (Sambrook and Russel, 2001). To do so, 2 ml of Nutrient Broth medium was placed into 15 ml falcons and each corresponding isolate was inoculated into the medium and incubated at 30°C overnight. Then, a sterile

labelled cryovial was used to mix 750 µl of the overnight growth culture and 250 µl of Glycerol 60% (Sambrook and Russel, 2001). The cryovials were taken to the vortex to ensure that the glycerol was evenly dispersed. Cultures were well mixed, if not, ice crystals would form decreasing the viability of the cells (Sambrook and Russel, 2001). Then the isolated cultures were stored at -80°C for future use.

2.3. Characterization of the isolated bacteria

Bacterial isolates were characterized according to their colony color and texture, cells shape and Gram staining. The colonies color and their texture were verified macroscopically. The bacterial shape was assessed in a microscopic slide with bacterial cells from each isolated bacteria. Isolates were assigned into *coccus*, *bacillus* and *cocobacillus* (Cabeen and Jacobs-Wagner, 2005).

Bacteria can be divided into two major groups, called Gram-positive and Gram-negative. Bacteria are grouped in each type of Gram accordingly with their cell wall structure and color reaction to Gram stain. Fig. 3 shows that the surface of Gram-positive and Gram-negative cells as viewed in the electron microscope differs markedly, whereas the Gram-positive cell wall is typically much thicker and consists primarily of a single type of molecule called peptidoglycan, as much as 90% of the cell wall (Madigan *et al.*, 2012), on the other hand, despite Gram-negative have peptidoglycan, this molecule on them is less thicker and they contain an outer membrane that lacks in Gram-positive bacteria (Fig. 3). In order to classify the isolates according to their Gram group, microscope slides of each isolate cells suspension was prepared by a Previ™ color Gram (Biomérieux) which is an automated Gram stainer system. This standardized coloration improved bacteria differentiation in comparison with manual and bath staining results.

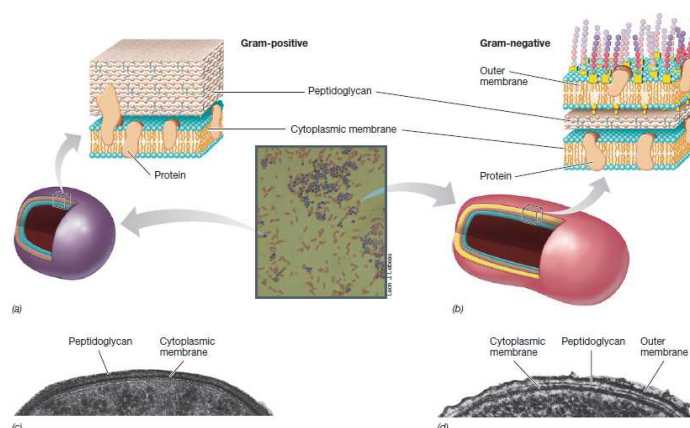


Figure 3 – Main differences from Gram-positive bacteria and Gram-negative bacteria. (From Madigan *et al.*, 2012)

2.4. Molecular identification of the isolates

Bacterial DNA extraction was performed by two different methods. Boiling method was performed for Gram-negative bacteria, whereas for Gram-positive bacteria an Invisorb® Spin Plant Mini Kit (INVITEK) was used for DNA isolation, following the manufacturer's instructions.

In respect to boiling method, bacterial cultures were collected into an eppendorf with 750 µl of apyrogenic water, which is water free from pyrogens (exotoxins and endotoxins) and particulate matter. These samples were centrifuged at 14000 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 500 µl of apyrogenic water with the vortex and centrifuged at 10000 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 300 µl of apyrogenic water, subjected to boiling at 100°C in a water bath for 15 min and centrifuged at 10000 rpm for 5 min. Supernatants were placed into a new eppendorf before they were stored at -20°C.

The nucleic acids concentration and purity was assessed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific) by pipetting 1 µl of sample.

Aliquots of 2 µl of template DNA were used for PCR amplification of 16S rRNA gene. PCR was performed in a 25 µl reaction mixtures containing 10x PCR buffer (Invitrogen), 1.25 mM dNTPs, 50 µM of each primer, 1 mg/ml BSA, 3 mM MgCl₂ (Invitrogen) and 1 U of Taq polymerase (Invitrogen).

The universal bacterial primers 104F and 907R were provided and designed by Chaves, (2005) and the expected amplified fragment has about 800 bp of length. The reactions were performed in a Tpersonal thermocycler (Biometra®) with hot lid (95°C). The temperature profile had five steps, an initial denaturation (94°C for 5 min); 40 cycles of denaturation (94°C for 1 minute), annealing temperature (variable for some isolates) for 1 minute, extension (72°C for 1 minute); and a final extension step (72°C for 5 min). The PCR products were resolved by electrophoresis in a 1% (w/v) agarose gel at 75 V for 45 min, using TAE 1x as buffer. GelRed, which is a safe fluorescent nucleic acid dye designed to replace the highly toxic ethidium bromide, was incorporated in the gel to allow the PCR amplicons visualization. The gel image was acquired using a gel transilluminator (UVITEC).

PCR products were purified with peqGOLD Cycle-Pure Kit (peqLab) and then a 10 µl pre-sequencing reaction, using BigDye terminator reaction was performed. The PCR temperature profile was constituted by 25 cycles of 96°C for 10 seconds, 50°C for 5

seconds and 60°C for 4 seconds. The samples were sent for sequencing at the Molecular Biology Laboratory of INSA. In some bacterial isolates, there were some nonspecific PCR products that could not be eliminated without concomitantly lose the amplicon of interest, and in those cases bands were extracted from the gel. In order to do so, the specific bands were cut from the 1% (w/v) agarose gel with a scalpel blade under UV light and purified with NucleoSpin® Gel and PCR clean-up kit (MACHEREY-NAGEL) according to the manufacturer's instructions.

Bacterial sequences were corrected using BioEdit program (Hall, 1999) and afterward compared to the GenBank nucleotide data library using Basic Local Alignment Search Tool (BLAST) software (Altschul *et al.*, 1990) at the National Center of Biotechnology Information Website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine their closest phylogenetic relatives.

2.5. Bacterial cell growth

Bacterial growth was assessed in a rapid 96-well microplate bioassay where each isolate was inoculated in a Nutrient Broth medium and the three variants of microcystin (MCLR, MCRR and MCYR) purified extracts (table 1) were added into the culture medium to yield a final concentration of 1 nM, 10 nM and 1 µM. The highest MCYR concentration used was 0.3 µM instead of 1 µM because it was the available stock in the laboratory. The concentrations used in the present study were selected from other studies currently being held at the LBE-INSA.

Pre-inoculums were prepared in 10 ml of Nutrient Broth medium in 100 ml Erlenmeyer flasks. Cells were incubated overnight at 20°C, on Orbital Shaker SO3 at 300 rpm. Growth experiments were initiated in the day after with the pre-inoculums of the cultured cells with an initial optical density of 0.05, measured by a colorimeter 257 (Sherwood) at 660 nm wavelength.

Microplates were inoculated as illustrated in Fig. 4. Thus, the blank was inoculated with 200 µl of Nutrient Broth medium and the negative control was constituted by Nutrient Broth medium and bacterial cells. The bacterial cultures were added to wells with microcystin in five replicates for each concentration of each variant of microcystin (table 1). The total volume in each microplate well was 200 µl. The microplates were incubated at 20°C with stirring.

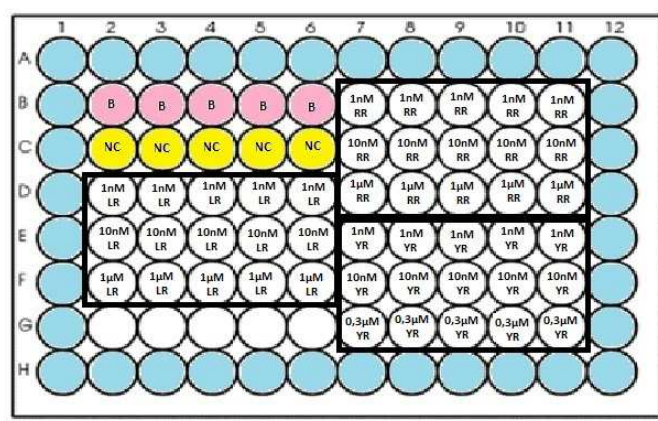


Figure 4 - Schematic representation of the microplate wells inoculation, containing microcystin exposure in five replicates. (B) - Blank. (NC) - Negative control. (LR) - Microcystin-LR concentrations, (RR) - Microcystin-RR concentrations and (YR) - Microcystin-YR concentrations.

Optical densities of the isolated bacteria on each microplate assay were measured at 600 nm reading from 30 to 30 min using a microplate absorbance Multiskan Ascent Thermo Labsystems, with fast shaking for 15 seconds, until stationary phase was achieved. Optical densities were measured according to each isolate growth rate and optical densities readings were made until 8h to 13 h. Growth curves of each tested isolate were made, after the data treatment with Excel™ program (Microsoft Office™). The results were expressed as means ± SE with the optical densities measured. All data were evaluated by F test and student's *t* test with a significant level of $p < 0.05$ (Fowler, 1998) to verify significant differences.

Table 1 - Concentrations of the microcystins extract variants used. These extracts were obtained from strains of cyanobacterium *Microcystis aeruginosa*

MCLR (LMECYA 110)	MCRR (LMECYA 103)	MCYR (LMECYA 179)
1 nM	1 nM	1 nM
10 nM	10 nM	10 nM
1 µM	1 µM	0.3 µM

2.6. Determination of the activity of the antioxidant system enzymes

The oxidative stress was assessed in some of the isolates with the determination of the activity of two antioxidant system enzymes, catalase (CAT) and superoxide dismutase (SOD). These isolates were chosen taking into account that they have reached a high OD (> 0.7) and there were growth effects when exposed to

microcystins. Thus, to determine enzymatic activities, the control group (not exposed to MCs) and cells exposed to microcystins at a concentration of 10 nM of each microcystin variant used, were grown overnight in 10 ml Nutrient Broth medium during 12 hours and the pellets were obtained by centrifugation at 15°C for 10 min at 112 g (1500 rpm) and then washed with sterile distilled water and the pellet was kept at -80°C. To extract the proteins, the pellets were thawed and resuspended in sodium phosphate buffer 0.08 M. Cells were disrupted using 100 µl microspheres (Sigma) with six alternate cycles of 1 minute vortex and 1 minute in ice. Cellular debris was removed by centrifugation for 20 min at 12000 rpm, the supernatant recovered and used to analyze the enzyme activities of CAT and SOD. The amount of total proteins in the samples was estimated by Lowry method, where the absorbance of the samples was read at 750 nm of the end product of the Folin reaction against a standard curve of a selected standard protein solution (BSA). The samples were prepared as Lowry *et al.*, (1951) described.

CAT activity was measured by the decomposition of H₂O₂, which was monitored directly by the decrease in absorbance at 240 nm. The reaction mixture of 3 ml contained 50 mM sodium phosphate buffer (pH 7.0); 1 ml of 0.2% H₂O₂ and 3.75 and 7.5 µg of the enzymatic extract of each isolate, respectively (Yang *et al.*, 2008).

SOD activity of SOD1 and SOD2 was measured by the inhibition of the rate of formation of NBT-diformazan using the Superoxide dismutase assay kit (Trevigen) according to the manufacturer's instructions. The samples supernatants were previously treated with ice-cold chloroform/ethanol, mixed for 30 seconds and centrifuged for 10 min at 10000 rpm. The aqueous phase was recovered without touching the interphase formed (Fig. 5) and placed into a new eppendorf (SOD1 + SOD2 fraction). To assess SOD2 activity, 50 µl were recovered from the aqueous phase and added KCN to a final concentration of 2 mM. The cyanide ion inhibits more than 90% of SOD1 activity, according Superoxide dismutase assay kit (Trevigen) manufacturer's instructions. To determinate each type of SOD activity, 5 µg of the enzymatic extract of each isolate was measured in the reaction mixture by a spectrophotometer (UNICAM UVNis Spectrometer UV4).



Figure 5 - Two phases of the samples treated with ice-cold chloroform/ethanol. The top phase is the aqueous phase, white in the middle is the interphase and the bottom phase is the organic phase.

3. RESULTS

3.1. Characterization of the heterotrophic bacteria isolated

For each reservoir, eight colonies were picked, except for Albufeira de Castelo de Bode where only four colonies were selected. Twenty eight colonies were picked taking into account the morphological features differences observed in R2A medium and LB medium. Bacterial isolates were classified with letters and numbers for further identification (B – from Albufeira de Monte da Barca; M – Albufeira de Magos; P – Albufeira de Patudos; C – Albufeira de Castelo de Bode).


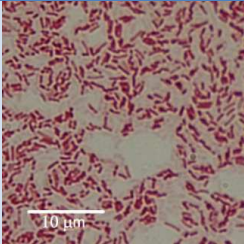
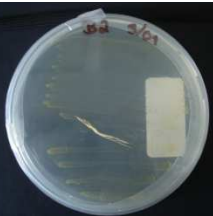
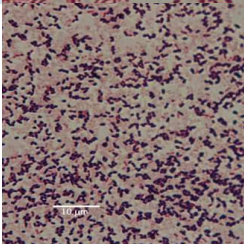

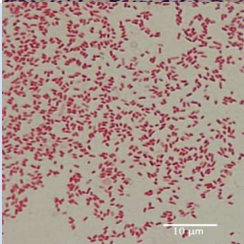
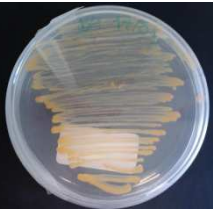
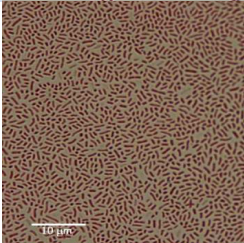
The colonies color and their texture were macroscopically verified and the bacterial isolates were assigned as white, whitish, pale yellow, yellow, brown, orange, pink, pinkish, or dark blue as showed in table 2 and table 3; and their texture was designated as mucous or very mucous and some isolates had individualized colonies. In C4 isolate a peculiar blue pigmentation was also observed (table 3).

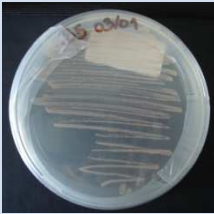
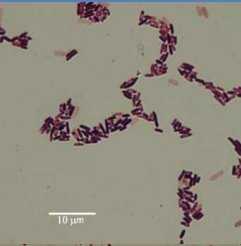

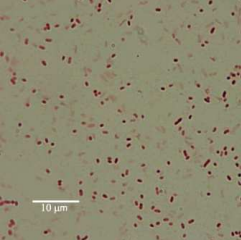

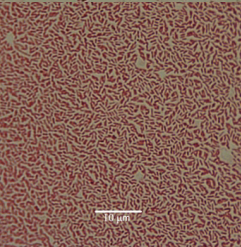

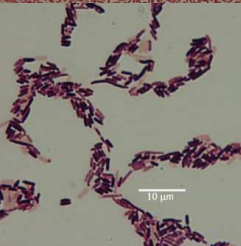
The bacterial shape was assessed in a microscopic slide with a suspension of bacterial cells from each isolated bacteria. Isolates were classified into *coccus*, *bacillus* and *cocobacillus* (table 2 and 3). One of the isolates was a “prosthecate” bacterium (table 3) and some isolates exhibited cells aggregation (table 2).


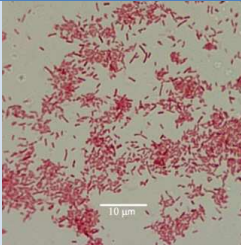
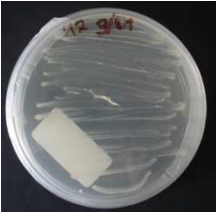
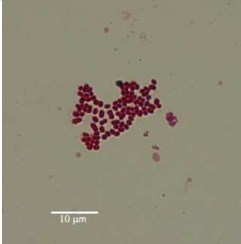
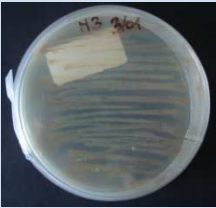
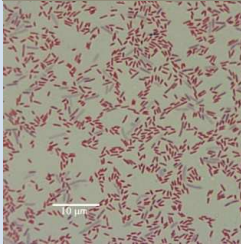

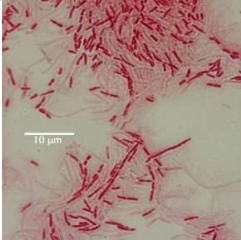
Furthermore, the isolates were divided into Gram-positive and Gram-negative using a microscope to observe the microscope slides prepared by a Previ™ color Gram (Biomérieux) which is an automated Gram stainer system (table 2 and 3).


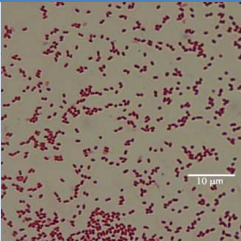

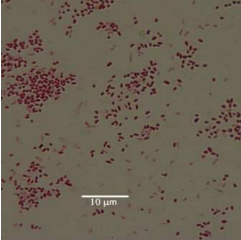
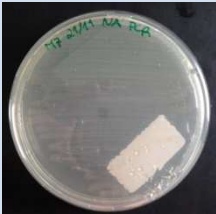
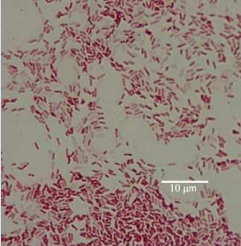

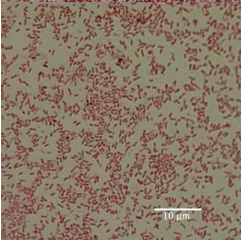
Bacterial sequences were compared in BLAST software and their molecular identification is showed in table 2 and 3.

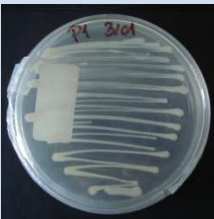
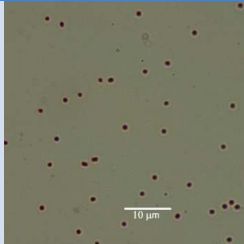
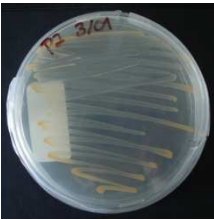
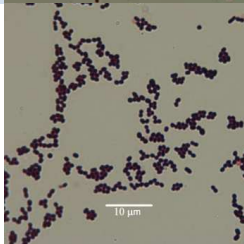

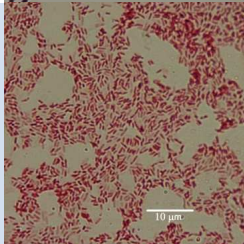
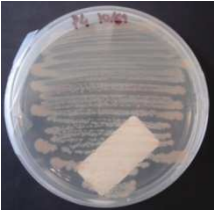
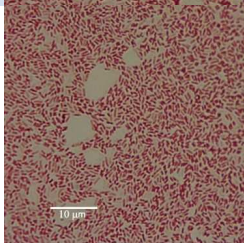
Table 2 - Major features of all 24 aquatic bacteria isolated from three Portuguese freshwater reservoirs: Albufeira de Monte da Barca (B), Magos (M) and Patudos (P). The isolates where the MCs impact on the bacterial antioxidant system enzymes was evaluated are highlighted. (*) – This parameter was registered after 8 days of growth in Nutrient Agar medium at 20°C. (**) - The white scale in the image indicates a length of 10 µm. (#) – BLAST molecular identification

Isolate	Molecular identification#	Colony color(*)	Macroscopic image(*)	Morphologic features(*)	Gram staining	Microscopic image(**)	Cell shape	Cells aggregation
B1	<i>Shewanella</i> sp.	Yellow		Mucous	–		<i>Bacillus</i>	No
B2	<i>Frigoribacterium</i> sp.	Pale yellow		Slightly mucous	+		<i>Coccus</i>	No
B3	<i>Aeromonas</i> sp.	White		Very mucous	–		<i>Coccobacillus</i>	No
B4	<i>Acidobacterium capsulatum</i>	Orange		Mucous	+		<i>Bacillus</i>	No

Isolate	Molecular identification#	Colony color(*)	Macroscopic image(*)	Morphologic features(*)	Gram staining	Microscopic image(**)	Cell shape	Cells aggregation
B5	<i>Bacillus vietnamensis</i>	Pale pink		Slightly mucous	+		<i>Bacillus</i> (spores observed)	Yes
B6	<i>Aeromonas veronii</i>	White		Mucous. Individualized colonies	-		<i>Coccus</i>	No
B7	<i>Anaeromyxobacter</i> sp.	Yellow		Mucous	-		<i>Bacillus</i>	No
B8	<i>Bacillus vietnamensis</i>	Pink		Slightly mucous	+		<i>Bacillus</i>	No

Isolate	Molecular identification#	Colony color(*)	Macroscopic image(*)	Morphologic features(*)	Gram staining	Microscopic image(**)	Cell shape	Cells aggregation
M1	<i>Bacillus vietnamensis</i>	Yellow		Mucous	-		<i>Bacillus</i>	No
M2	<i>Shewanella</i> sp.	White		Mucous	+		<i>Coccus</i>	No
M3	<i>Flavobacterium</i> sp.	Brown		Very mucous	-		<i>Bacillus</i>	No
M4	<i>Thioalkalivibrio nitratireducens</i>	White		Mucous	-		<i>Bacillus</i>	Yes

Isolate	Molecular identification [#]	Colony color(*)	Macroscopic image(*)	Morphologic features(*)	Gram staining	Microscopic image(**)	Cell shape	Cells aggregation
M5	<i>Aeromonas veronii</i>	Pale yellow		Very mucous. Individualized colonies	+		<i>Coccus</i>	No
M6	<i>Aeromonas</i> sp.	White		Mucous	+		<i>Coccus</i>	Yes
M7	<i>Shewanella xiamenensis</i>	Whitish		Mucous	-		<i>Coccus</i>	No
M8	<i>Amycolatopsis mediterranei</i>	Yellow		Mucous	-		<i>Bacillus</i>	No

Isolate	Molecular identification [#]	Colony color(*)	Macroscopic image(*)	Morphologic features(*)	Gram staining	Microscopic image(**)	Cell shape	Cells aggregation
P1	<i>Raoultella terrigena</i>	White		Mucous	+		<i>Coccus</i>	No
P2	<i>Exiguobacterium acetylicum</i>	Orange		Mucous	+		<i>Coccus</i>	Yes
P3	<i>Shewanella</i> sp.	Pale yellow		Mucous	-		<i>Bacillus</i>	No
P4	<i>Shewanella putrefaciens</i>	Pinkish		Mucous	-		<i>Bacillus</i>	No


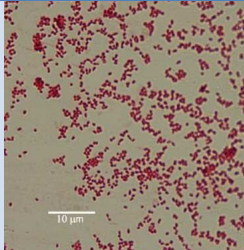
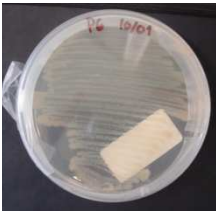
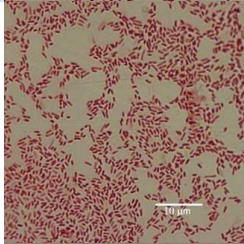

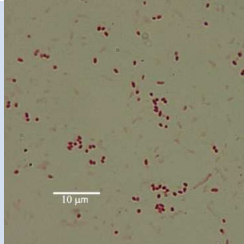
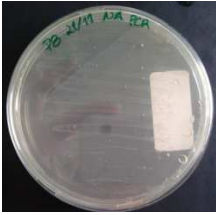
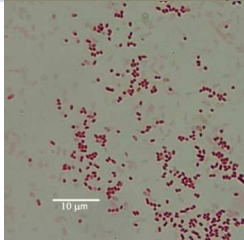

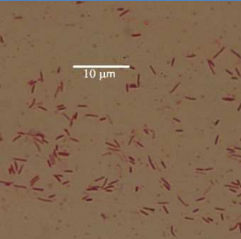

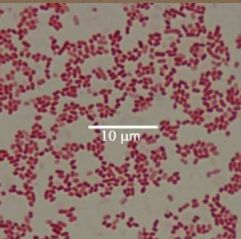

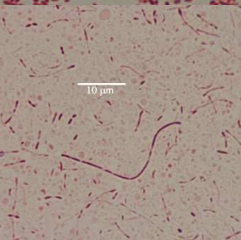

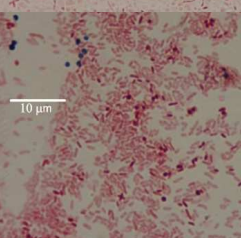
Isolate	Molecular identification [#]	Colony color(*)	Macroscopic image(*)	Morphologic features(*)	Gram staining	Microscopic image(**)	Cell shape	Cells aggregation
P5	<i>Sorangium cellulosum</i>	Yellow		Mucous	–		Coccus	No
P6	<i>Shewanella</i> sp.	Pale yellow		Mucous	–		Bacillus	No
P7	<i>Aeromonas jandaei</i>	Pale yellow		Mucous	–		Coccus	No
P8	<i>Pectobacterium carotovorum</i>	Pale yellow		Slightly mucous	–		Coccus	No

Table 3 - Major features of the four aquatic bacteria isolated from Albufeira de Castelo de Bode (C). The isolates where the MCs impact on the bacterial antioxidant system enzymes was evaluated are highlighted. (*) – This parameter was registered after 8 days of growth in Nutrient Agar medium at 20°C. (**) - The white scale in the image indicates a length of 10 µm. (#) – BLAST molecular identification.

Isolate	Molecular identification [#]	Colony color(*)	Macroscopic image(*)	Morphologic features(*)	Gram staining	Microscopic image(**)	Cell shape	Cells aggregation
C1	<i>Bradyrhizobium</i> sp.	Yellow		Mucous	–		<i>Bacillus</i>	No
C2	<i>Pseudomonas alkylphenolia</i>	White		Mucous	–		<i>Cocobacillus</i>	No
C3	<i>Flavobacterium</i> sp.	Yellow		Mucous	–		"Prosthecate" bacterium	No
C4	<i>Vogesella</i> sp.	Dark blue		Slightly mucous Blue pigmentation	–		<i>Bacillus</i>	No

3.2. Effects of microcystins on the bacterial growth

The isolates were exposed to three different concentrations (1 nM, 10 nM, 1 μ M or 0.3 μ M in MCYR cases)) of each microcystin variant (MCLR, MCRR and MCYR) and displayed several behaviors such as a growth reduction, no growth effect, different effects according to each concentration on the same variant and a growth stimulation compared to the control group, where no microcystins were added.

As evident from the growth graphs, there is no significant difference between the control cells and the microcystin exposure cells until they've reached late exponential phase, where it can be observed some effects on the growth, however in contrast, with the others isolates, B3 isolate was the only bacterium who had significantly statistic meaning ($p < 0.05$) since the beginning of the growth experiment.

In Fig. 6 is represented the isolates where a reduction on the growth was observed when compared to their control group for all the MCs concentrations tested. The isolates where a reduction on the growth was observed were B3, B6 and P1 in all the three MC variants. Isolates B1, M8, P3, P5, and P6 had a growth reduction with MCLR and MCRR concentration, and M1 and C2 with MCLR and MCYR. In all of these isolates, the reduction is little but significantly ($p < 0.05$). These cited isolates grown until reached an OD between 0.7 and 1.0 at 600 nm in the control group.

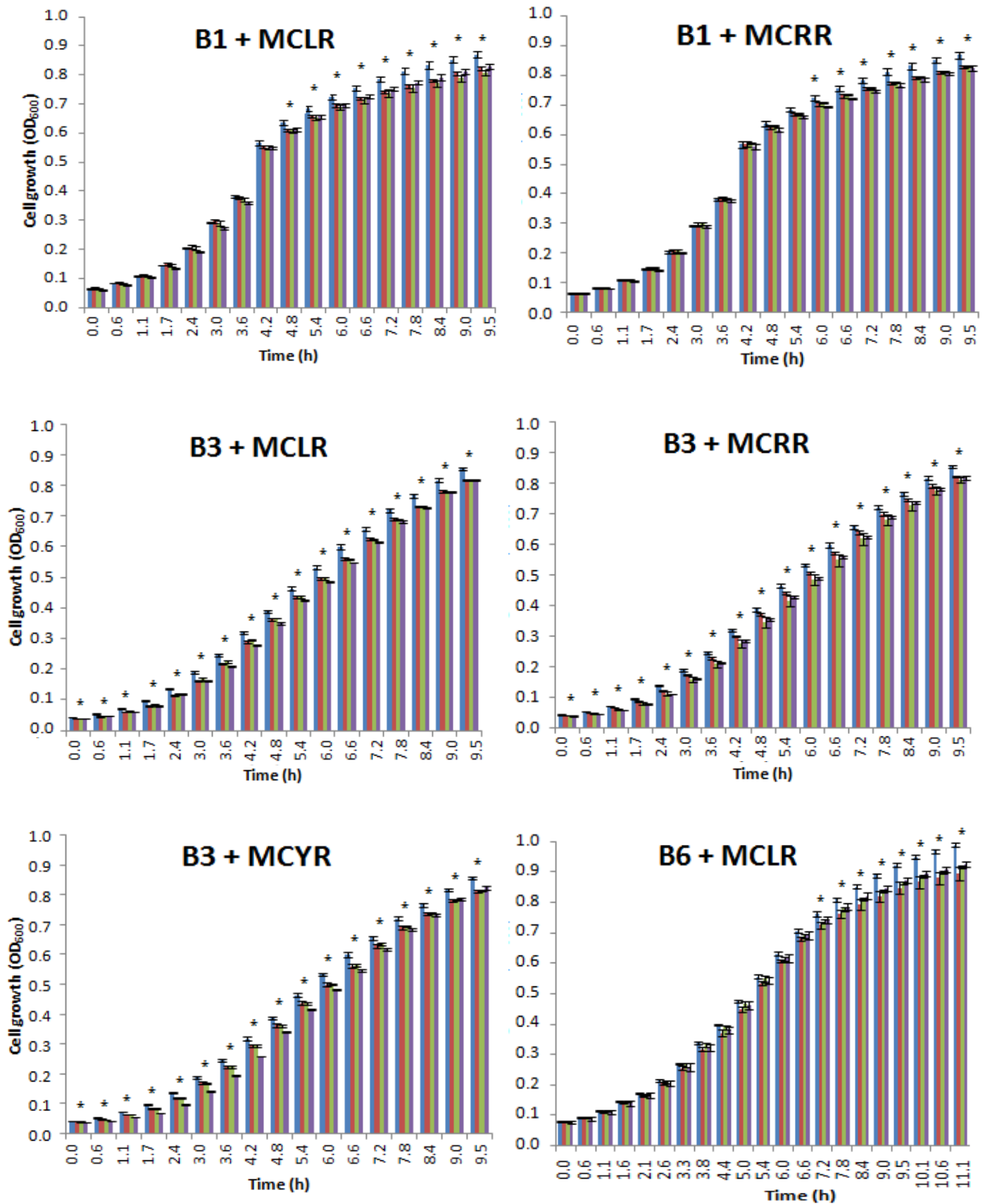


Figure 6 - Isolates where a growth reduction was observed in all the MCs concentrations tested. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD₆₀₀) and each bar represents means from five replicates with the respective standard error (means ± SE). (*) indicates a significant difference from the control (p < 0.05). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μM or 0.3 μM (in MCYR cases) exposure cells.

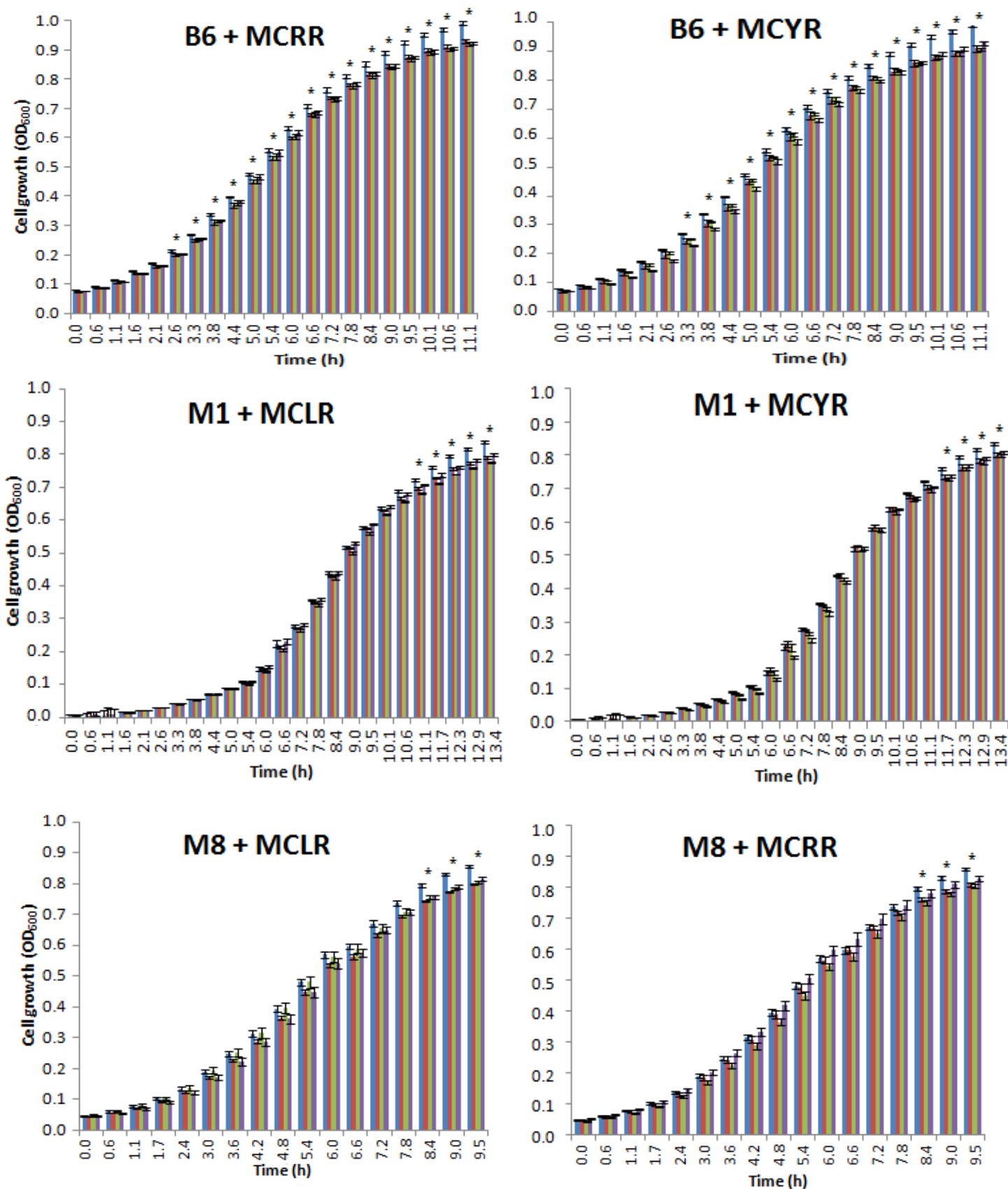


Figure 6 - Continuation. Isolates where a growth reduction was observed in all the MCs concentrations tested. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD₆₀₀) and each bar represents means from five replicates with the respective standard error (means \pm SE). (*) indicates a significant difference from the control ($p < 0.05$). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μ M or 0.3 μ M (in MCYR cases) exposure cells.

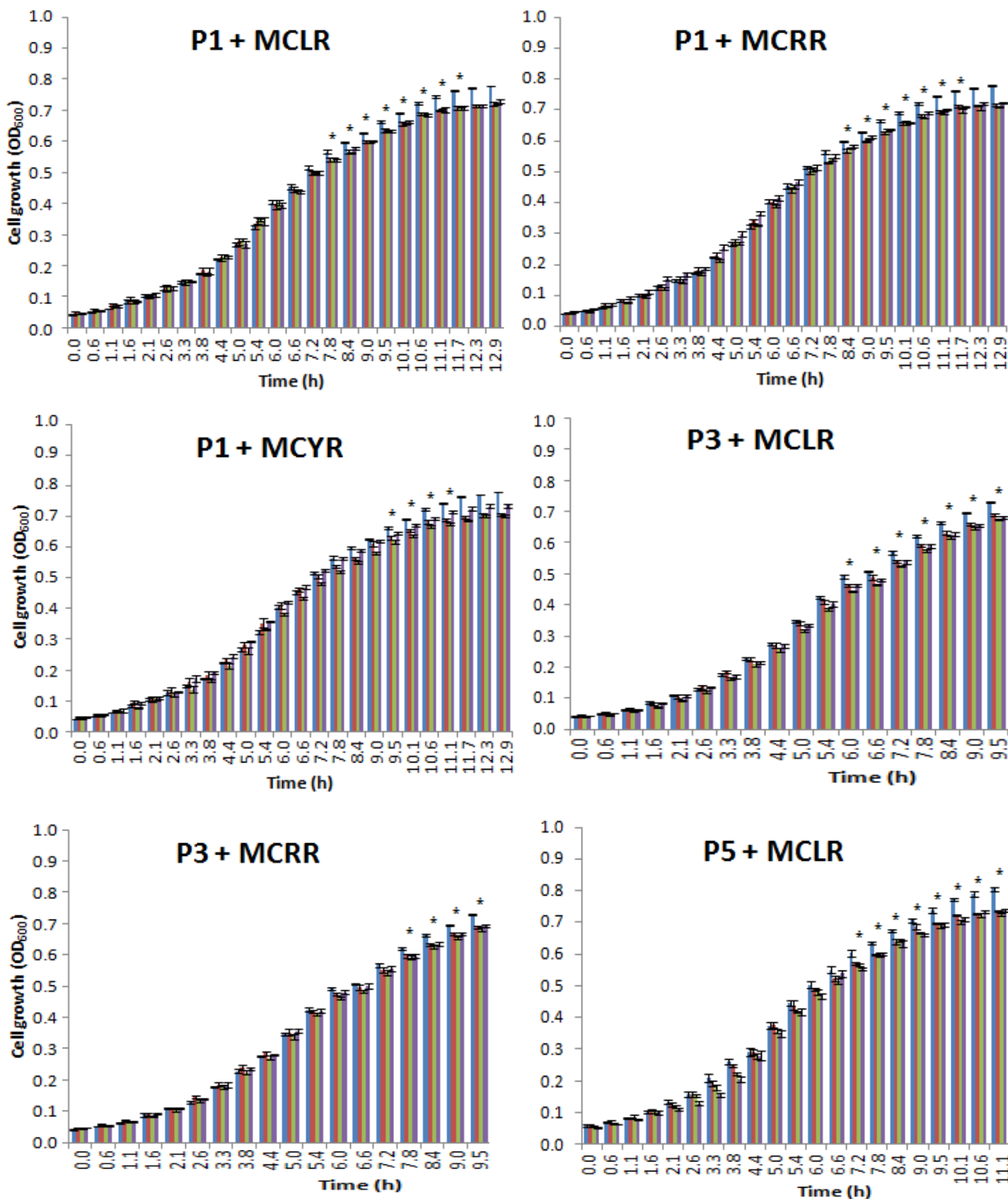


Figure 6 - Continuation. Isolates where a growth reduction was observed in all the MCs concentrations tested. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD₆₀₀) and each bar represents means from five replicates with the respective standard error (means ± SE). (*) indicates a significant difference from the control (p < 0.05). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μM or 0.3 μM (in MCYR cases) exposure cells.

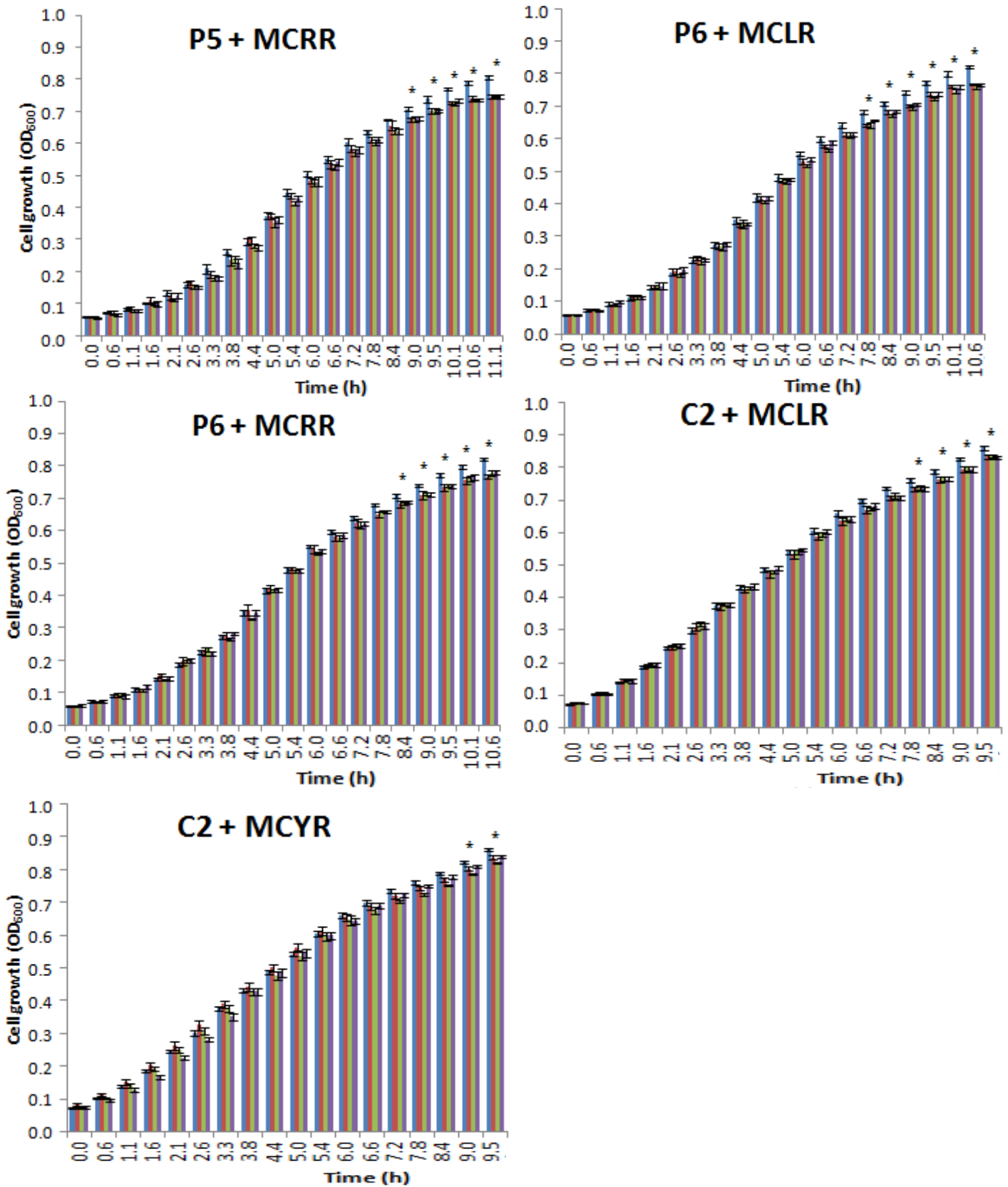


Figure 6 - Continuation. Isolates where a growth reduction was observed in all the MCs concentrations tested. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD₆₀₀) and each bar represents means from five replicates with the respective standard error (means \pm SE). (*) indicates a significant difference from the control ($p < 0.05$). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μ M or 0.3 μ M (in MCYR cases) exposure cells.

There were some isolates where no effects on the growth were observed. The isolates where there was no effect verified were B7, M5 and C4 for all the MCs variants tested; B4 and M2 with MCLR and MCRR, and C2 with MCRR (Fig. 7).

All of these bacterial isolates reached an optical density between 0.7 and 1.0, except M2 with MCLR and MCRR and M5 with all three variant microcystin that reached an optical density between 0.2 and 0.3 in the control group.

M2 isolated tested with MCLR was the only isolate in this category that had some significant meaning.

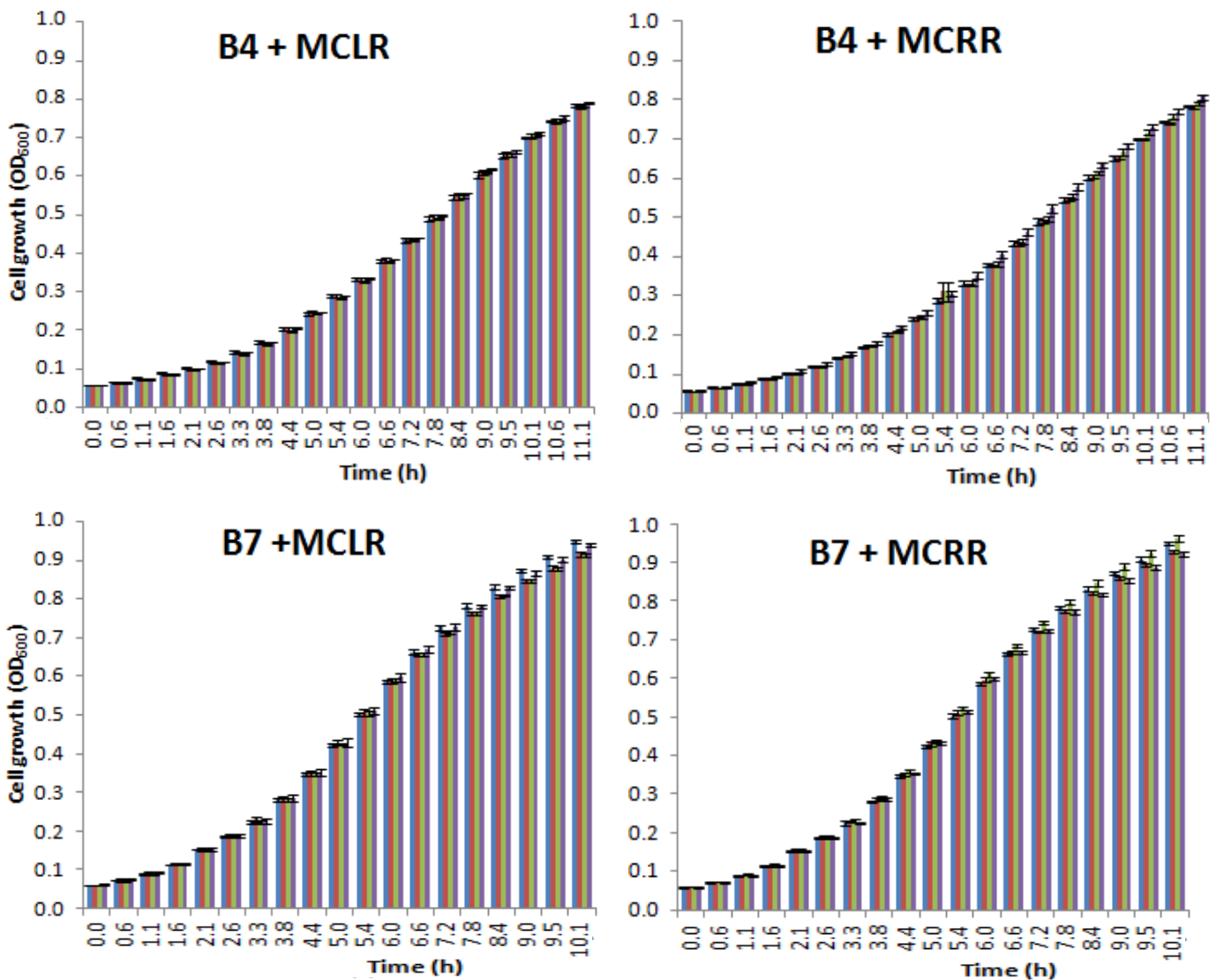


Figure 7 - Isolates where no effects on the growth rate has been observed after exposure to MCs. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD_{600}) and each bar represents means from five replicates with the respective standard error (means \pm SE). (*) indicates a significant difference from the control ($p < 0.05$). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μ M or 0.3 μ M (in MCYR cases) exposure cells.

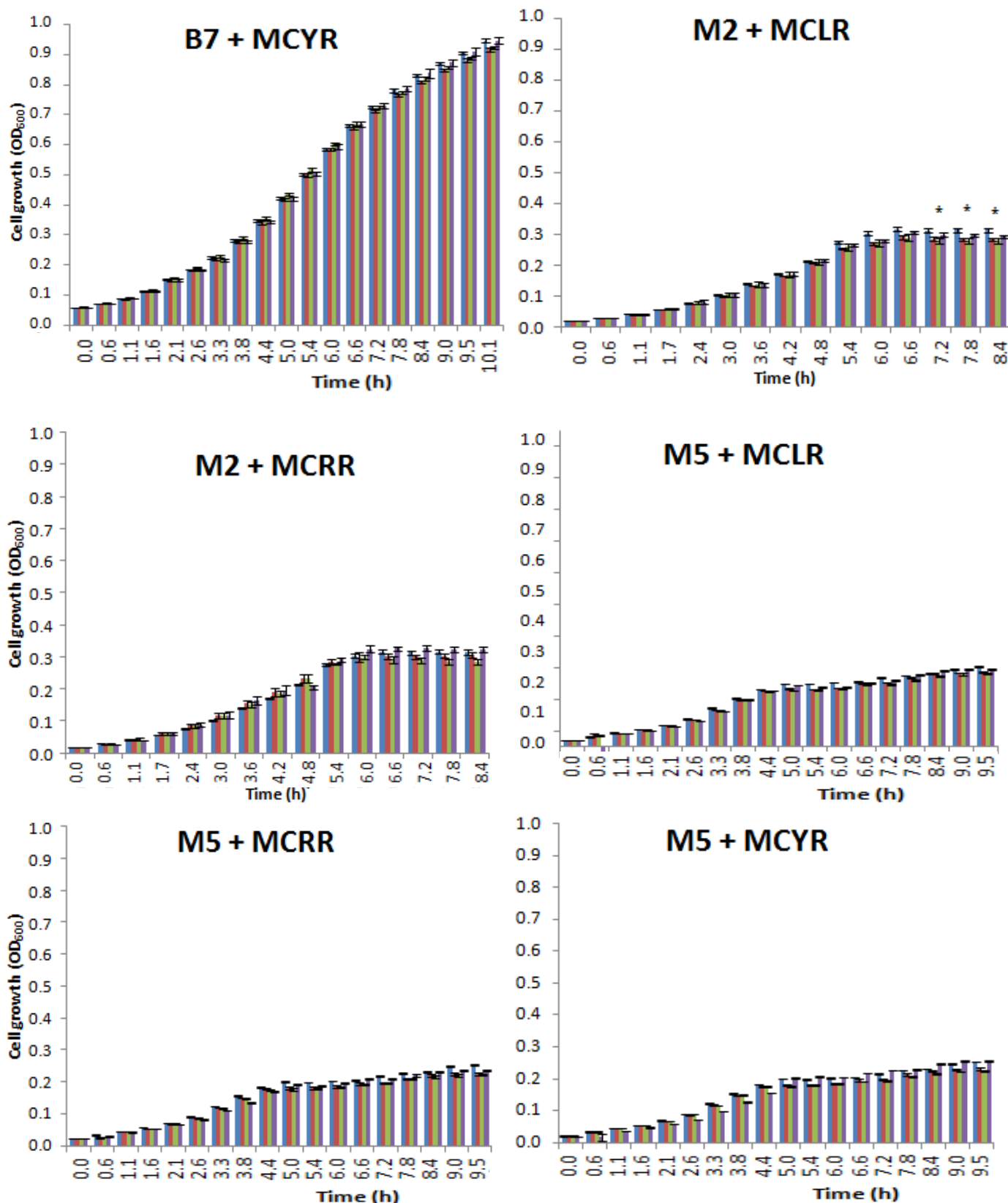


Figure 7 – Continuation. Isolates where no effects on the growth rate has been observed after exposure to MCs. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD₆₀₀) and each bar represents means from five replicates with the respective standard error (means ± SE). (*) indicates a significant difference from the control (p < 0.05). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μM or 0.3 μM (in MCYR cases) exposure cells.

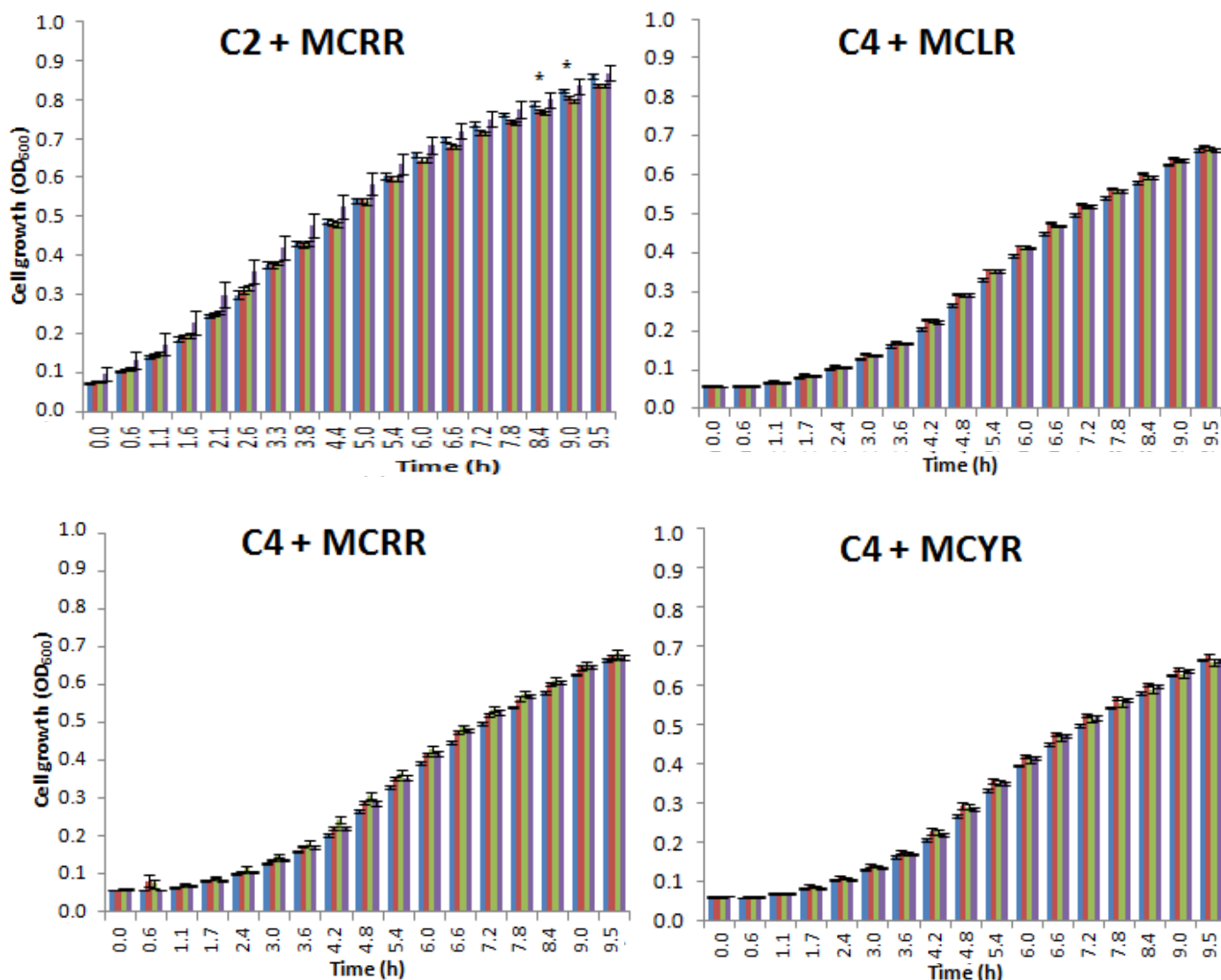


Figure 7 - Continuation. Isolates where no effects on the growth rate has been observed after exposure to MCs. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD₆₀₀) was measured and each bar represents means from five replicates with the respective standard error (means \pm SE). (*) indicates a significant difference from the control ($p < 0.05$). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μ M or 0.3 μ M (in MCYR cases) exposure cells.

Not all of the isolates respond to microcystin exposure with the same behavior in the three concentrations used for each microcystin variant. As so, some isolates showed different effects within the microcystin variant used, depending on the concentration exposure. The different effects showed were a growth reduction or no effect on growth (Fig. 8). Except for M1 with MCRR who showed no effects with 1 nM and 10 nM, and a reduction growth when exposed to the highest concentration (1 μ M) with significant meaning ($p < 0.05$), the others isolates B1, M8, P3, P5 and P6, all with MCYR showed that the highest concentration (0.3 μ M) produced no effect on the growth while the cells exposed to the other two concentrations had a reduction on the growth, with significant difference ($p < 0.05$).

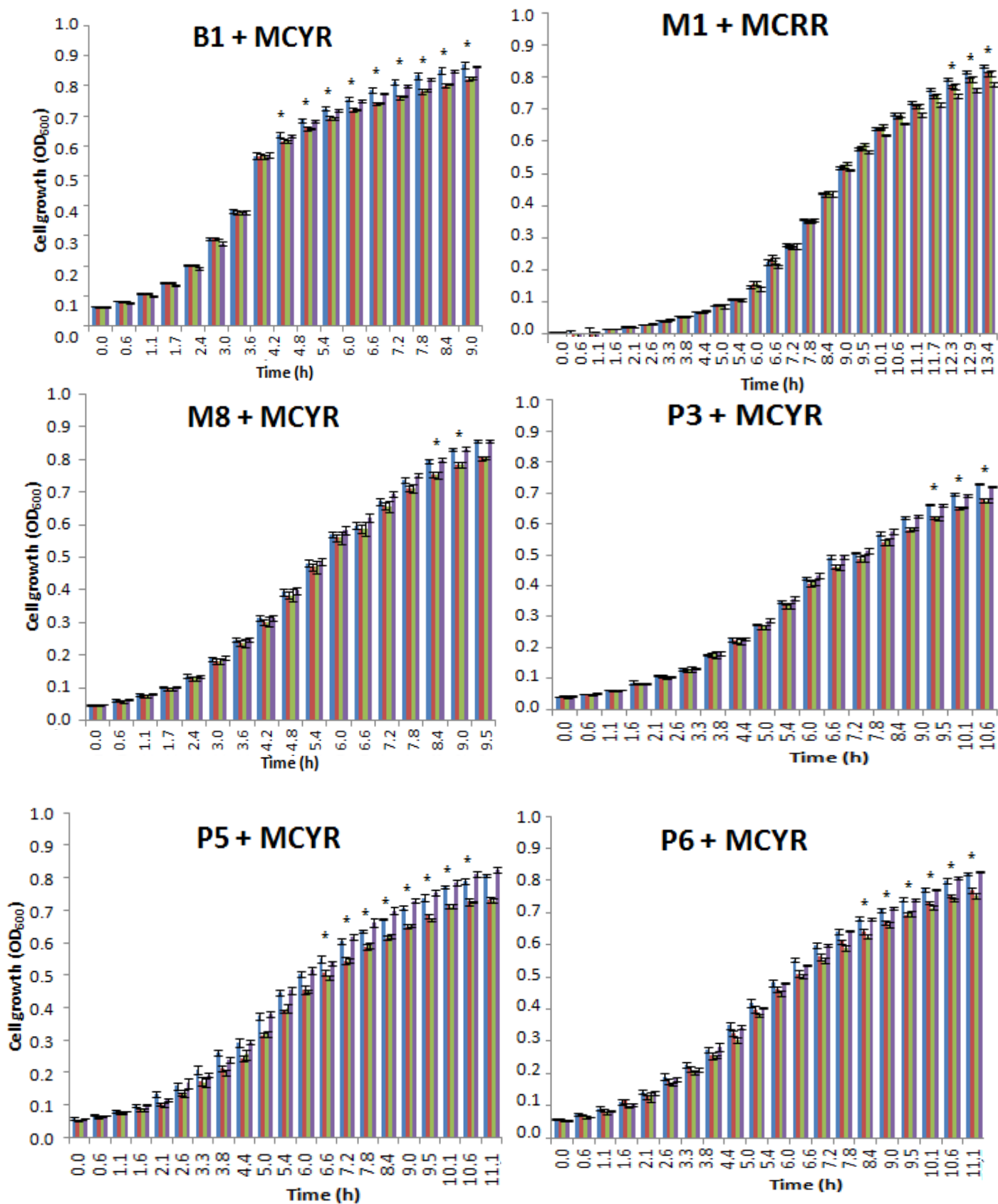


Figure 8 - Isolates where different effects on the growth rate have been observed according to the concentrations tested. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD₆₀₀) and each bar represents means from five replicates with the respective standard error (means ± SE). (*) indicates a significant difference from the control (p < 0.05). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μM or 0.3 μM (in MCYR cases) exposure cells.

There were two isolates where some growth stimulation was observed. In Fig. 9 are represented B4 with MCYR and M2 with MCYR which had a small growth stimulation when exposed to 0.3 μM . Nevertheless, these measurements had no significant meaning. The other two concentrations had no effects on the growth comparing to group control.

In respect to the optical densities reached, isolate B4 grown until 0.8 and isolate M2 grown until almost 0.4, both in the higher concentration where a stimulation growth is observed.

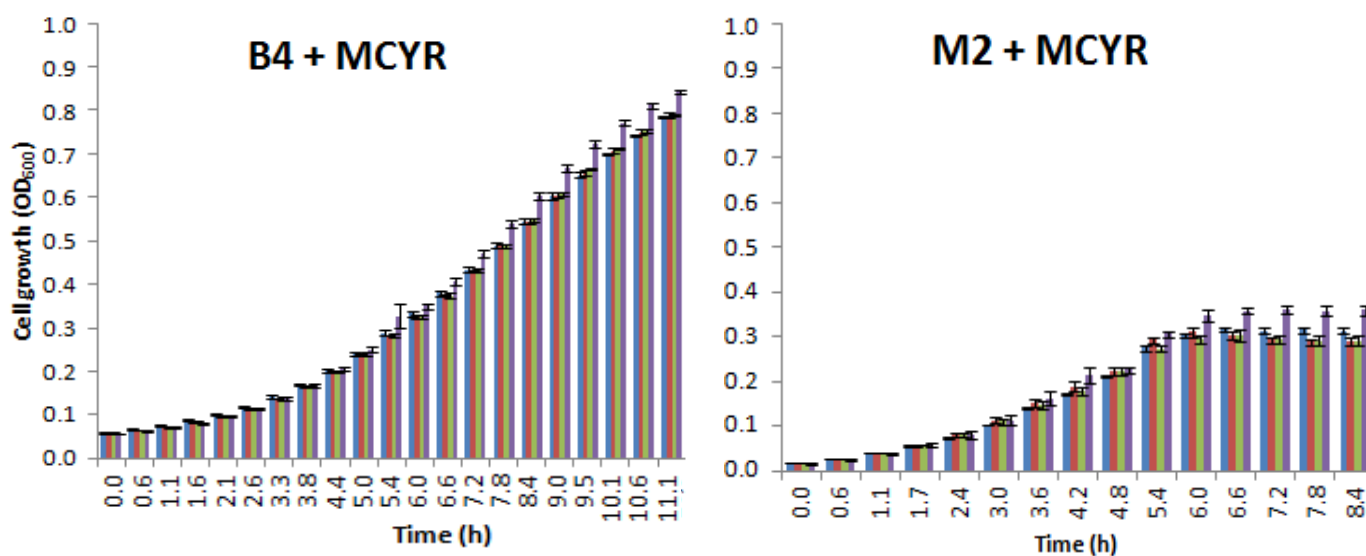


Figure 9 - Isolates where growth stimulation was observed in one of the MCs concentrations tested. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD_{600}) and each bar represents means from five replicates with the respective standard error (means \pm SE). (*) indicates a significant difference from the control ($p < 0.05$). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μM or 0.3 μM (in MCYR cases) exposure cells.

Some of the bacterial isolates didn't grow overnight in the liquid Nutrient broth medium or when inoculated in the microplate didn't development any growth. Those bacterial isolates were B2 (Fig. 10), B5, B8, M3, M4, M6, M7, P2, P4, P7, C1 and C3. All of them were tested twice in independent experiments, but either ways no growth development was obtained.

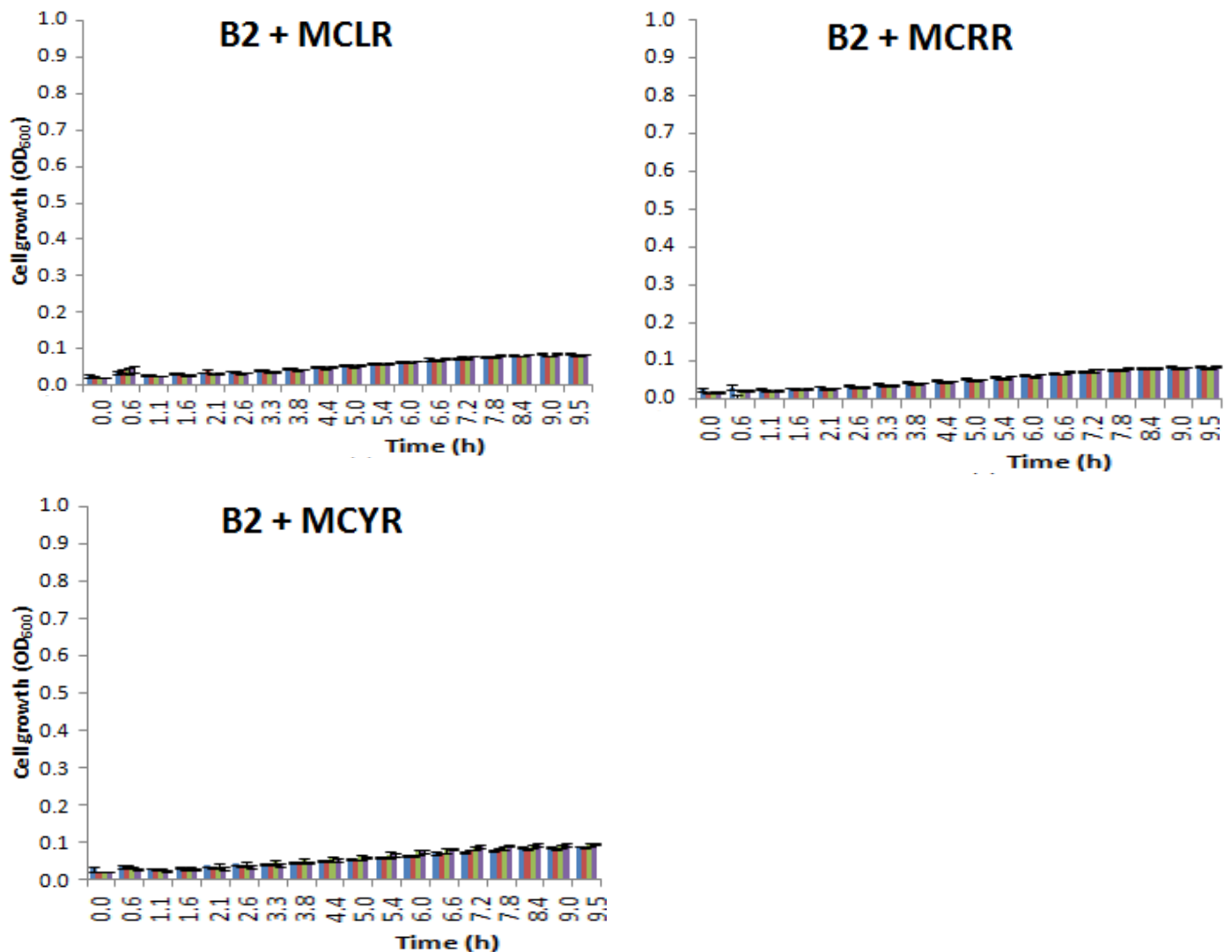


Figure 10 - Exemplification of an isolates that did not grow in the inoculated microplate. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD_{600}) and each bar represents means from five replicates with the respective standard error (means \pm SE). (*) indicates a significant difference from the control ($p < 0.05$). (■) Control bacterial group, (■) 1 nM microcystin exposure cells, (■) 10 nM microcystin exposure cells and (■) 1 μ M or 0.3 μ M (in MCYR cases) exposure cells.

After the growth experiments were performed for all of the isolates, two isolates were selected (B1 and M1), because these two have displayed exponential growth curves with clear lag, exponential and stationary phases and a significant growth effect when exposed to MC. These isolates were chosen to test if different initial optical densities (0.02, 0.05 and 0.1) would provide different effects on the growth. In Fig. 11 are represented the isolates B1 and M1 growth with MCLR. In both cases there are no significant differences on the effect observed between each initial OD bars. M1 and B1 isolates grew until OD measured passed 0.7 and the effect observed was always a growth reduction with significant meaning ($p < 0.05$).

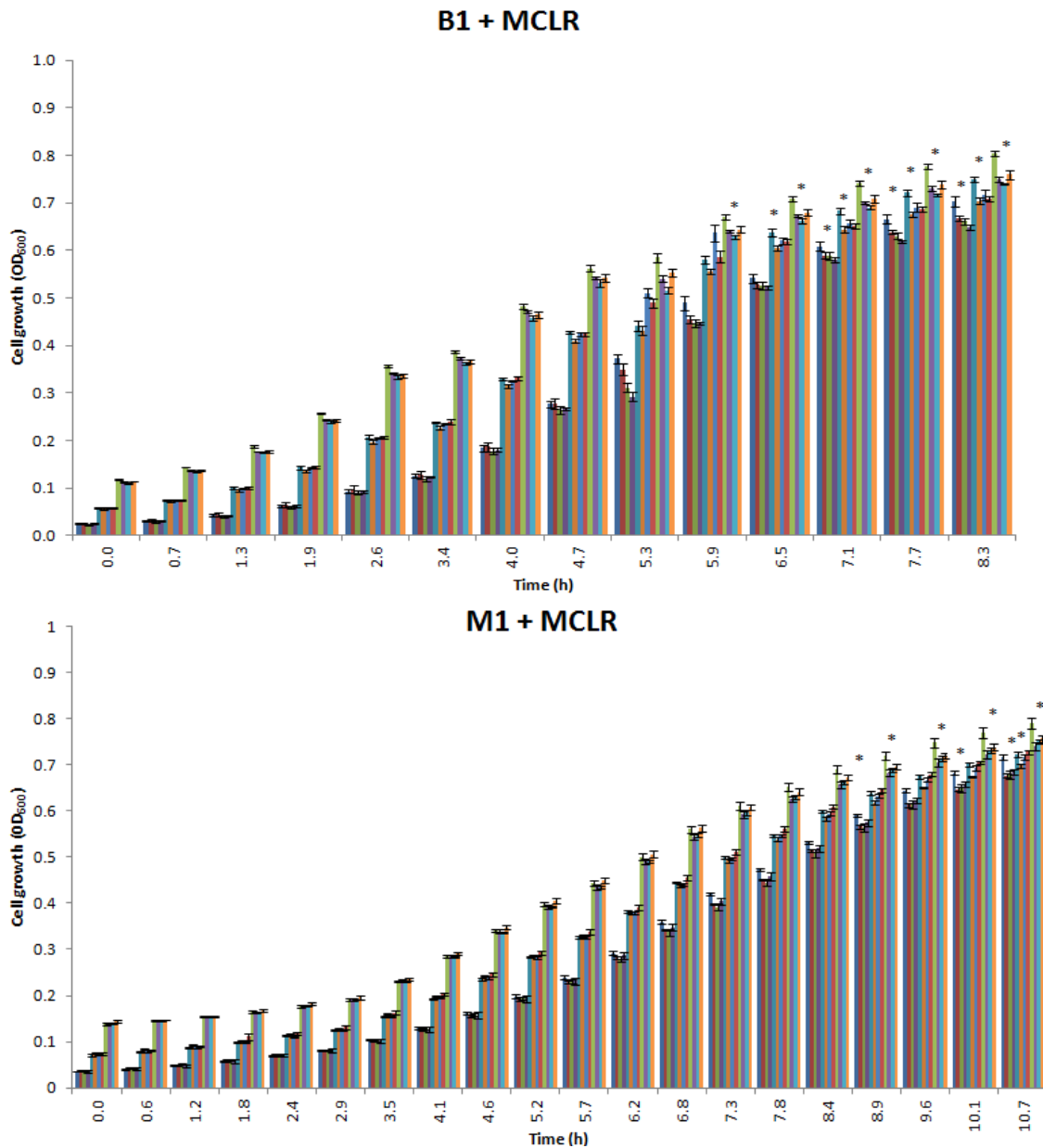


Figure 11 - Isolates where three different initial OD were tested with MCLR. Growth bars obtained for the bacterial isolates exposed to three different concentrations of each microcystin variant used. The optical densities were measured at 600nm (OD₆₀₀) and each bar represents means from five replicates with the respective standard error (means \pm SE). (*) indicates a significant difference from the control ($p < 0.05$). (■) Control bacterial group (ODi = 0.02), (■) 1 nM MCLR exposure cells (ODi = 0.02), (■) 10 nM MCLR exposure cells (ODi = 0.02) and (■) 1 μ M MCLR exposure cells (ODi = 0.02); (■) Control bacterial group (ODi = 0.05), (■) 1 nM MCLR exposure cells (ODi = 0.05), (■) 10 nM MCLR exposure cells (ODi = 0.05) and (■) 1 μ M MCLR exposure cells (ODi = 0.05); (■) Control bacterial group (ODi = 0.1), (■) 1 nM MCLR exposure cells (ODi = 0.1), (■) 10 nM MCLR exposure cells (ODi = 0.1) and (■) 1 μ M MCLR exposure cells (ODi = 0.1).

In order to evaluate if a higher concentration of MC could provide a higher impact on the bacterial growth, a growth experiment using 10 μ M of MCLR was conducted, using isolate B1. This had to be done with pure MCLR to obtain such a high concentration. As the results are displayed on Fig. 12, where we can observe that isolate B1 had no significant effect on the growth when exposed to this higher concentration.

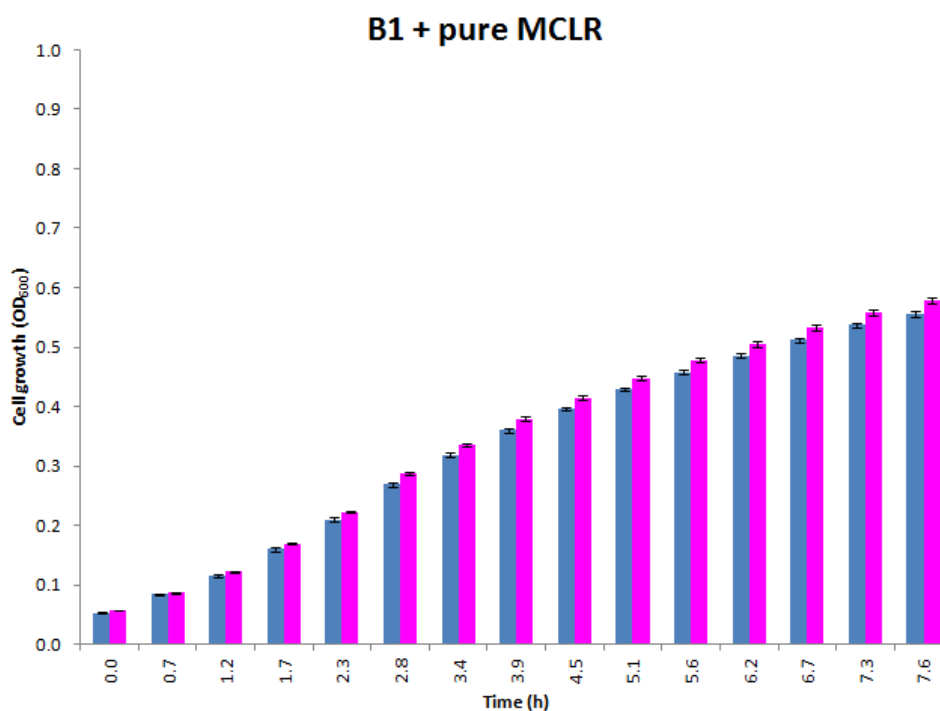


Figure 12 – B1 isolates exposed to 10 µM of pure MCLR. The optical densities were measured at 600nm (OD₆₀₀) and each bar represents means from five replicates with the respective standard error (means ± SE). (*) indicates a significant difference from the control (p < 0.05). (■) Control bacterial group, (■) 10 µM pure MCLR exposure cells.

3.3. Effects of microcystins on the bacterial antioxidant system

After 12 hours of incubation with each microcystin variant at a concentration of 10 nM, the CAT activity was measured as well as in control cells. In Fig. 13 is represented % of CAT activity increase relative to the control cells (not exposed to MCs) for the eight isolates chosen to perform this assay. All of the isolates tested had an increased CAT activity when exposed to each variant microcystin but all with different values between each other. B1, P6 and C2 when exposed to MCLR the CAT activity increase was higher when compared to the two other variants. B3, M1 and P1 showed the highest increase when exposed to MCRR. M8 and C4 had the exact same increase in cells exposed to two variants. M8 had the same increased value with MCLR and MCRR (1000%), however C4 and the same effect but with MCLR and MCYR. P6 and C2 when exposed to MCRR showed the lowest (< 50%) CAT activity increased when compared to the other two variants. However, P1 with same concentration of MCRR had the highest increase (2500%) when compared to the other to variants, and when compared with all the isolates tested.

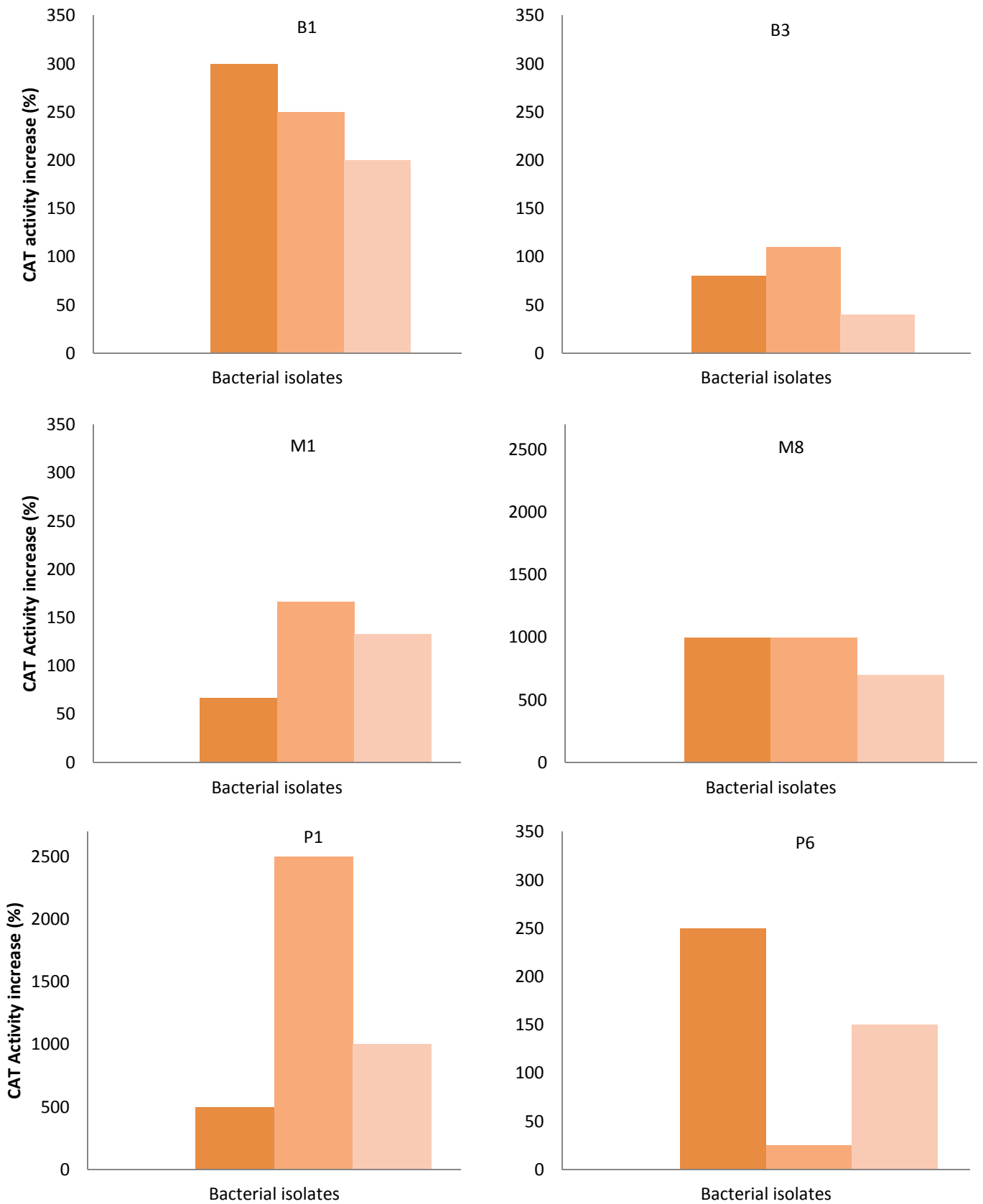


Figure 13 - Isolates where CAT activity was measured according to the variant microcystin tested at 10 nM concentration. The bars obtained for the bacterial isolates indicate the increase % of CAT activity when exposed to each microcystin variant relative to the control group. (■) 10 nM MCLR exposure cells, (■) 10 nM MCRR exposure cells and (■) 10 nM MCYR exposure cells.

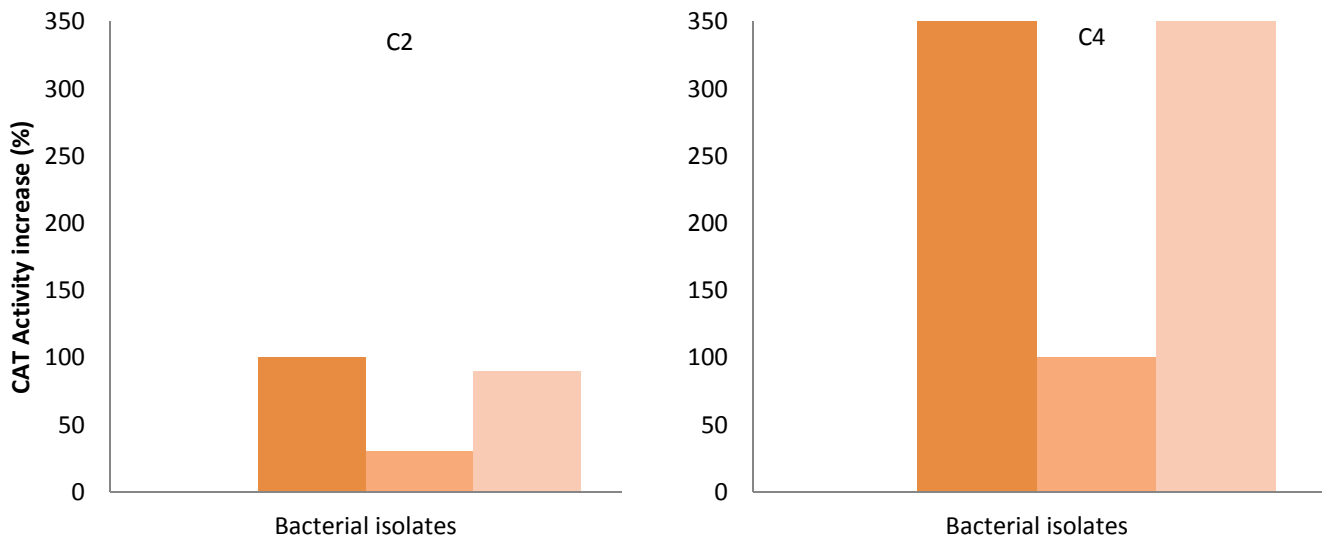


Figure 13 – Continuation. Isolates where CAT activity was measured according to the variant microcystin tested at 10 nM concentration. The bars obtained for the bacterial isolates indicate the increase % of CAT activity when exposed to each microcystin variant relative to the control group. (■) 10 nM MCLR exposure cells, (■) 10 nM MCRR exposure cells and (■) 10 nM MCYR exposure cells.

After 12 hours of incubation with each variant microcystin at a concentration of 10 nM, the SOD1 and SOD2 activity inhibition were measured and in Fig. 14 and in Fig 15 are represented % of SOD1 activity and of SOD2 activity for the eight isolates chosen to do this assay, respectively. In SOD1 and SOD2 activities were observed two effects that could be a negative SOD inhibition which means an increase in the ROS content, and a positive SOD inhibition which means an increase of SOD.

In respect to SOD1 activity inhibition, isolates who had only a positive % inhibition were B1 and C2. B1 had an inhibition effect with MCLR but no effect with MCRR and MCYR on SOD1 activity. C2 isolate had inhibition effect with MCLR and MCRR and no effect with MCYR.

M1 and P1 isolates had only a negative % inhibition of SOD1 activity. M1 had effect with the three microcystin variants. However, P1 only had an effect with MCLR.

The remaining isolates, B3, M8 and P6 had negative and positive inhibitions effects on SOD1 activity. B3 had the same negative effect with MCLR and MCRR, but with MCYR had a positive inhibition effect. M8 had a negative inhibition with MCLR and MCYR, and a positive with MCRR. P6 isolate had the same positive inhibition % effect with MCLR and MCRR and with MCYR had a negative inhibition effect.

In C4 isolate it was not possible to determine SOD1 activity inhibition because the isolate did not grow enough overnight in NB medium and the volume of the sample was less than 15 µl (which was not enough to perform the assay).

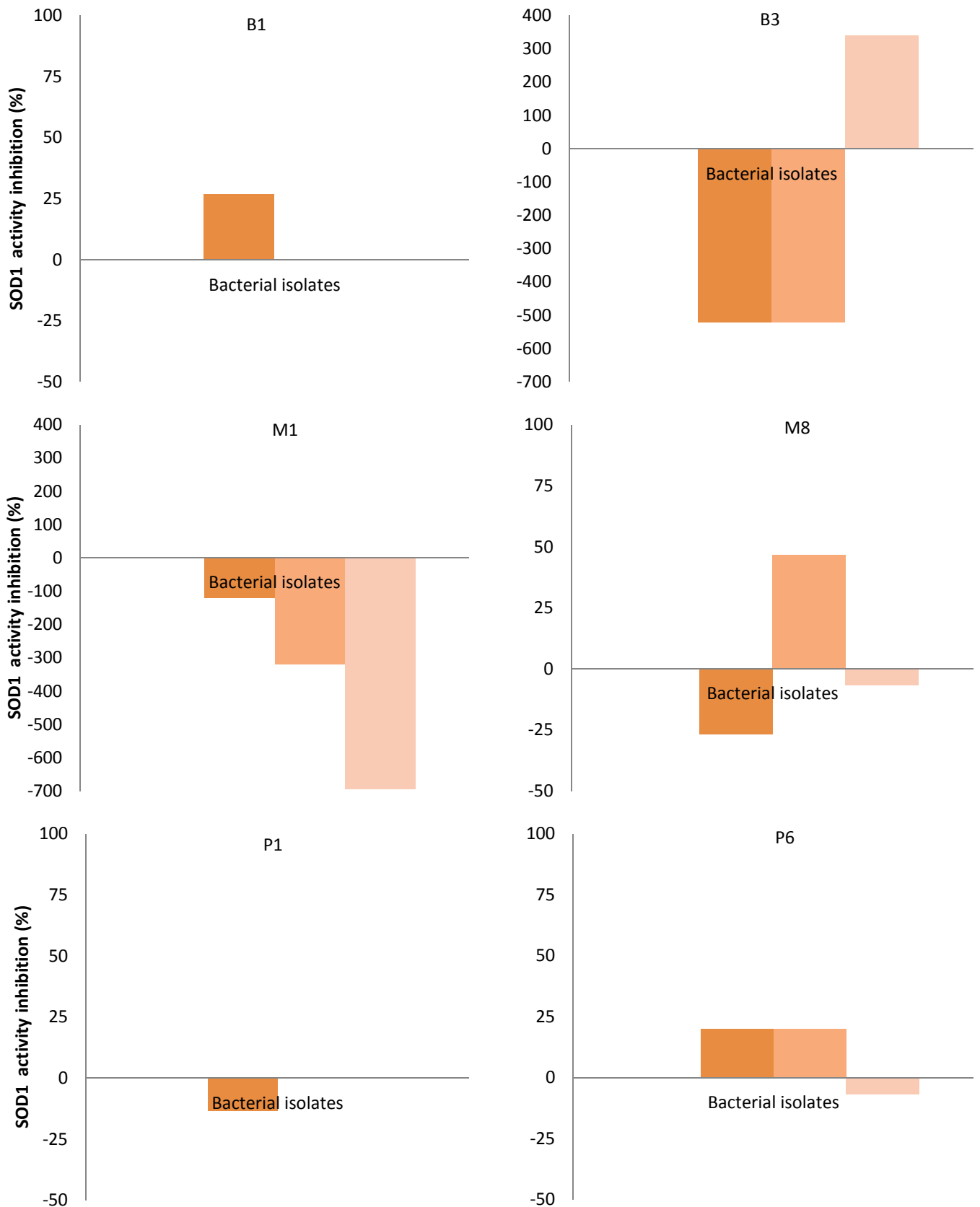


Figure 14 – Isolates where SOD1 activity was measured according to the variant microcystin tested at 10 nM concentration. The bars obtained for the bacterial isolates indicate the inhibition % of SOD1 activity when exposed to each microcystin variant relative to the control group. (■) 10 nM MCLR exposure cells, (■) 10 nM MCRR exposure cells and (■) 10 nM MCYR exposure cells.

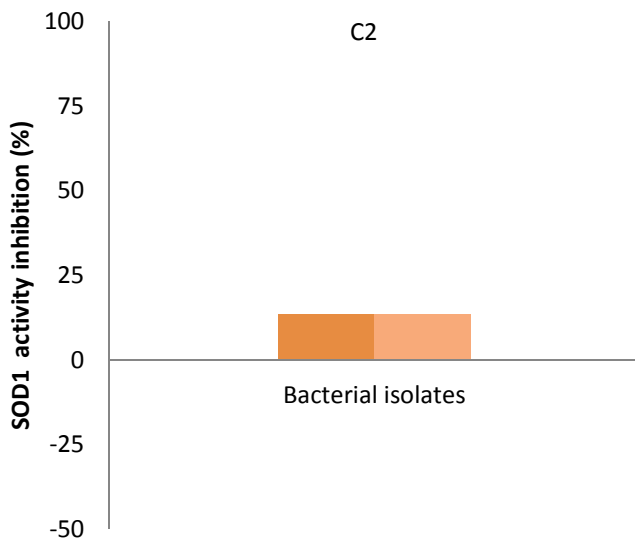


Figure 14 – Continuation. Isolates where SOD1 activity was measured according to the variant microcystin tested at 10 nM concentration. The bars obtained for the bacterial isolates indicate the inhibition % of SOD1 activity when exposed to each microcystin variant relative to the control group. (■) 10 nM MCLR exposure cells, (■) 10 nM MCRR exposure cells and (■) 10 nM MCYR exposure cells.

For SOD2 activity inhibition, isolates who had only a negative % inhibition were B1, M8, P1 and C2. B1 had an inhibition effect with MCLR and with MCRY and no effect with MCRR on SOD2 activity. M8 isolate had also negative %inhibition with MCRR and MCYR, and no effect with MCLR. P1 isolate had a negative effect on SOD2 activity with MCRR and MCYR and no effect with MCLR. C2 isolate had the same rate of inhibition with all three variants.

M1 had only a positive % inhibition of SOD2 activity. M1 had effect with the three variant microcystin.

The remaining isolates, B3 and P6 had negative and positive inhibitions effects on SOD2 activity. B3 had a negative effect on SOD2 activity with MCRR and a positive one with MCYR, and with MCLR it had no inhibition effect. P6 had the same rate of negative inhibition with MCLR and MCRR, and a positive one with MCYR.

In C4 isolate it was not possible to determine SOD2 activity inhibition because the isolate did not grew enough overnight in NB medium and the volume of the sample was not enough to perform the assay.

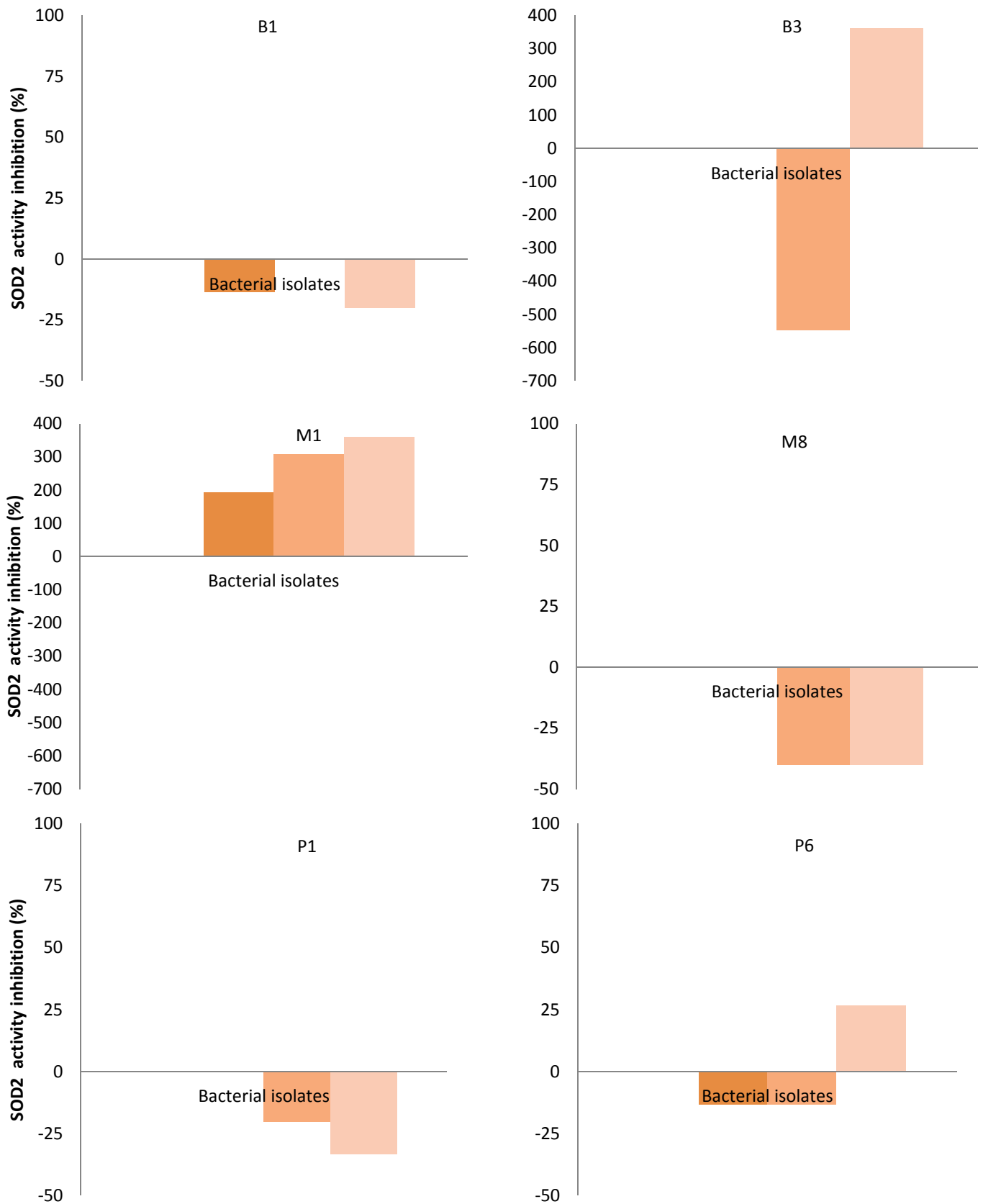


Figure 15 – Isolates where SOD2 activity was measured according to the variant microcystin tested at 10 nM concentration. The bars obtained for the bacterial isolates indicate the inhibition % of SOD2 activity when exposed to each microcystin variant relative to the control group. (■) 10 nM MCLR exposure cells, (■) 10 nM MCRR exposure cells and (■) 10 nM MCYR exposure cells.

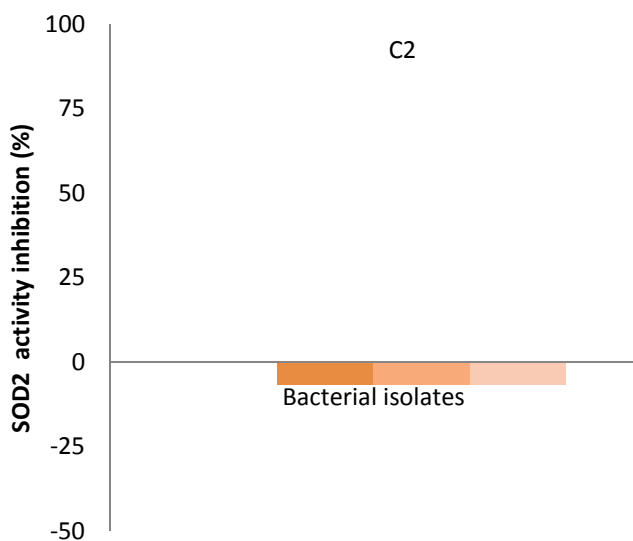


Figure 15 – Continuation. Isolates where SOD2 activity was measured according to the variant microcystin tested at 10 nM concentration. The bars obtained for the bacterial isolates indicate the inhibition % of SOD2 activity when exposed to each variant microcystin relative to the control group. (■) 10 nM MCLR exposure cells, (■) 10 nM MCRR exposure cells and (■) 10 nM MCYR exposure cells.

4. DISCUSSION

4.1. Characteristics of the isolated bacteria

The bacterial isolates were morphologically and molecularly characterized. These bacteria were picked randomly taking into account the morphological features and color of the colonies revealed in R2A agar medium and LB agar medium as showed in table 2 and 3, in order to obtain a manageable number of isolates to perform all the subsequent analyses but that could be representative of the macroscopic diversity observed.

Colony colors observed were yellow in B1, B7, M1, M8 P5, C1 and C3; pale yellow in isolates B2, M5, P3, P6, P7 and P8; white in B3, B6, M2, M4, M6, P1 and C2; orange in B4 and P2; pale pink in B5; Pink in B8; brown in M3; whitish in M7; pinkish in P4 and dark blue in C4. It was observed that this C4 isolate when grown in NB medium formed some blue pigments that were also observed microscopically (table 3).

From the 28 isolates, 32% were Gram-positive bacteria which are isolates B2, B4, B5, B8, M2, M5, M6, P1 and P2. The remaining 68% were Gram-negative bacteria which are B1, B3, B6, B7, M1, M3, M4, M7, M8, P3, P4, P5, P6, P7, P8, C1, C2, C3 and C4 (table 2 and 3). These isolates were classified into *coccus* (11 isolates), *bacillus* (14

isolates) and *cocobacillus* (2 isolates) and C3 isolate was a “prosthecate” bacterium (table 3). This “prosthecate” bacterium has appendages, termed *prosthecae* which are not neither pili nor flagella, as they are extensions of the cellular membrane and contain cytosol (Madigan *et al.*, 2012).

Bacterial sequences were compared with the available ones in public databases using BLAST software and their molecular identification assessed. The major bacterial divisions of freshwater heterotrophic bacteria are *Alpha*- and *Betaproteobacteria*, *Actinobacteria*, *Flavobacteria*, *Verrumicrobia* and *Gammaproteobacteria* (Berg, 2009). Thus, the majority of the bacteria isolated from the four Portuguese reservoirs belong to these Classes. According to Boone *et al.*, (2001) isolates B1 (*Shewanella* sp.), B3 (*Aeromonas* sp.), B6 (*Aeromonas veronii*), M2 (*Shewanella* sp.), M4 (*Thioalkalivibrio nitrareducens*), M5 (*Aeromonas veronii*), M6 (*Aeromonas* sp.), M7 (*Shewanella xiamenensis*), P1 (*Raoultella terrigena*), P3 (*Shewanella* sp.), P4 (*Shewanella putrefaciens*), P6 (*Shewanella* sp.), P7 (*Aeromonas jandaei*), P8 (*Pectobacterium carotovorum*) and C2 (*Pseudomonas alkylphenolia*) belong to *Phylum Proteobacteria*, *Class Gammaproteobacteria*; Isolate C1 (*Bradyrhizobium* sp.) to class *Alphaproteobacteria*; C4 (*Vogesella* sp.) to class *Betaproteobacteria*; B2 (*Frigoribacterium* sp.) and M8 (*Amycolatopsis mediterranei*) to *Phylum Actinobacteria*; M3 (*Flavobacterium* sp.) and C3 (*Flavobacterium* sp.) to *Phylum Bacteroidetes*, class *Flavobacteria*. All of the cited isolates belong to bacterial group divisions which are dominant in freshwater reservoirs, except for B4 (*Acidobacterium capsulatum*) which belongs to *Phylum Acidobacteria*, isolates B7 (*Anaeromyxobacter* sp.) and P5 (*Sorangium cellulosum*) that belong to class *Deltaproteobacteria* and B5 (*Bacillus vietnamensis*), B8 (*Bacillus vietnamensis*), M1 (*Bacillus vietnamensis*) and P2 (*Exiguobacterium acetylicum*) which belong to *Phylum Firmicutes*.

In summary, five bacterial isolates belong to genus *Aeromonas*, six to genus *Shewanella*, three isolates to genus *Bacillus*, two isolates to genus *Flavobacterium* and the remaining isolates belong to a different genus each one. *Aeromonas* and *Shewanella* were found in three of the four reservoirs sampled.

4.2. Effects of microcystins on the bacterial growth

Except B3 who displayed differences on the growth curves with significantly statistic meaning ($p < 0.05$) since the beginning of the growth experiment, the growth

graphs only presented significant differences between the control cells and the microcystin exposed cells after they have reached late exponential phase.

It was found that MC can reduce the growth of most bacteria tested (62.5%) when compared to their control group for all the MCs concentrations tested (Fig. 6). These results showed that MC can reduce growth on these bacteria tested, but in comparison with other authors' results, these ones are not as drastic as observed by Hu *et al.* (2005) that showed that MCRR (100 nM) drastically inhibited the growth of cyanobacterium *Synechococcus elongates*. The variant MCRR also made the cyanobacterial cells display a prolonged lag growth phase when compared with the control (Hu *et al.*, 2005). However in the present study, MCs did not affect the lag phase of any of the isolates tested. M1 with MCLR and with MCRR had a longer lag phase because cells were inoculated, by accident, with an initial OD < 0.05. Begot *et al.*, (1996) showed that bacteria adapted their cellular components to the new temperature during latency, and this explains why some bacteria have a longer lag phase than other bacteria.

There were some isolates (37.5%), where no effects on the growth were observed (Fig. 7). These isolates were not affected by MCs maybe because they have genetic features that prevent the MC entrance in the bacterial cell, which is not directly related to this isolates type of wall, since 50% are Gram – and the other 50% are Gram +, not to these isolates genera, since they all belong to different ones. C2 is an isolate from a freshwater reservoir where it is not frequent to observe cyanobacterial blooms. However, in this bacterium there was also no effect on the growth when exposed to MC, exactly the same effect has other bacteria isolated from a freshwater reservoir where cyanobacterial blooms are frequent.

So, we did not observe significant differences of MCs effect on the growth between bacteria that co-inhabit with cyanobacterial toxins from bacteria that do not inhabit ecosystems that usually have MC-producing cyanobacteria.

All of these bacterial isolates reached an optical density between 0.7 and 1.0, except M2 with MCLR ($p < 0.05$) and MCRR and M5 with all three variant microcystin that reached an optical density between 0.2 and 0.3 in the control group. That fact can be explained by a lack of some kind of nutrient that would be essential to these bacterial growths.

Yang *et al.* (2008) observed that *E. coli* only showed growth inhibition at the initial growth phase when cells were treated with MCRR (1, 5, 10 and 15 μM). The normal rate of growth was re-established and the growth curves of treated and untreated

bacteria became parallel, only showing a reduction of the growth when exposed to 1 and 5 μM , and displaying a marked inhibition for the higher concentrations tested. In this study, MC did not inhibit bacterial growth, but only reduced it, probably because the bacteria tested are less susceptible to MC as the *E. coli* tested by Yang *et al.* (2008). Moreover, except for isolate B1, where a higher MCLR concentration was tested (10 μM) without differences from the control (Fig. 12), all the other growth experiments were made with MCs closest to the lowest one tested by Yang *et al.* (2008), which also did not display marked differences at the end of the growth curves.

Not all of the isolates respond to microcystin exposure with the same behavior in three concentrations used for the microcystin variants -RR and mostly -YR. As so, some isolates showed different effects within the variant microcystin used, usually a growth reduction and no effect on growth (Fig. 8). Isolates B1, M8, P3, P5 and P6, all with MCYR showed that the highest concentration (0.3 μM) produced no effect on the growth and the other two concentrations exposed cells had a reduction growth with significant difference ($p < 0.05$). These results point out that MCRR and MCYR may produce different effects depending on the concentration used. Nevertheless, to clarify this issue more research is needed to prove if, in fact, higher concentrations of MCRR and MCYR don't affect these bacteria growth.

There were two isolates where some growth stimulation was observed (Fig. 9). Isolates B4 and M2 with MCYR which had a small growth stimulation when exposed to 0.3 μM . Nevertheless, this growth stimulation was observed with no significant meaning. The other two concentrations had no effects on the growth comparing to group control. There are known strains of bacteria which are able to degrade cyanobacterial toxins and belong to the class *Alphaproteobacteria* and *Betaproteobacteria* (Jones *et al.*, 1994; Park *et al.*, 2001; Saito *et al.*, 2003; Ishii *et al.*, 2004; Rapala *et al.*, 2005; Amé *et al.*, 2006) and especially the *Flavobacterium* strains (Berg *et al.*, 2009). Although B4 (*Acidobacterium capsulatum*) and M2 (*Shewanella* sp.) isolates do not belong to any of those known MC-degrading classes, there is a remote hypothesis that these isolates could also degrade MC and, so that MC could stimulate bacterial growth. However to elucidate this issue, it would be necessary to verify these strains ability to degrade MC

Some of the bacterial isolates (12) didn't grow overnight in the liquid Nutrient broth medium or when inoculated in the microplate didn't development any growth (Fig. 10). One can speculate that this was because the isolates can't grow in liquid

medium and it has been verified the same problem with other microorganism such as yeast (personal communication, E. Valério). They probably have some problem growing in liquid medium or in the microplates plastic material because these bacteria grew in NA.

In order to evaluate if the absence of a marked effect on the bacterial growth could be somehow related to the inoculum, two isolates were selected (B1 and M1) to test different initial optical densities (0.02, 0.05 and 0.1). In both cases there are no significant differences on the effect observed between each initial OD bars (Fig. 11), thus, showing that the results displayed were not affected by the inoculum concentration.

4.3. Effects of microcystins on the bacterial antioxidant system

In previous studies, it has been found that microcystins could induce oxidative stress in animals, plants and few microorganisms (Jos *et al.*, 2005; Hu *et al.*, 2005; Yin *et al.*, 2005; Vassilakaki and Pflugmacher, 2008; Yang *et al.*, 2008). In this study, to assess enzymatic CAT and SOD activities a 10 nM concentration of each microcystin variant was chosen, because it was the concentration that produces the most pronounced growth effect on these bacteria.

Some evidence point to alternative mechanisms of toxicity for microcystins, including oxidative stress (Hu *et al.*, 2005). Thus, several studies showed that microcystin act via oxidative stress toxicity mechanisms in some organisms as green algae (Bártová *et al.*, (2010), rat hepatocytes (Guzman and Solter, 1999; Ding *et al.*, 2003), fish hepatocytes (Li *et al.*, 2003), watercress and water moss (Gehring *et al.*, 2003; Wiegand *et al.*, 2002) and in bacteria (Yang *et al.*, 2008). In the present study it was also observed that MC extract could also induce oxidative stress in the isolates tested.

CAT activity increased relative to the control cells (not exposed to MCs) for the eight isolates chosen. All of the isolates tested had an increased CAT activity when exposed to each variant microcystin but all with different values between each other. An opposite effect was observed by Mittler and Tel-Or, (1991) which declared that CAT plays only a minor role in preventing photo-oxidative damage during exponential growth of *Synechococcus* R-2 cells. However, the results here presented showed that CAT activity was always increased, confirming that CAT might have a preventing role in

oxidative stress as Vassilakaki and Pflugmacher, (2008) and Jos *et al.*, (2005) showed in their studies.

These results showed that all of the bacteria tested were in oxidative stress when exposed to MCs and CAT activity was enhanced, probably for scavenging ROS and prevent cellular damage. Relating these results with the growth results we can verify that except for C4 with all of three MC variant, the other seven bacterial isolated had a reduction growth effect when exposed to one or two MC variants. So if MC reduces the growth, it makes sense to induce, as well, oxidative stress in these bacteria.

CAT activity increased in Vassilakaki and Pflugmacher, (2008) study after exposure to 0.5 nM crude extracts, but not with 0.05 and 1.0 nM. This is very interesting because stress oxidative was induced in 0.5 nM so it would be expected to have the same or higher effect with 1.0 nM, however, that didn't occurred. In the present work only one concentration (10 nM) was tested, yet with different microcystin variants that produced different increments in CAT activity. The crude extract used includes other compounds such as lipopolysaccharides, and these additional compounds may be able to influence enzymes activities (Vassilakaki and Pflugmacher, 2008). Those compounds are probably not the same in MCLR, MCRR and in MCYR. So, when the increment of CAT activity was little in comparison with the other cells exposed, as showed with P6 and C2 when exposed to MCRR, these results might be explained with reason cited above relative to other compounds present in the extract.

Similar effect was observed by Yang *et al.*, (2008) but with higher concentrations of MCRR. The authors showed that CAT activity of 5, 10 or 15 μM toxin-treated *E. coli* was also significantly increased after 1 hour exposure, which is similar to that of SOD results.

In respect to SOD1 activity inhibition, isolates who had a positive % inhibition, that is, an increase in SOD, were B1 and C2 with all three MC variant, B1 with MCLR, B3 with MCYR, M8 with MCRR and P6 and C2, both isolates with MCLR and MCRR. No effects in B1 with MCRR and MCYR, P1 with MCRR and MCYR, and C2 with MCYR were observed on SOD1 activity. Isolates B3 with MCLR and MCRR, M1 with the three MC variants, M8 with MCLR and MCYR, P1 with MCLR and P6 with MCYR had negative % inhibition of SOD1 activity, which means that was a ROS content increment.

One of the possible reasons for the increased hydrogen peroxide concentration is also the activity of SOD. SOD converts the superoxide anion radical, one of the possible generated ROS, to hydrogen peroxide (Vassilakaki and Pflugmacher, 2008).

Thus, when the SOD inhibition was increased which means, that SOD is probably active for scavenging ROS such as superoxide anion radical. Hydrogen peroxide is the most stable ROS and, to avoid damaging consequences of hydrogen peroxide in cyanobacteria, the cells have evolved various enzymes that are able to detoxify this compound (Vassilakaki and Pflugmacher, 2008). As cyanobacteria evolved to prevent cellular damage, bacteria also have those mechanisms that prevent ROS from damaging cellular structures.

For SOD2 activity inhibition, isolates who had a negative % inhibition were B1 with MCLR and MCYR, B3 with MCRR, M8 with MCRR and MCYR, P1 with MCRR and MCYR, P6 with MCLR and MCRR and C2 with all three MC variants.

No effect in B1 with MCRR, B3 with MCLR, M8 with MCLR and P1 with MCLR was observed. Isolates where a positive SOD2 inhibition was observed were B3 with MCYR, M1 with all three MC variants and P6 with MCYR.

Yang *et al.* (2008) showed that SOD activity of toxin-treated *E. coli* was significantly increased when exposed to MCRR of 5 μ M or above for one hour relative to the control and reached four times higher than that in the control when exposed to 10 μ M or above. It indicated that SOD might play an important role in scavenging ROS. However, when the toxin treatment prolonged, SOD activities of the treatment group decreased, and it had almost no difference from the control after three hours exposure. The decrease of SOD activities may attribute to the increase of cell number and the clearance of ROS (Yang *et al.*, 2008).

Yang *et al.* (2008) also verified that with the increment of toxin-treated time, there was almost no difference between the treatment group and the control in *E. coli*. However, in the present study after 12 hours of exposure, the bacteria tested presented always an increase of SOD inhibition, except for P1 which had always a negative inhibition in both SOD1 and SOD2 activity which means that the ROS content was increased and SOD was not active or was not present.

Both SOD and CAT were involved in the defense against the stress caused by MCRR (Yang *et al.*, 2008). The same authors showed that SOD and CAT had almost little difference from that of the control when exposed to 1 μ M microcystin. MCRR had no lethal effect on *E. coli* and could induce the accumulation of ROS in *E. coli* for a short period. On the other hand, it could use the increase of antioxidant system enzymes activities to scavenge the ROS and, so could prevent the cells from being damage (Yang *et al.*, 2008).

The results showed in this study suggest that MCs (MCLR, MCRR and MCYR) cause oxidative stress on bacterial cells and the antioxidant system enzymes CAT and SOD were induced as defense mechanisms, similarly to Jos *et al.*, (2005) in their study with tilapia fish.

As far as SOD and CAT activities are concerned, a simultaneous induction response is usually observed after exposure to pollutants (Dimitrova *et al.*, 1994). However in the present study no such relationship was observed in all of bacteria tested because as cited above, some MC variant did not enhance SOD activity.

5. CONCLUSION

In this study we intended to investigate if three of the most common variants of microcystin were able to influence the growth and promote oxidative stress in heterotrophic bacteria that co-inhabit with microcystin producing cyanobacteria. The major effect observed in these bacteria tested was a reduction on the growth. In respect to the antioxidant system enzymes, all results point out that microcystins can induce oxidative stress in the bacteria tested and that CAT and SOD activities were activated as a defense mechanism to scavenge ROS increment. To our knowledge this is the first study where the impact of microcystins was tested in an extensive and diverse number of heterotrophic bacteria. Although the MCs did not seem to have a huge impact on the bacterial growth, they were able to induce an increase of the intracellular ROS levels, which is one of the most common effects of MC on eukaryotic organisms. This study paves the way to elucidate the molecular mechanisms of MC toxicity also in prokaryotes.

6. REFERENCES

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