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Evaluation of the physiologic response of heterotrophic bacteria present in aquatic environments to the presence of microcystins

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Abstract

Microcystins (MC) are the hepatotoxins produced by cyanobacteria, which are photosynthetic organisms that usually colonize aquatic environments. Climate change has had a significant impact on the increased frequency of cyanobacterial blooms, which are often associated with the production of cyanotoxins. Studies show that the growth of aquatic heterotrophic bacteria that co-occur with cyanobacteria may not be affected by the presence of MC, or, on the other hand they may present a reduction in growth, never being totally inhibited by their presence (Miguéns, 2013; Pinto, 2016).

The aim of this study was to examine the effects of three microcystin variants (MCLR, MCRR, MCYR) on heterotrophic aquatic bacteria living in the same ecosystem as cyanobacteria. In particular, (1) to observe the impact of microcystins on the growth of heterotrophic bacteria, (2) the impact on the enzymes of the antioxidant system of these bacteria and (3) to screen the presence of *mlr* genes.

For this purpose, we performed the morphological and molecular characterization of 22 bacteria isolated from two reservoirs. It was intended to analyze the growth of bacterial isolates exposed to different concentrations of extract of each variant of MCs, and also to two concentrations of pure microcystin-LR. The search for the presence of MCs degradation genes (*mlrA-D*), was done using PCR. In order to analyze the effect of microcystins on the antioxidant system of the isolates, catalase (CAT) and superoxide dismutase (SOD1 and SOD2) activities were determined spectrophotometrically, at 240 nm and 550 nm, respectively, in the cells exposed to the different variants of microcystin.

None of the isolates grown in liquid medium was completely inhibited by the exposure to microcystins, however, it was found that MCs can reduce the growth of most bacteria tested. While some bacteria grew without any induced effect, others reacted differently according to the variant and the concentration (in the same isolate). In some cases a slight growth stimulation was observed. In most cases the growth stimulation appears to be related with other molecules from the MC extracts. The results of the determination of CAT and SOD activities revealed that only three isolates have catalase, namely M17F, M17K and 594196, and only three isolates have superoxide dismutase, M17C, M17D and 594196. In this study, a heterotrophic bacteria, M17C, *mlr*+, was also isolated, and it's *mlr* genes were amplified and sequenced. A new primer pair for the *mlrA* was also proposed in this study.

In summary, the results from this study, showed that there is not a pattern characteristic of the species or genera analyzed, thus indicating that the response of the heterotrophic bacteria isolates to MCs is mainly related to strain characteristics. They corroborate previous studies, which indicate that the cluster of *mlr* genes is not the only cause to allow bacteria to grow in the presence of MC without being affected by them, even with no alteration in the main enzymes from the antioxidant system (CAT and SOD).

Keywords: Microcystins, heterotrophic bacteria, catalase, superoxide dismutase, *mlr*

Resumo

As cianobactérias são organismos fotossintéticos capazes de colonizar uma grande variedade de habitats, em que se destacam os ambientes aquáticos. Nestes ambientes aquáticos as cianobactérias habitam a zona fótica, que corresponde à camada superficial da água que recebe luz solar. Em ambientes hídricos eutrofizados e em condições ambientais favoráveis, como temperaturas elevadas e grande disponibilidade de nutrientes como fosfatos e nitratos, as cianobactérias podem atingir elevadas densidades celulares, um fenómeno conhecido por *blooms* ou florescências. As alterações climáticas têm tido um impacto significativo no aumento de frequência de *blooms* de cianobactérias.

Estes *blooms* podem levar a um decréscimo de oxigénio e à produção de toxinas por cianobactérias (metabolitos secundários, normalmente designados cianotoxinas). Hoje sabe-se que, até 70% dos *blooms* de cianobactérias são tóxicos (Kazuya *et al*, 2011).

O efeito das cianotoxinas é já bem conhecido em mamíferos, permitindo uma divisão das cianotoxinas pelo seu alvo principal, nomeadamente: (1) neurotoxinas, cujo principal alvo é o sistema nervoso, (2) dermatotoxinas, de que o principal alvo é a pele e (3) hepatotoxinas, em que o fígado é o alvo principal (Zanchett *et al*, 2013).

As microcistinas (MCs) são o tipo de hepatotoxinas mais produzidas por cianobactérias, sendo a microcistina-LR, microcistina-RR e microcistina-YR as variantes mais comuns destas toxinas. Os efeitos tóxicos destas cianotoxinas encontram-se amplamente estudados em organismos eucariotas, contudo os efeitos em microrganismos são ainda escassos.

As cianobactérias encontradas em ambientes aquáticos convivem com outros microrganismos, nomeadamente bactérias heterotróficas como *Aeromonas* spp. e *Flavobacterium* spp. Nos últimos anos, tem-se procurado perceber de que forma é que bactérias heterotróficas, existentes em águas superficiais onde ocorrem habitualmente *blooms* de cianobactérias se comportam perante a presença de MCs. Verificou-se que estas toxinas, em alguns casos não têm qualquer efeito no crescimento das bactérias, enquanto que noutros provocam uma diminuição do crescimento de vários isolados de espécies diferentes, não o inibindo totalmente (Miguéns, 2013).

Estes estudos levaram à descoberta de bactérias heterotróficas aquáticas degradadoras de MCs. A primeira via de degradação de MCs foi descoberta por Bourne *et al* (2001) em bactérias do género *Sphingomonas*. Mais tarde, Bourne *et al* (2001), percebeu que o cluster de genes envolvido nesta via de degradação era composto por quatro genes, *mlrA, mlrB mlrC* e *mlrD.* Novos isolados de outras espécies foram identificados como detentores do cluster *mlr*. No entanto, o número de bactérias *mlr+* tem-se mostrado reduzido. Mais recentemente bactérias heterotróficas aquáticas degradadoras de MCs, *mlr-*, foram detetadas (Manage *et al*, 2009) e o seu estudo parece indicar que, apesar de serem menos eficientes na degradação de MCs do que as bactérias *mlr+*, estas estão presentes em maior número. Em 2016, Lezcano *et al*, propôs que a degradação de MCs por bactérias *mlr-* poderá estar associada ao metabolismo xenobiótico, ideia que veio reforçar a observação feita por Kormas e Lymperopoulou (2013) segundo a qual a maioria das bactérias degradadoras de MCs consegue normalmente "biodegradar" outros produtos. Em 2017, Dziga *et al* propôs a primeira via de degradação de MCs *mlr-*, que se pensa envolver um consórcio de bactérias heterotróficas aquáticas. Não estão ainda descritos os genes associados, apenas os locais de corte da MCs são para já conhecidos.

Estudos mostram que a exposição de bactérias heterotróficas aquáticas a MCs induz, entre outros efeitos, stresse oxidativo (Li *et al*, 2009), induzindo a formação de espécies reativas de oxigénio (ROS), como resultado do metabolismo oxidativo (Pflugmatcher, 2004). Os ROS podem causar sérios danos nas células, como a peroxidação de membranas lipídicas, genotoxicidade, apoptose e necrose (Ding e Ong, 2003).

O objetivo deste estudo foi examinar os efeitos de três variantes de MCs (MCLR, MCRR, MCYR) em bactérias heterotróficas aquáticas que vivem no mesmo ecossistema que as cianobactérias. Nomeadamente (1) observar o impacto das MCs no crescimento das bactérias heterotróficas, (2) o impacto destas nas enzimas do sistema antioxidante das bactérias e (3) rastrear a presença de genes *mlr*.

Para tal, procedeu-se à caracterização morfológica e molecular das bactérias isoladas. Pretendeu-se analisar o crescimento de isolados bacterianos expostos a diferentes concentrações de extrato de cada variante de MCs, e ainda a duas concentrações de microcistina-LR pura. Pesquisou-se a presença de genes de degradação de MCs (*mlrA*-D), recorrendo a PCR e confirmando quais os produtos amplificados por sequenciação. Analisou-se o efeito das MCs no sistema antioxidante dos isolados, nomeadamente, a atividade enzimática da catalase (CAT) e da superóxido dismutase (SOD) sendo estas determinadas espectrofotometricamente a 240 nm e 550 nm, respetivamente, nas células expostas às variantes da MCs.

Neste estudo, das 22 bactérias isoladas das Albufeiras de Magos e Roxo, oito são *Aeromonas* spp. e cinco são *Flavobacterium* spp.

Nenhum dos isolados que cresceu em meio líquido foi totalmente inibido pela exposição a MCs, no entanto, verificou-se que as MCs podem reduzir o crescimento da maioria das bactérias testadas, sendo que algumas bactérias cresceram sem efeito algum induzido, enquanto outras reagiram de forma diferente consoante a variante e a concentração usada no mesmo isolado. Em alguns casos observou-se ainda uma ligeira estimulação do crescimento. A comparação de respostas a extratos de MCLR e MCLR pura parece indicar que outras moléculas presentes no extrato estão a influenciar o crescimento das bactérias heterotróficas. Por exemplo, na maioria dos casos em que houve uma aparente estimulação do crescimento do isolado causado pela MC, correspondeu à adição de extratos, verificando-se que a mesma estimulação não ocorria quando era adicionada microcistina pura ao meio.

Os resultados da determinação das atividades da catalase (CAT) e superóxido dismutase (SOD) revelaram que apenas três isolados têm catalase, nomeadamente, M17F, M17K e 594196, e apenas três isolados têm superóxido dismutase, M17C, M17D e 594196. Juntando estes resultados aos resultados anteriores (Miguéns, 2013 e Pinto, 2016) algumas conclusões foram possíveis. Em relação à catalase, (1) a maioria das bactérias estudadas não apresentam atividade CAT nestes três estudos (19 isolados foram testados e apenas em seis foi detetada atividade deste enzima). (2) A maioria dos isolados apresentou redução de atividade quando expostos ao extrato de MCLR e a maioria dos isolados apresentou aumento de atividade CAT quando expostos ao extrato de MCRR, podendo esta resposta ser causada pela diferente hidrofobicidade das variantes MC (sendo MCLR mais hidrofóbica que a MCRR) o que vai facilitar a sua entrada na célula causando assim mais dano. (3) *Sphingomonas* sp. isolado 594196, também parece ter uma resposta completamente diferente à exposição de variantes de MCs, em comparação com os outros isolados. Em relação à atividade SOD, (1) os resultados suportam a ideia de que a resposta da atividade SOD às MCs é uma característica de estirpe. (2) Outros mecanismos podem estar envolvidos na degradação de $O_2^{\bullet-}$. Neste estudo foi ainda isolada uma bactéria heterotrófica *mlr*+, denominada M17C, cujos quatro genes *mlr* foram sequenciados. Um novo par de primers *mlrA* foi ainda proposto neste estudo.

Em resumo, os resultados apontam para que a resposta dos isolados às MCs esteja relacionada com as características de cada estirpe e corrobora estudos anteriores que indicam que o cluster de genes *mlr* não é a única via que permite que bactérias cresçam na presença de MC sem serem afetadas por estas, mesmo quando não há resposta alterada nas principais enzimas do sistema antioxidante. Conclui-se ainda que outros componentes do extrato de MC podem estar a afetar as bactérias de diferentes maneiras, interferindo nos ensaios.

Palavras-chave: Microcistinas; bactérias heterotróficas, catalase, superoxide dismutase, *mlr*

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Abbreviations

ASBE	Biology and Ecotoxicology lab
BSA	Bradford protein assay
CAT	Catalase
GR	glutathione reductase
GST	glutathione transferase
DSA	Environmental Health Department
INSA	National Institute of Health Doctor Ricardo Jorge
MCs	microcystins
MCLR	microcystin-LR (Leucine; Arginine)
MLRR	microcystin-RR (Arginine; Arginine)
MCYR	microcystin-YR (Tyrosine; Arginine)
NBT- diformazan	nitro blue diformazan
OATPs	organic anion transport system
OD	optical density
PCR	polymerase chain reaction
PP1	protein phosphatases 1
P2A	protein phosphatases 2A
ROS	reactive oxygen species
SOD	superoxide dismutase
SOD_t	the sum of SOD_1 and SOD_2
SOD_1	Cu/Zn-SOD
SOD_2	Mn-SOD

1. Introduction

1.1. Cyanobacteria, blooms and its geographical localization

Cyanobacteria are a ubiquitous diverse group of phototrophic prokaryotes which inhabits mainly aquatic ecosystems, such as fresh and brackish water, oceans and hot springs and also terrestrial environment, like soils, deserts and glaciers. They also form symbiotic relations with fungi (lichens) and plants. Thus, cyanobacteria have a large geographical distribution (Sivonen and Jones,1999). In aquatic ecosystems, they live in community with other organisms, such as aquatic heterotrophic bacteria.

In optimal environmental conditions, high temperature and high availability of nutrients, such as phosphorus or nitrates, a rapid increase in cyanobacteria population leads to *bloom* formation. That is, a high biomass development that may lead to a thick horizontal and vertical layer of cells through the water column. These *blooms* may lead to oxygen decrease and toxin production by cyanobacteria (commonly nominated as cyanotoxins) causing severe biological impacts in the environment.

Eutrophication is an enrichment of water with limiting resources (like nitrogen and phosphorus) that causes structural changes to the ecosystem, for example: increased production of algae and aquatic plants, hypoxia and dead of fish and other animals. Eutrophication of natural and artificial water bodies has become a big concern over the last years. As high availability of nutrients entering the water course, mainly from runoffs from farm lands combined with waste waters both from urban and industrial activities (Giaramida *et al*, 2013), has increased, also because of higher temperatures and the construction of water barriers that raise the probability of water eutrophication (Churro *et al*, 2012).

With it, the occurrence and persistence of blooms of cyanobacteria has increased and raise concerns, as many of these microorganisms can produce toxins.

It is now known that up to 70% of cyanobacterial blooms are toxic (Kazuya *et al*, 2011). Cyanotoxin contamination of water occurs mostly when the bloom enters in decline and the cyanobacteria cell walls burst, releasing its contents, namely the cyanotoxins, into the water (Blom *et al*, 2001).

Cyanotoxins are currently divided according to the main target organ: neurotoxins, dermatoxins and hepatotoxins such as microcystins (MCs) and nodularins (Zanchett, 2013). The most widespread and frequently found cyanotoxins are MCs.

Hepatotoxic cyanobacterial blooms have been found practically everywhere in the world, Europe, mainly in Denmark, Finland, France, Germany, Ireland, Norway, Portugal, America in Brazil, Canada, USA, Asia in China and Japan, Africa and Australia (Sivonen and Jones, 1999)

1.2. Microcystins

MCs represent a high risk to human and animal health, as such molecules have been shown to cause liver damage (MacKintosh *et al*, 1990) and tumour activity (Zhou *et al*, 2002). Its effects on eukaryotic cells are already well studied, but on the other hand, the effects of cyanotoxins on heterotrophic bacteria are still scarce (Christoffersen *et al*,2002).

As previously mentioned, microcystins are one of the main cyanotoxins and microcystin-LR (MCLR), microcystin-RR (MCRR) and microcystin-YR (MCYR) are the major isoforms and most studied MCs (Li *et al*, 2009). These are cyclic peptides mostly produced by *Microcystis aeruginosa, Planktothrix, Anabaena* and *Nostoc* (Silvonen and Jones, 1999). MCs are especially capable of inhibiting the protein phosphatases 1 and 2A (PP1 and P2A) of both mammals and

higher plants (Mackintosh *et al*, 1990). They are hepatotoxins, meaning that, their main target is the liver by specific binding to the organic anion transport system (OATPs) in hepatocyte cell membranes, inhibiting type 1 and type 2A eukaryotic serine/threonine protein phosphatases (Valerio *et al*, 2009). They also target cells from the intestines and kidney and can be found in small amounts in the heart, spleen, brain, gonads and stomach (Wang *et al*, 2008).

1.2.1. Chemistry and structure

There are up to 90 microcystin isoforms known presently. These vary in the degree of methylation, hydroxylation, epimerization, peptide sequence and toxicity (Pearson *et al*., 2010).

MC consist in a cyclic heptapeptide of cyclo-D-Ala-R1-D-MeAsp(iso)-R2-Adda-DGlu(iso)-Mdha (figure 1.1). Adda, a β-amino acid characteristic to microcystins and nodularins, is a (2S, 3S, 8S, 9S)-3-amino-9methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid, Mdha is N-methyldehydroalanine, and D-MeAsp is erythro-β-methyl-D-Asp (Imanishi *et al*, 2005). And two 'non-conserved' L-amino acids at positions Z and X (figure 1.1). These L-amino acids differ in isoforms and as amino acids have different polarity, their combination in MCs will affect MCs hydrophobicity. For instance, the MCs variants used in this study all have an arginine (R) on position Z. However, in position X, MCLR has a leucine (L), MCYR has a tyrosine (Y) and MCRR has a arginine (R). As leucine and tyrosine are both hydrophobic amino acids and arginine is a hydrophilic amino acid, MCLR and MCYR are both more hydrophobic than MCRR.

Most microcystins molecular weight vary between 909 and 1115 Da, *e.g.*, MCLR weights 994 Da, MCRR weights 1037 Da and MCYR weights 1094 Da (Duy *et al*, 2000).

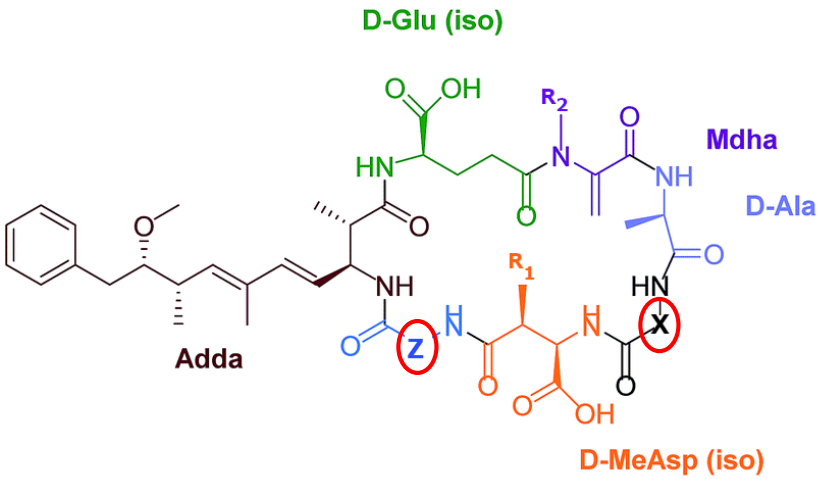


Figure 1.1. Microcystin chemical structure (adapted from Neumann *et al*, 2016).

1.2.2. Degradation of microcystins

MCs are chemically stable in water bodies due to its stable cyclic structure. This makes them resistant against physical and chemical processes such as high temperatures, above 40 ˚C, or extreme pH, above nine or bellow one (Rastogi *et al*, 2014; Harada *et al*, 1996).

Thus, photolysis (chemical reaction in which a chemical compound is broken down by photons) and biodegradation are the best processes known to inactivate cyanotoxins (Chen *et al*, 2010).

1.3. Heterotrophic bacteria

As referred before, cyanobacteria and aquatic heterotrophic bacteria share the same habitat, they represent the basic unit of the trophic web in these environments. Heterotrophic bacteria have a special role in several geochemical cycles in their aquatic habitats (Figueiredo *et al*., 2007). Therefore, it became important to understand how this tendency of more frequent and intense blooms of toxin producing cyanobacteria will affect these microorganisms. Furthermore, studies show that aquatic heterotrophic bacteria are able to degrade MCs, and a degradative pathway of microcystin-LR (*mlr* gene cluster) that can degrade MCs has already been described (Bourne *et al*, 2001).

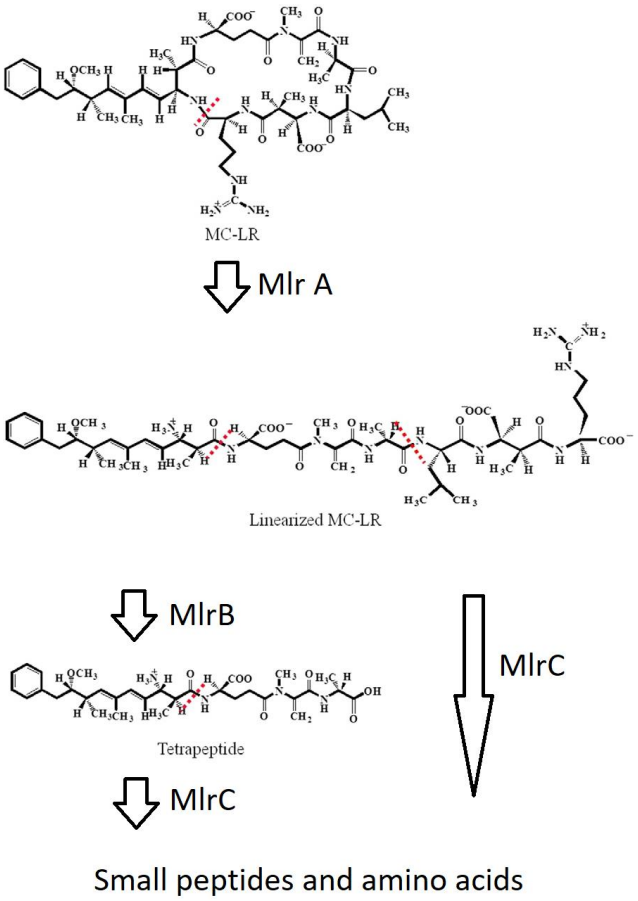


Figure 1.2. The degradative pathway of microcystin LR (adapted from Li *et al*, 2017).

1.4. Biodegradation and MC-degrading aquatic heterotrophic bacteria

As MC-degrading bacteria were discovered it became important to understand how they work, since such bacteria could be the answer to the water treatment problem.

In 2001, Bourne *et al*, presented a pathway through which a *Sphingomonas* strain could degrade MCLR and use it as its sole carbon source. This pathway comprised three hydrolytic enzymes (Mlr A, B, C) (figure 1.2) and a transporter (MlrD). In this pathway it is believed MlrA has the most important function as this enzyme cleaves the aromatic ring of MCLR at the Arg-Adda bond, linearizing the molecule and reducing its toxicity by 160-fold. MlrB and MlrC cleave the molecule further until small peptides and amino acids. These enzymes were then found to belong to the same cluster now named *mlr* (Bourne *et al*, 2001).

After the discovery of this gene cluster, it became more usual to identify MC-degrading bacteria by screening the existence of *mlrA* gene in their genome (Jiang *et al*, 2011). However, through these last years it has been demonstrated that some bacteria are able to degrade MCs even in absence of *mlr* cluster (Manage *et al*, 2009).

These MC-degrading bacteria *mlr*- displayed a reduced efficiency in degrading MCs when compared to MC-degrading bacteria *mlr*+, having a longer lag phase and therefore taking more days to degrade the same amount of MCs. A study published in 2017 (Lezcano *et al*) suggested that in these bacteria lacking the *mlr* gene cluster, the MC degrading ability may be linked with their xenobiotic metabolism.

For instance, *Sphingomonas acidaminiphila* strain MC-LTH2 has been shown to degrade not only MCLR and MCRR but also completely degrade other compounds containing an Adda

residue, under various conditions, even though *mlrA* wasn't detected, suggesting another path for Adda compounds degradation (Yang *et al*, 2013).

Recently, Dziga *et al* (2017) presented a new pathway of MC degradation that does not involve the Mlr pathway. Although, the enzymes involved in the process haven't yet been identified, the places of cleavage of the MC molecule differ significantly from *mlr* pathway can be observed. In the pathway, described by Dizga *et al*, the cleavage of the MC cyclic ring does not occur in the Adda-Arg bond but on the Leu-Asp-Arg bond.

Recalling the concerns about increasing blooms associated with climate change, Morón-López *et al* (2017) showed in a recent study that not only previous exposure to MCs, but also a higher intake of phosphorus, nitrate, and carbon sources, stimulates the MCs degradation both in *mlr*+ and alternative biodegradation pathways. Besides testing MC degradation relation with these abiotic factors, they also tested how temperature could affect MC degradation. After testing MC degradation at 22 ˚C, 27 ˚C and 32 ˚C, they reached the conclusion that temperature isn't a major factor in MC degradation by alternative biodegradation pathways.

Although *mlr*- MCs degrading bacteria are not the most efficient ones in MCs degradation, their study is still rather important as it has been shown that these *mlr*- bacteria are in high abundance in nature (Lezcano *et al*, 2016) and that members from *Sphingomonadales* order (the one with more known *mlr*+ strains so far) are not the most abundant in this aquatic ecosystems (Mou *et al*, 2013), making it essential to know the full microbial diversity.

1.5. Bacterial antioxidant system and oxidative stress

Studies show that exposure to MCs induce, among other effects, oxidative stress (Li *et al*, 2009). This exposure may induce the 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 (Pflugmatcher, 2004). ROS can cause serious damage in cells such as peroxidation of lipid membranes, genotoxicity, apoptosis and necrosis (Ding and Ong, 2003). Oxidative stress is characterized by a disruption of balance between the oxidative impact and the antioxidant defense system (Pflugmatcher, 2004). This disruption can be imposed on cells in one of three ways: (1) increase of the oxidants generation, (2) decrease in the antioxidant protection, or (3) failure to repair oxidative damage (Vassilakaki and Pflugmacher, 2008).

Under normal circumstances enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione transferase (GST) and glutathione reductase (GR), would clear ROS before any damage occurred in the cell. However, in case of oxidative stress lipid peroxidation, DNA damage and mutation may occur (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 unstable superoxide radicals to H_2O_2 and molecular oxygen (O_2). H_2O_2 is harmful to the cell but is the most stable form of ROS, avoiding greater damage to the cell until catalases and peroxidases (hydrogen peroxide removing enzymes) are available to further degrade it (Vassilakaki and Pflugmacher, 2008). In animal cells different types of SOD act in different cell compartments. For instance, there are three types of SOD containing Mn, Fe or Cu and Zn as prosthetic metals (Rahda, 2010). They are SOD_1 (cytosolic Cu/Zn-SOD), SOD_2 (mitochondrial Mn-SOD), and SOD_3 (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).

Catalase (CAT) is an enzyme whose function is to decompose H_2O_2. When H_2O_2 is in high concentrations, CAT acts catalytically and converts H_2O_2 in O_2 and H_2O (Radha, 2010), but when in low concentration CAT acts peroxidically using suitable hydrogen donors such as etanol,

removing H_2O_2 but oxidizing its substrate (Turkseven *et al*., 2005). Most of the catalases characterized until now can be classified in two types: typical catalases and bifunctional catalase-peroxidases and both have been found in bacteria, such as *Escherichia coli, Bacillus subtilis* and *Klebsiella pneumoniae,* simultaneously (Kim *et al*., 1994). The typical CAT is active in a high pH range, i.e. 5-10, and is resistant to ethanol/chloroform (Kim *et al*., 1994). On the other hand, bifunctional catalase-peroxidase (only detected in bacteria) is pH dependent, with an optimal pH of 6-6.5 and is more sensitive to temperature, ethanol/chloroform and H_2O_2 then the typical CAT (Kim *et al*., 1994).

1.6. Aim of this study

The aim of this study was to examine the effects of three variants of microcystins (MCLR, MCRR, MCYR) on aquatic heterotrophic bacteria that live in the same ecosystem as cyanobacteria, namely:

(1) isolate and identify heterotrophic bacteria from reservoirs where cyanobacterial blooms are observed;

(2) assess microcystins impact on bacterial growth;

(2) observe their impact on enzymes of the antioxidant system (catalase and superoxide dismutase) of these heterotrophic bacteria;

(3) screen for the presence of *mlr* genes on these heterotrophic bacteria.

2. Methods

2.1. Sampling reservoirs

Sampling was performed on May 24th, October 3rd and October 23rd 2017 using 1000 mL sterile bottles. The sampling occurred at Albufeira de Roxo, Albufeira de Magos and Albufeira de Roxo, respectively.

Water samples were transported either in a cooler box, in the dark to prevent cyanobacteria growth and the increase of water temperature.

The isolates were named, for this study, with letters and numbers. The first letter represented the place of isolation (M – from Albufeira de Magos, R – from Albufeira de Roxo). The number, 17, represents the year of isolation, therefore, isolates are either M17 or R17. To differentiate each isolate from the same reservoir, another letter was added to the end, for instance the first isolate from Albufeira of Magos was named M17A.

2.2. Isolation of bacteria

The bacteria were isolated from water samples from each reservoir by plating beads method, where 100 µL of each sample was spread using sterile glass beads in non-selective medium plates, Reasoner'2A medium (R2A medium) with 1.5% agar. The plates were then incubated at 20 ˚C ± 2 ˚C in the dark, until colonies were observed, for two days (R17E; R17I; R17K; R17O; R17Q-R17T), three days (M17A-F) and six days (M17G-N).

Among the colonies present in the plates, white and mucus (possible *Aeromonas*) and strong yellow pigmentation (possible *Flavobacterium)* were preferably selected, however, some other (nine isolates) with pink and softer yellow pigmentation were also selected.

2.3. Phenotypic characterization of the isolated bacteria

Bacterial isolates were characterized according to their colony color and texture at macroscopic level and cell shape and gram staining at microscopic level.

In order to classify the isolates according to their Gram group, microscope slides of each isolated bacteria were prepared using an automated Gram stainer system (Previ™ color Gram, Biomerieux). This standardized coloration improved bacteria differentiation in comparison with manual staining method.

The microscope slides were also used to assess bacterial shape. The isolates were assigned to the following possible shapes: coccus, bacillus and coccobacillus (Cabeen and Jacobs-Wagner, 2005). Cell observation was performed under an Olympus BX60 fluorescence microscope coupled with a CCD camera (Olympus DP11).

2.4. Molecular analyses

2.4.1. Molecular identification of the isolates

Bacterial DNA extraction was performed with Invisorb® Spin Plant Mini Kit (INVITEK) following the manufacturer's instructions. The nucleic acids concentration and purity was assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) using 1.5 µL of each sample. Then an aliquot of all samples was prepared to achieve a DNA concentration of 50 ng/µL. Aliquots of 3 µL of template DNA were used for PCR amplification of 16S rRNA gene. PCR was performed in a 25 µL reaction mixtures containing 1x PCR buffer (Invitrogen), 0.05 mM dNTPs, 0.52 mM of each primer, 0.05% W-1 detergent, 3 mM $MgCl_2$ (Invitrogen) and 0.06 U of Taq polymerase (Invitrogen). The universal bacterial primers 16S_8F and 16S_1492R were designed by Chaves (2005) and the expected amplified fragment has about 1500 bp of length. The reactions

were performed in a Tpersonal thermocycler (Biometra®) with a hot lid (95 ˚C). The temperature profile had five steps, an initial denaturation (94˚C for 5 min); 35 cycles of denaturation (94 ˚C for 45 s), annealing temperature (49 ˚C for 45 s), extension (72 ˚C for 50 s); and a final extension step (72 ˚C for 5 min). The PCR products were separated by electrophoresis in a 1% (w/v) agarose gel at 85 V for 45 min, using TBE 1x as buffer. GelRed, which is a safer fluorescent nucleic acid dye than ethidium bromide, was incorporated in the gel to allow the PCR amplicons visualization. The gel image was acquired using a gel transilluminator system (UVITEC).

2.4.2. Microcystin degradation genes (*mlr*) amplification

Bacterial DNA extraction was performed as described in the previous section. Aliquots of 3 µL of template DNA were used for PCR amplification of the different *mlr* genes. PCRs were performed in a 25 µL reaction mixtures containing 1x PCR buffer (Invitrogen), 0.05 mM dNTPs, 0.52 µM of each primer, 0.05% W-1 detergent, 3 mM $MgCl_2$ (Invitrogen) and 0.06 U of Taq polymerase (Invitrogen). The primers presented below in table 2.1 were designed by Saito *et al*, 2003; Ho *et al* 2007; Yang *et al* 2014. The reactions were performed in a Tpersonal thermocycler (Biometra®) with a hot lid (95 ˚C). The temperature profile included an initial denaturation (94 ˚C for 5 min); 35 cycles of denaturation (94 ˚C for 30 s), annealing temperature (specified to each primer pair and presented in table 2.1), extension (72 ˚C for 60 s); and a final extension step (72 ˚C for 5 min). The PCR products were separated by electrophoresis in a 1% (w/v) agarose gel at 85 V for 45 min, using TBE 1x as buffer. GelRed was incorporated in the gel to allow the PCR amplicons visualization. The gel image was acquired using a gel transilluminator system (UVITEC).

Table 2.1. Primers list used in this study

Primer	Sequence (5' - 3')	Annealing temperature	Fragment size	Reference
mlrAF_Saito	5´-GACCCGATGTTCAAGATACT- 3´	47 ˚C	807 bp	Saito *et al*, 2003
mlrAR_Saito	5´-CTCCTCCCACAAATCAGGAC- 3´			
mlrBf1_Ho	5´-CGACGATGAGATACTGTCC- 3´	47 ˚C	448 bp	Ho *et al, 2007*
mlrBr1_Ho	5´-CGTGCGGACTACTGTTGG- 3´			
mlrCf1_Ho	5´-TCCCCGAAACCGATTCTCCA- 3´	59 ˚C	666 bp	Ho *et al, 2007*
mlrCr1_Ho	5´-CCGGCTCACTGATCCAAGGCT- 3´			
mlrDf1_Ho	5´-GCTGGCTGCGACGGAAATG- 3´	59 ˚C	671 bp	Ho *et al, 2007*
mlrDr1_Ho	5´-ACAGTGTTGCCGAGCTGCTCA- 3´			

2.4.3. Sequencing

To prepare the PCR products for sequencing, two steps were performed. The first had the purpose of neutralizing/removing unincorporated primers and nucleotides. Thus, illustra™ ExoProStar™ 1-Step Kit was utilized by adding 2 µL of illustra™ ExoProStar 1-Step to 5 µL of PCR mix, which was incubated for 15 min at 37 ˚C and then incubated at 80 ˚C for 15 min (the product was then stored at -20 ˚C until the pre-sequencing step). The second step was a pre-sequencing reaction using BigDye terminator, it was performed in a 10 µL reaction mixture

containing 1 µL of BigDye; 1 µL of BigDye Buffer (10x); 0.4 µL of primer (for the desired gene); 2 µL of purified PCR product, and 5.6 µL ultrapure water. The PCR temperature profile was constituted by 25 cycles of 96 ˚C for 10 s, 50 ˚C for 5 s and 60 ˚C for 4 s. The samples were sent for sequencing at the UTI-DGH Laboratory of INSA. In some bacterial isolates, there were some nonspecific PCR products amplification that could not be eliminated using column-based purification kits without concomitant lost of the amplicon of interest. Therefore, in those cases the ilustra™ ExoProStar™ 1-Step step wasn't performed, and the bands of interest were excised 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 ilustra™ GFX™ PCR DNA and Gel Band Purification Kit, according to the manufacturer's instructions.

Bacterial sequences obtained were corrected using BioEdit program (Hall, 1999) and afterwards 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). This step allowed to determine the sequences with higher similarity and other close sequences to be selected for the posterior phylogenetic analyses. Furthermore, sequences from previous studies (Miguéns, 2013; Pinto, 2016) were also used.

2.4.4. Phylogenetic Analyses

An alignment of approximately 800 bp nucleotides of the 16S rRNA gene from 23 isolates was used in the phylogeny. Alignments were made with BioEdit Sequence Alignment Editor version 7.0 (Hall, 1999) and visually corrected.

Phylogenetic trees were constructed based on Maximum likelihood statistical method with a bootstrap of 1000 replications using Mega6 software version 1.0 (Tamura, 1993). The sequence from *Saccharomyces cerevisiae* was used as outgroup.

2.5. Bacterial Cell Growth

Bacterial growth was assessed in a 96-well microplate bioassay. Each isolate was inoculated in Nutrient Broth medium and each of the three variants of microcystins (MCLR, MCRR and MCYR) purified extracts, or a pure MCLR was added into the culture medium to yield a final concentration of 1 nM, 10 nM, 100 nM and 1 µM (MCLR and MCRR extracts); 1 nM and 1 µM (MCLR pure) and 1 nM of MCYR. For the MCYR extracts, it was only possible to test one concentration (1 nM) due to stock limitation in the laboratory. The concentrations used in the present study were selected from previous studies held at the DSA-ASBE (INSA).

Pre-inoculums were prepared in 20 mL of Nutrient Broth medium in 100 mL Erlenmeyer flasks. Cells were incubated overnight at 20 ˚C, on Orbital Shaker SO3 at 300 rpm. The growth experiments were initiated the day after. Pre-inoculums initial optical density (OD) was measured in a colorimeter 257 (Sherwood) at 660 nm wavelength. Microplates were inoculated as illustrated in figure 2.1. Thus, the blank was inoculated with 200 µL of Nutrient Broth medium. Each MC three replicates were inoculated, with an initial cell density of 0.1 and a final volume of 200 µL (nutrient broth + cell suspension + 20 µL of toxin). The control condition (negative control) was inoculated with nutrient broth + cell suspension (OD_i of 0.1). The microplates were incubated at 20 ˚C with stirring.

Optical densities of the isolated bacteria on the microplate assay were measured at 600 nm, each 30 min during ten hours, using a microplate absorbance Multiskan Ascent Thermo Labsystems, with a previous slow shaking for 5 s prior to measurement. Growth curves of each isolate were made with the optical densities measurements, with Excel™ program version 16.0

(Microsoft Office™). The results were expressed as means ± SD. 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.

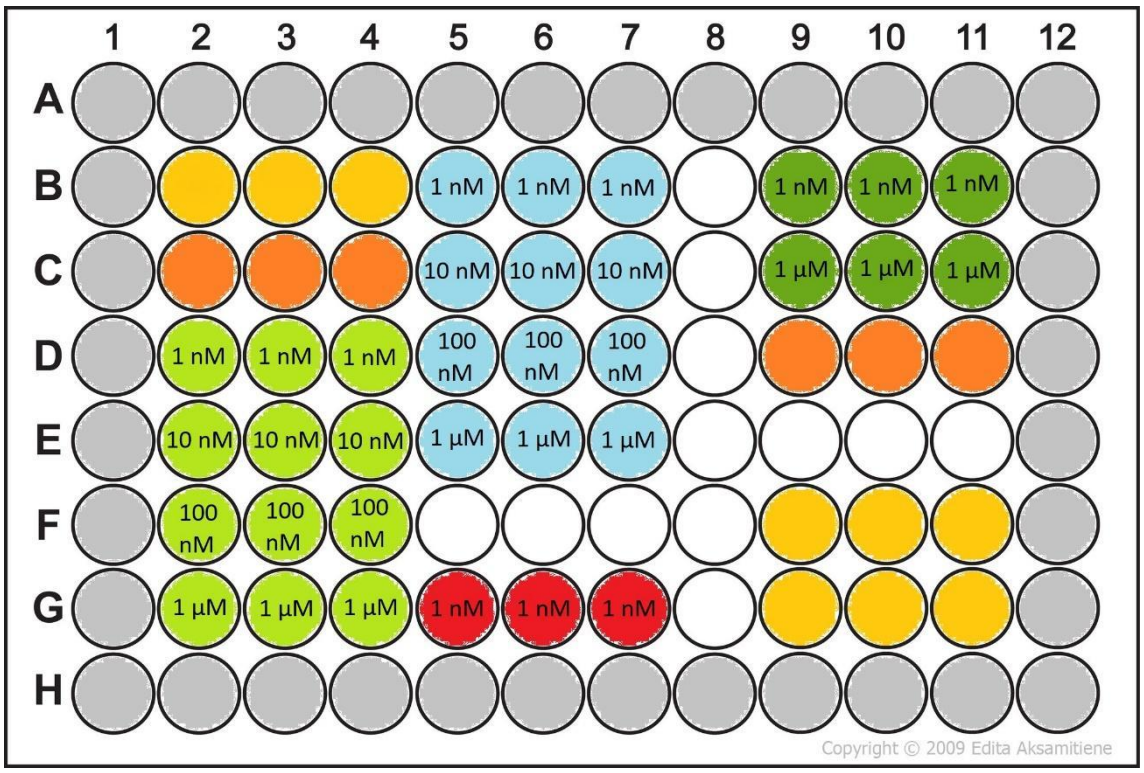


Figure 2.1. Schematic representation of the microplate wells inoculation, containing microcystin exposure in three replicates. Yellow - Blank. Orange - Negative control. Soft green - Microcystin-LR extract concentrations, Blue - Microcystin-RR extract concentrations. Red – Microcystin-YR extract concentrations. Dark green – Pure microcystin-LR concentrations. Grey – water.

2.6. Determination of activities of antioxidant system enzymes

The oxidative stress was assessed for some representative isolates with the determination of the activity of two antioxidant system enzymes, catalase (CAT) and superoxide dismutase (SOD). The isolates were chosen taking into account some factors, *Sphingomonas paucimobilis* 594196 (isolated in May 2017) was chosen since microcystins degradation was primarily identified in the *Sphingomonas* genera. The isolates M17C and M17D belong to the same genera, *Flectobacillus,* but one presents the *mlr* cluster and the other doesn't, so both were tested to see if there were differences between them. Besides these three, three *Flavobacterium* and three *Aeromonas* where chosen since both genera were favored in the initial selection of this work, with allow the recovery of several isolates to compare. Thus, to determine enzymatic activities, the control group (not exposed to MCs) and cells exposed to microcystins at a concentration of 10 nM of MCLR extract, or MCRR extract or purified MCLR, were grown overnight in 15 mL Nutrient Broth medium during 12 hours. The small amount of MCYR made it impossible to test this variant in this trial. The pellets, primarily divided in two tubes, (one with 10 mL for the SOD trial and other with 5 mL for the CAT trial) were obtained by centrifugation at 4 ˚C for 20 min at 4500 rpm, washed with PBS pH 7.4 (1x) (Gibco®), and kept at - 80 ˚C until use. To extract the proteins, the pellets were thawed in ice and resuspended in 300 µL of sodium phosphate buffer 50 mM. Cells were disrupted using 100 µL microspheres (Sigma) with six alternate cycles of 1 min in ice and 1 min vortex.

For SOD trial, ice-cold chloroform/ethanol was added to the samples, and mixed for 30 s. By using this technique, many proteins precipitated during cellular debris removal.

Cellular debris were removed by centrifugation for 20 min at 9000 rpm, the supernatant was recovered (note: SOD samples formed 2 phases – as icecold chloroform/ethanol was added – and the aqueous phase (on top) was recovered without touching the interphase).

The amount of total protein in the samples was estimated by Bradford method in microplate, where the absorbance of the samples was read at 600 nm and compared against a standard curve of a standard protein solution (BSA), with the following concentrations 0.125, 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00 mg/mL. The samples were prepared according to the Quick Start™ Bradford Protein Assay Manual instructions (BioRad).

CAT activity was measured by the decomposition of H_2O_2, which was monitored directly by the absorbance decrease at 240 nm. The reaction mixture of 3 mL contained 50 mM sodium phosphate buffer (pH 7.0); 1 mL of 0.2% H_2O_2 and 5 µg or 10 µg or15 µg of the enzymatic extract of each isolate.

SOD activity of Cu/Zn-SOD (SOD_1) and Mn-SOD (SOD_2) was measured by the inhibition of the rate of formation of nitro blue diformazan (NBT-diformazan) using the Superoxide Dismutase assay kit (Trevigen) according to the manufacturer´s instructions**.** To assess SOD2 activity, an aliquot of 100 µL of sample was separated to a new Eppendorf and KCN was added to a final concentration of 2 mM. The cyanide ion inhibits more than 90% of SOD_1 activity, according Superoxide dismutase assay kit (Trevigen) manufacturer's instructions. To determinate the absorption of SOD activity, 5 µg of the enzymatic extract of each isolate was used in the reaction mixture and followed in a spectrophotometer (UNICAM UVNis Spectrometer UV4), to determine the rate at 550 nm.

3. Results

3.1. Characterization and identification of the heterotrophic bacteria isolated

The colonies' color and texture were macroscopically verified, and summarized in table 3.1. The bacterial isolates assigned as either white, pale white, pale yellow, yellow or pink regarding color and as either mucous, very mucous or slightly mucous regarding texture.

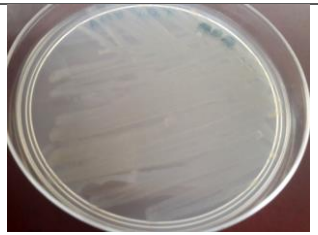
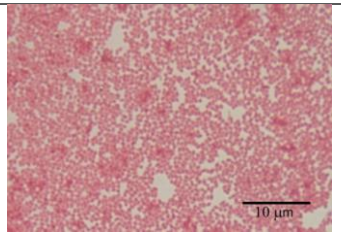
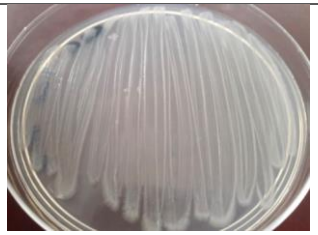
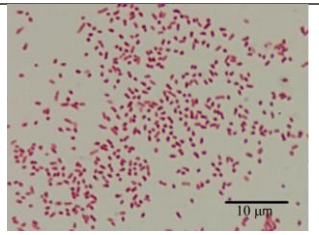
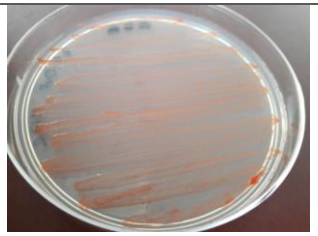
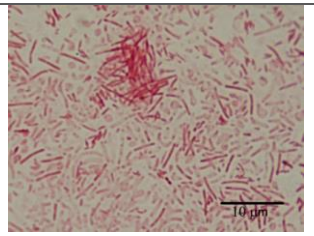
The bacterial shape was assessed in a microscopic slide with a suspension of bacterial cells from each isolate. They were classified into coccus, bacillus, coccobacillus and long rods (table 3.1).

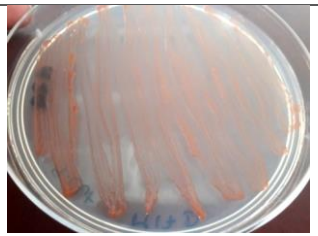
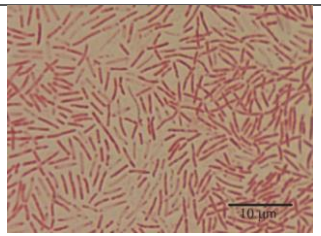
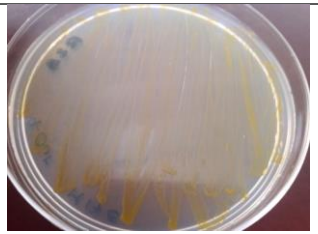
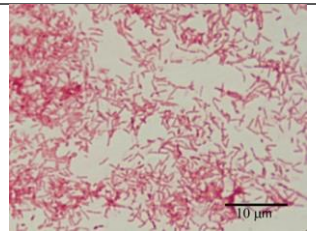
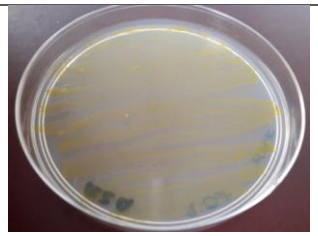
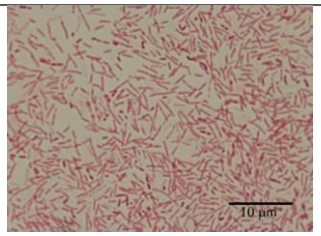
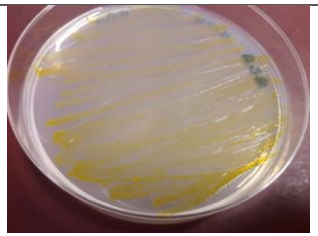
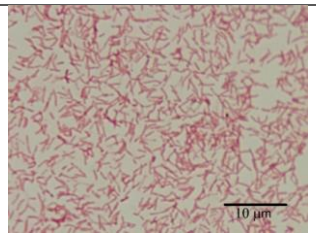
Furthermore, the isolates were divided into Gram-positive and Gram-negative using a light microscope to observe the microscope slides prepared in the automatized system Previ™ color Gram (Biomerieux) (table 3.1).

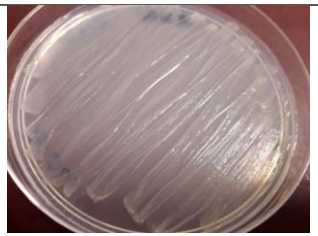
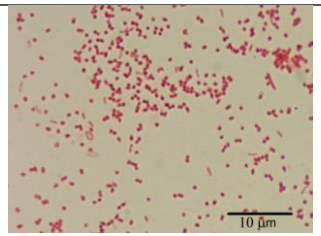
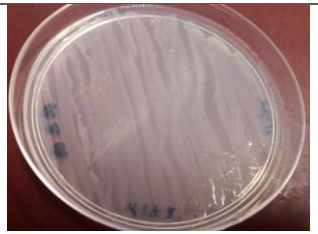
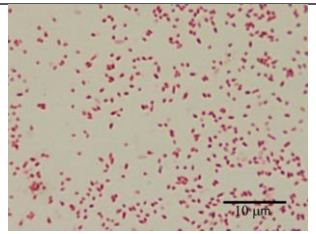
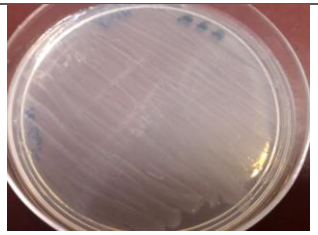
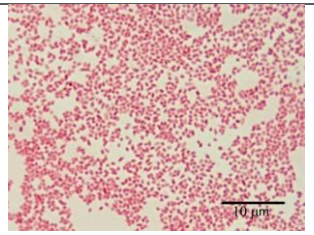
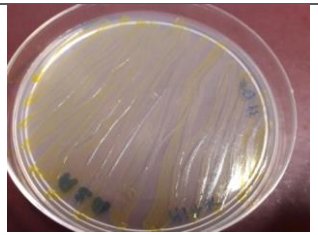
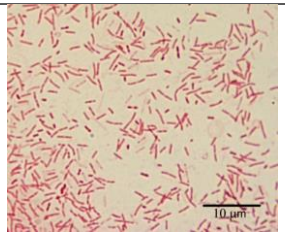
Bacterial sequences were corrected with BioEdit software (Hall, 1999) and then compared in BLAST software at https://blast.ncbi.nlm.nih.gov/Blast.cgi. In figure 3.1 is the phylogenetic tree produced in MEGA6 (Tamura *et al*, 2013), to further assess the isolates phylogenetic position and obtain their molecular identification, which is also showed in table 3.1.

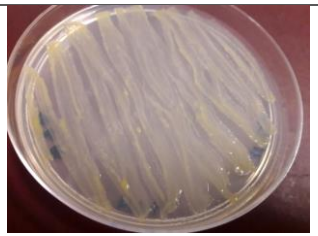
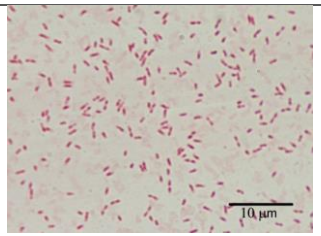
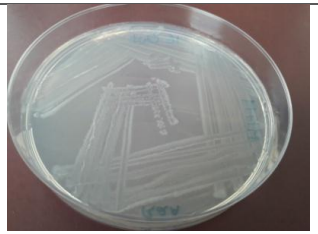
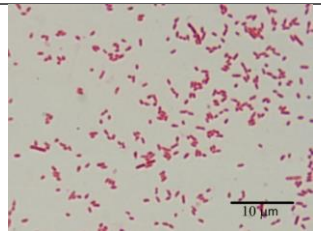
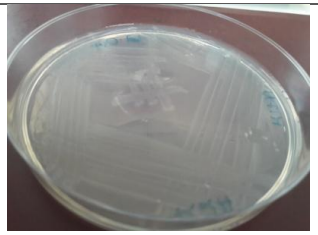
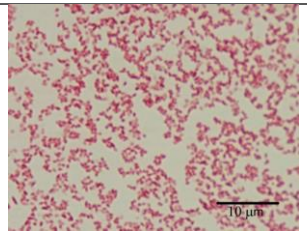
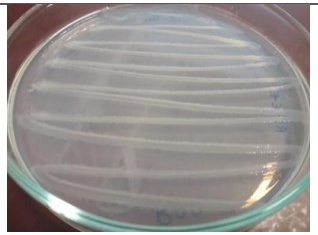
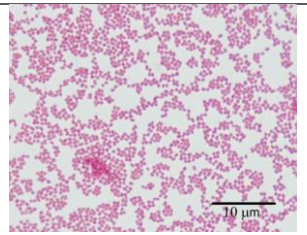
Note: some previously isolated and identified bacterial isolates (not present in table 3.1) were also used in this study. Namely, *Sphingomonas* sp. isolate 594196; *Aeromonas* sp. isolate B3 (Miguéns, 2013); *Aeromonas* sp. isolate M6 (Miguéns, 2013); *Flavobacterium* sp. isolate M3 and *Shewanella* isolate B1 (Miguéns, 2013).

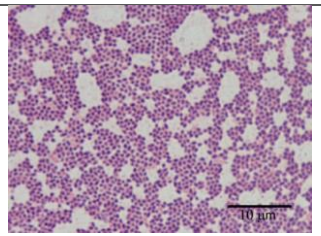
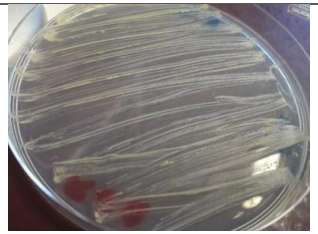
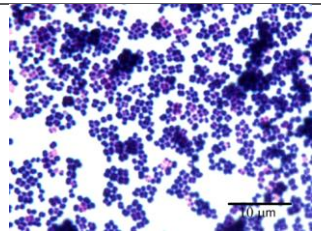
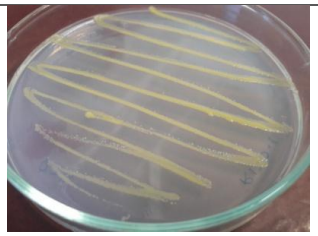
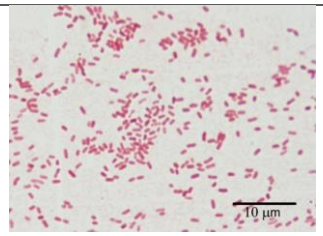
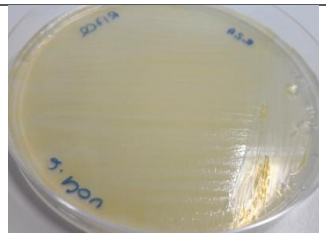
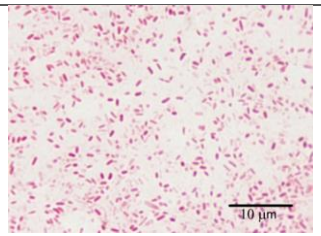
Table 3.1. Major features of all 22 aquatic bacteria isolated from two Portuguese freshwater reservoirs: Albufeira of Magos (M) and Roxo (R). (*) - The black scale in the image indicates a length of 10 μm.

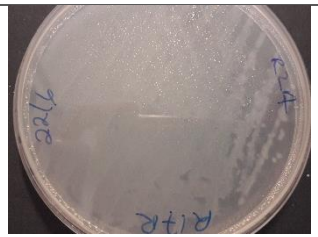
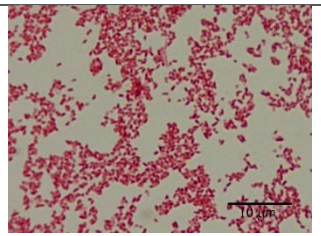
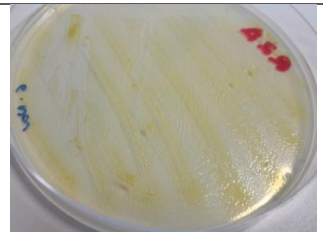
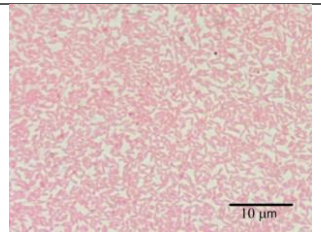
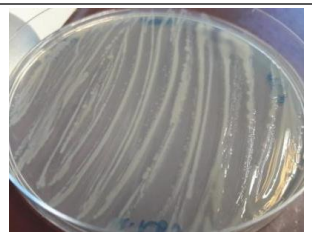
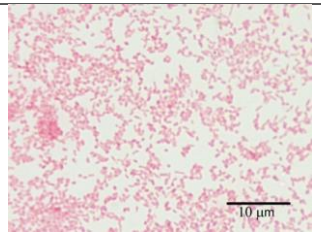
Isolate	Molecular identification	Macroscopic image	Colony coloration	Morphologic features	Microscopic image*	Gram staining	Cellular shape
M17A	*Aeromonas* sp.		Pale white	Mucous		Gram -	Cocobacilli
M17B	*Aeromonas* sp.		Pale white	Mucous		Gram -	Cocobacilli
M17C	*Flectobacillus* sp.		Pink	Mucous		Gram -	Long rods

Isolate	Molecular identification	Macroscopic image	Colony coloration	Morphologic features	Microscopic image*	Gram staining	Cellular shape
M17D	*Flectobacillus* sp.		Pink	Mucous		Gram -	Long rods
M17E	*Flavobacterium* sp.		Yellow	Mucous		Gram -	Bacilli
M17F	*Flavobacterium* sp.		Yellow	Mucous		Gram -	Bacilli
M17G	*Flavobacterium* sp.		Yellow	Slightly mucous		Gram -	Bacilli

Isolate	Molecular identification	Macroscopic image	Colony coloration	Morphologic features	Microscopic image*	Gram staining	Cellular shape
M17H	*Rahnella aquatilis*		White	Mucous		Gram -	Cocci
M17I	*Aeromonas* sp.		White	Slightly mucous		Gram -	Cocobacilli
M17J	*Aeromonas* sp.		White	Slightly mucous		Gram -	Cocobacilli
M17K	*Flavobacterium* sp.		Yellow	Mucous		Gram -	Bacilli

Isolate	Molecular identification	Macroscopic image	Colony coloration	Morphologic features	Microscopic image*	Gram staining	Cellular shape
M17L	*Rheinheimera aquatica*		Yellow	Very mucous		Gram -	Bacilli
M17M	*Aeromonas* sp.		White	Slightly mucous		Gram -	Cocobacilli
M17N	*Aeromonas* sp.		White	Slightly mucous		Gram -	Cocobacilli
R17E	*Acinetobacter* sp.		White	Mucous		Gram -	Cocci

Isolate	Molecular identification	Macroscopic image	Colony coloration	Morphologic features	Microscopic image*	Gram staining	Cellular shape
R17I	*Micrococcus luteus*		White	Mucous		Gram +	Cocci
R17K	*Arthrobacter globiformis*		Pale yellow	Slightly mucous		Gram +	Cocci
R17O	*Sphingomonas* sp.		Yellow	Mucous		Gram -	Bacilli
R17Q	*Flavobacterium* sp.		Yellow	Slightly mucous		Gram -	Bacilli

Isolate	Molecular identification	Macroscopic image	Colony coloration	Morphologic features	Microscopic image*	Gram staining	Cellular shape
R17R	*Aeromonas* sp.		White	Mucous		Gram -	Cocobacilli
R17S	*Rheinheimera aquatica*		Yellow	Very mucous		Gram -	Bacilli
R17T	*Aeromonas* sp.		White	Mucous		Gram -	Cocobacilli

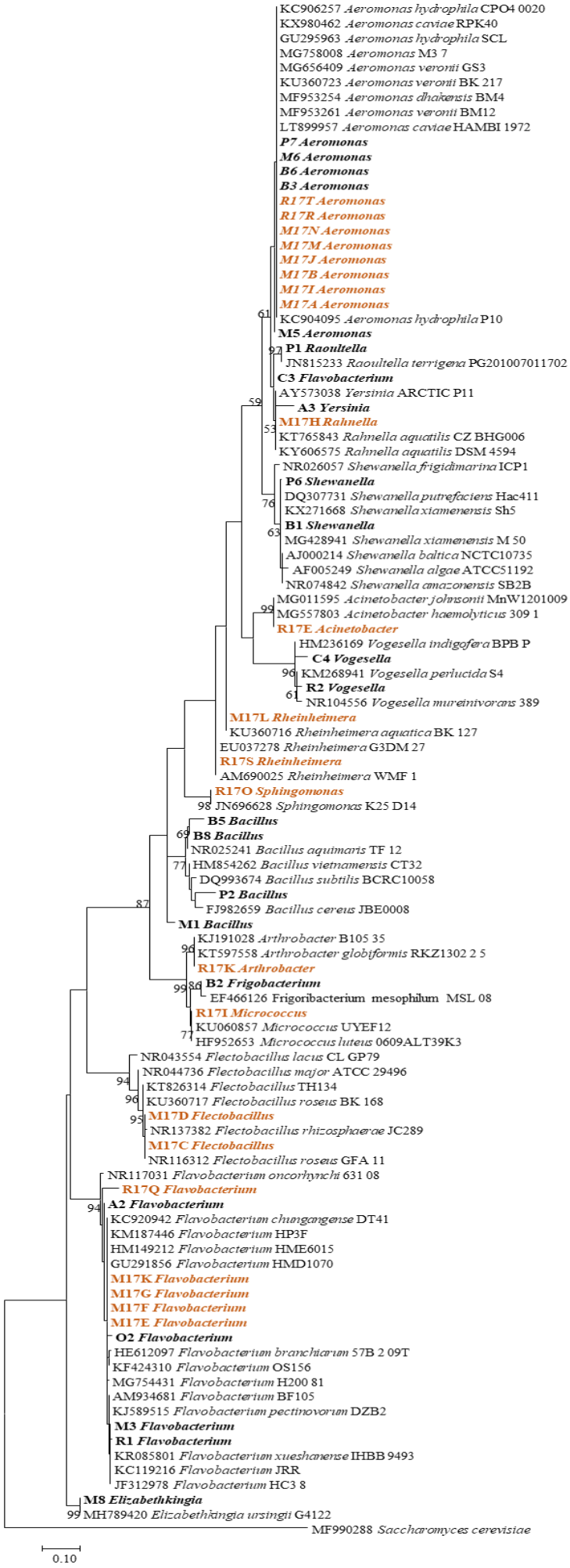


Figure 3.1. Phylogenetic analysis obtained using Maximum Likelihood method with 1000 Bootstrap. *Saccharomyces cerevisiae* was used as outgroup. In orange are the bacteria isolated in this study, and in bold are bacteria isolated in DSA-ASBE previously to this study. Each isolate only has the genera identification.

3.2. *mlr* gene identification

The presence of *mlr* gene cluster was screened in all isolates. All the genes of *mlr* cluster (*mlrA; mlrB; mlrC and mlrD*) were only identified in isolate M17C. However, none of the other isolates presented *mlr* genes amplification. Some other amplifications were verified, but after sequencing, Blastn (nucleotide-nucleotide) and Blastx (nucleotide-protein) verification, it was confirmed that these weren't, in fact, *mlr* or possible related genes (data showed in table 3.2).

Table 3.2. Unspecific genes amplified with *mlr* primers. (ND – not detected)

		mlrA	***mlrB***	***mlrC***	***mlrD***
		Similarity with			
Aeromonas	**M17I**	ND	Chromosome partitioning protein ParB	ND	Non-specific amplification
	M17J	ND	Non-specific amplification	ND	Non-specific amplification
	M17N	ND	ND	ND	Non-specific amplification
	R17R	ND	ND	ND	Non-specific amplification
Acinetobacter	**R17E**	DNA-binding response regulator	ND	ND	MFS – transporter
Rahnella	**M17H**	ND	MATE family afflux transporter	ND	ND
Rheinheimera	**M17L**	ND	No significant similarity found	ND	ND
Shewanella	**B1**	Potassium transporter	ND	ND	ND
Sphingomonas	**594196**	ND	Hypothetical protein	ND	ND

3.3. Effects of microcystins on the bacterial growth

The isolates were exposed to four different concentrations (1 nM, 10 nM, 100 nM and 1 µM) of two microcystin variant extract (MCLR, MCRR), one concentration of MCYR (1 nM), and to two concentrations of pure MCLR (1 nM and 1 µM). Several behaviors were displayed such as a growth reduction, growth increase or no growth effect and different effects according to each concentration of the same variant when compared to the control group, where no microcystins were added.

Initial tests showed that some of the studied bacteria took too many hours to reach stationary phase, so in order to make this study possible a period was defined for the trial: 10 hours from t_0 to t_{20} (interval between t_x and t_{x+1} was 30 min). All graphics presented in the growth trial results were the mean of two biological replicates (duplicates). These duplicates were performed in different days with different pre-inoculums. The (*) indicates a significant difference between growth with MC stress and control ($p<0.05$). Yet, in some measured times, the difference between stress condition and control condition, didn't translate into a statistically significant difference due to biological replicates differences.

In *Aeromonas* spp. isolates, M17I, M17M, R17R, R17T and B3 (figure 3.2; 3.3; 3.4) most stress provoked a slight decrease in growth but, only in M17M with 1 µM (t_{19}, t_{20}), R17R with 100 nM and 1 µM (after t_{12}) and B3 with 1000 nM (after t_9) with MCLR extract, and in R17R with MCRR extract (100 nM – after t_9; and 1000 nM – t_{20}) and MCYR extract (after t_{12}) the decrease in growth was statistically significant ($p < 0.05$). The growth of R17T, when exposed to MCRR, shows a different response to the stress, as it shows a slight increase in growth (not statistically significant).

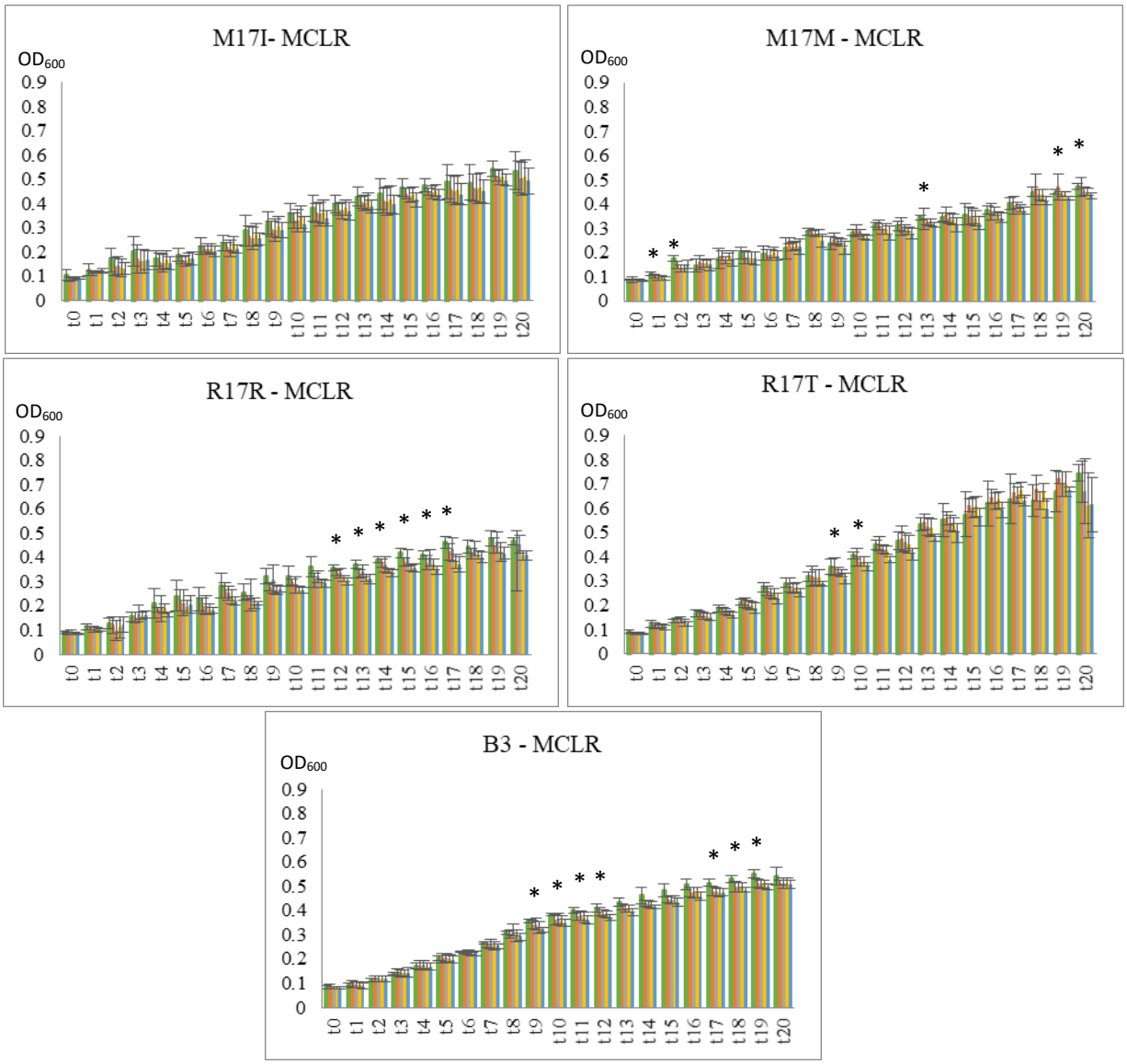


Figure 3.2. Graphs of growth curves from *Aeromonas* spp. isolates with MCLR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

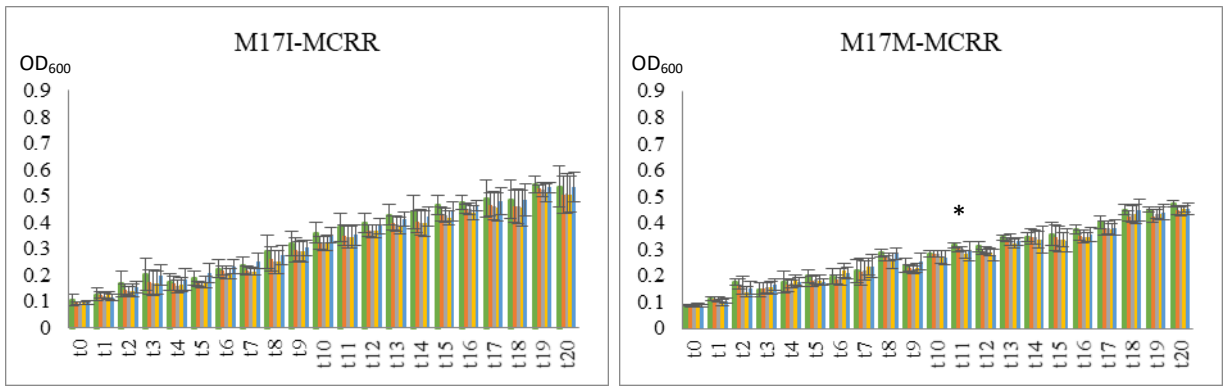


Figure 3.3. Graphs of growth curves from *Aeromonas* spp. isolates with MCRR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

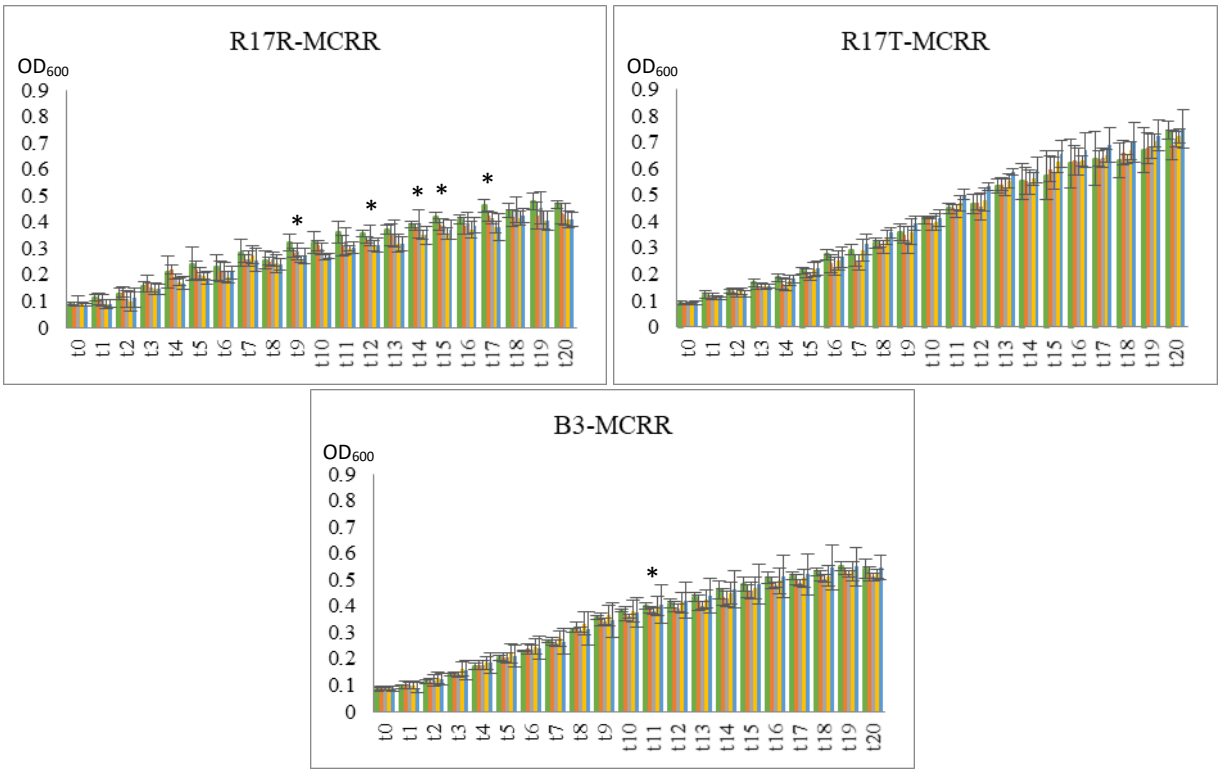


Figure 3.3. (continuation). Graphs of growth curves from *Aeromonas* spp. isolates with MCRR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

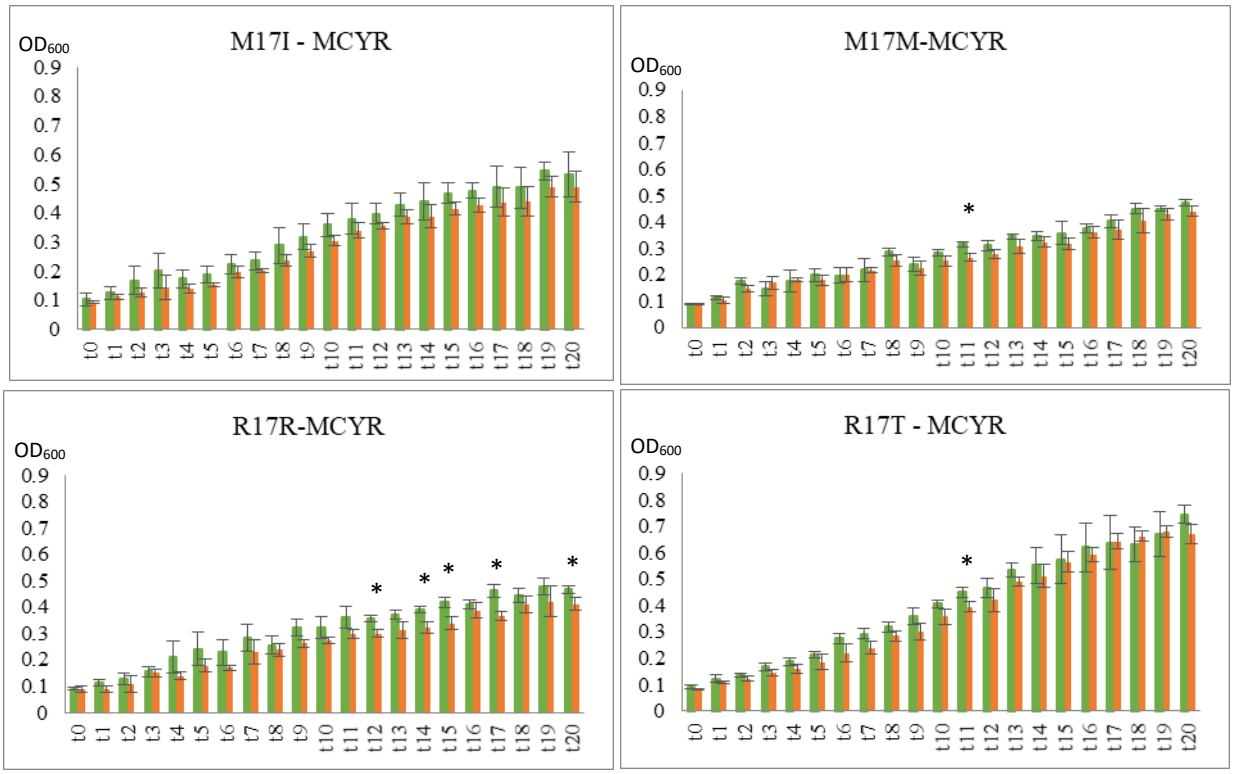


Figure 3.4. Graphs of growth curves from *Aeromonas* spp. isolates with MCYR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin.

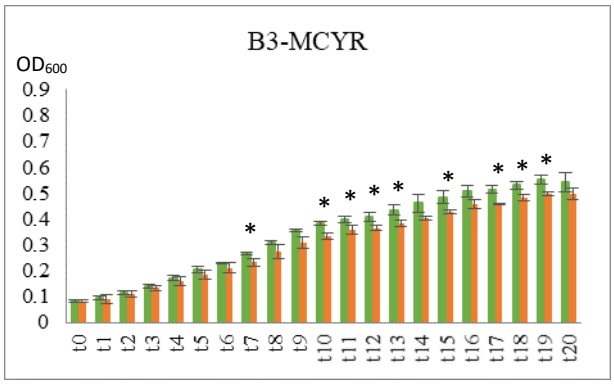


Figure 3.4. (Continuation) Graphs of growth curves from *Aeromonas* spp. isolates with MCYR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin.

In *Flavobacterium* spp. isolates, M17E, M17F, M17K and R17Q, the results diverged more (figure 3.5; 3.6; 3.7). For instance, M17F exposed to MCLR extract shows a slight increase in growth when compared to control conditions both with 1 nM and 10 nM (not statistically significant) and no difference at 100 nM or 1 µM. When exposed to MCRR there is a slight decrease in growth, with significantly statistic meaning ($p < 0.05$), at a 100 nM concentration starting at point t_{17}, and the same occurs with the exposure to MCYR. When exposed to microcystins extract, M17E showed no relevant difference in growth compared to control with all the three MC variants. M17K and R17Q appear to have no relevant difference in growth when exposed to MCYR extract and also MCLR and MCRR extract (M17K). However, when exposed to MCLR or MCRR extract R17Q seems to reveal a slight increase in growth.

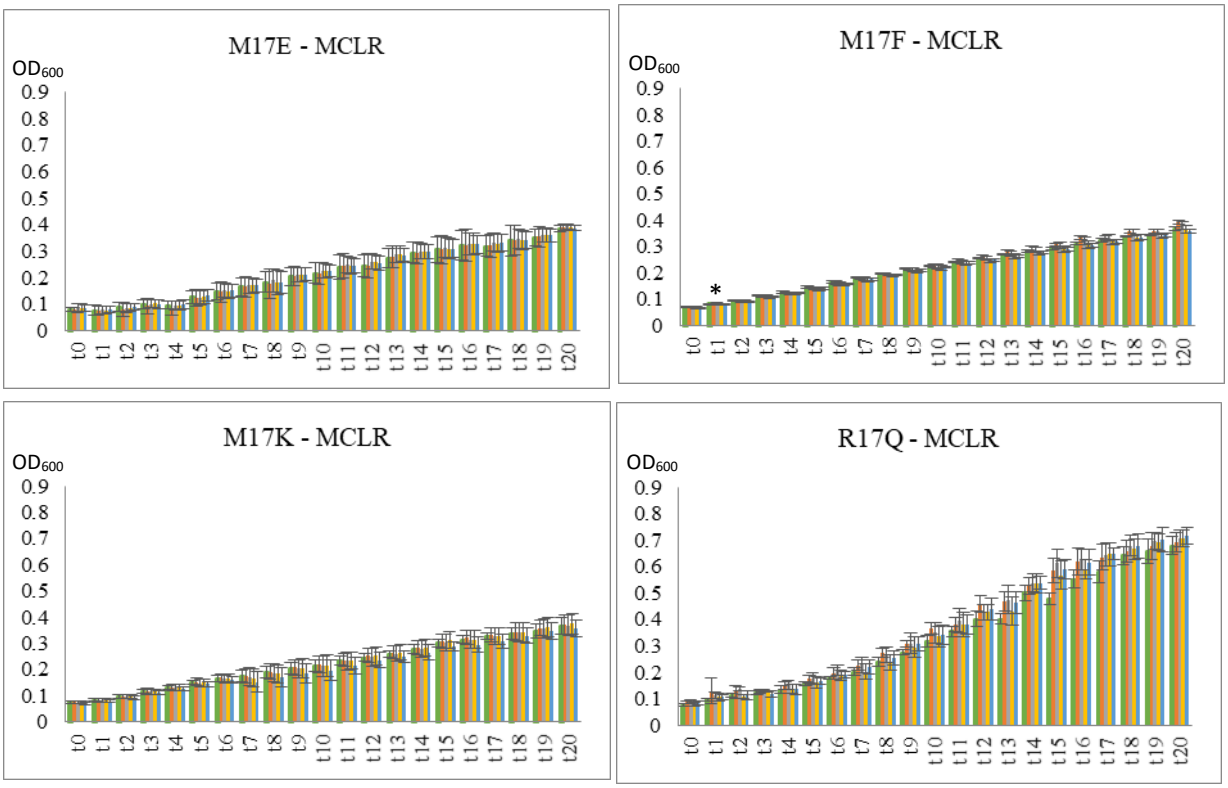


Figure 3.5. Graphs of growth curves from *Flavobacterium* spp. isolates with MCLR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

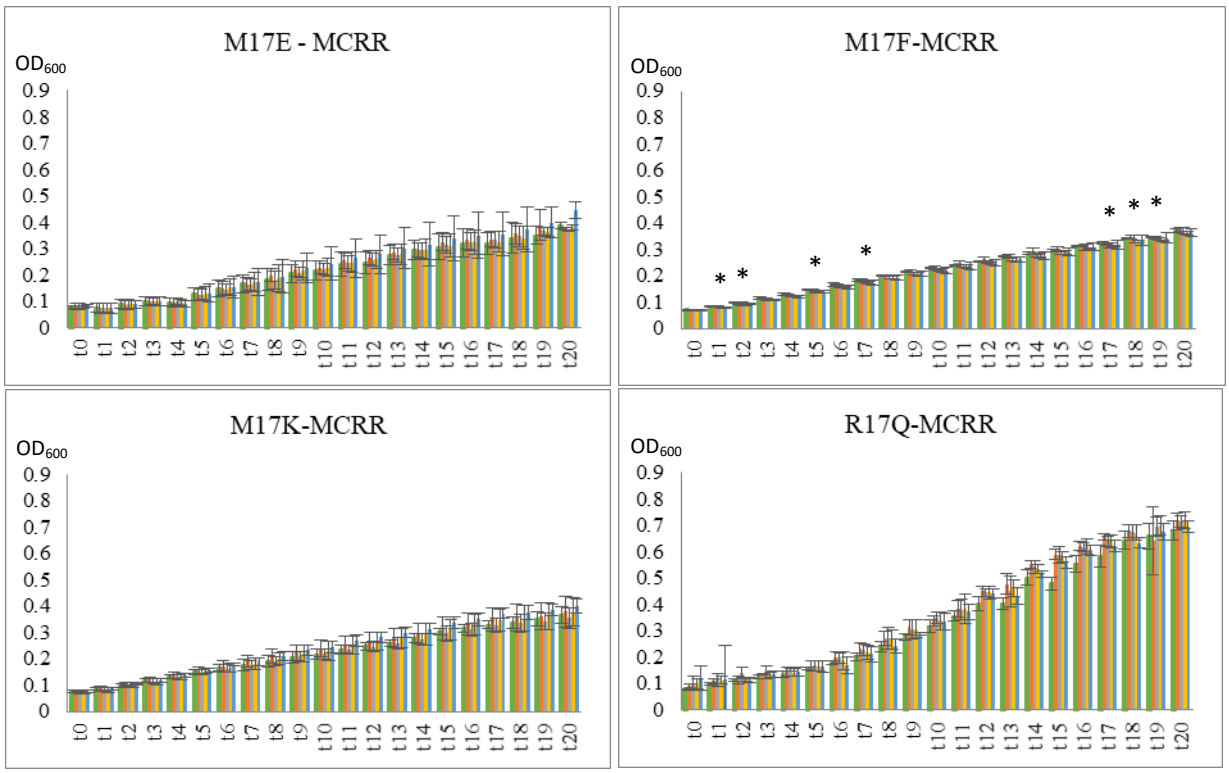


Figure 3.6. Graphs of growth curves from *Flavobacterium* spp. isolates with MCRR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

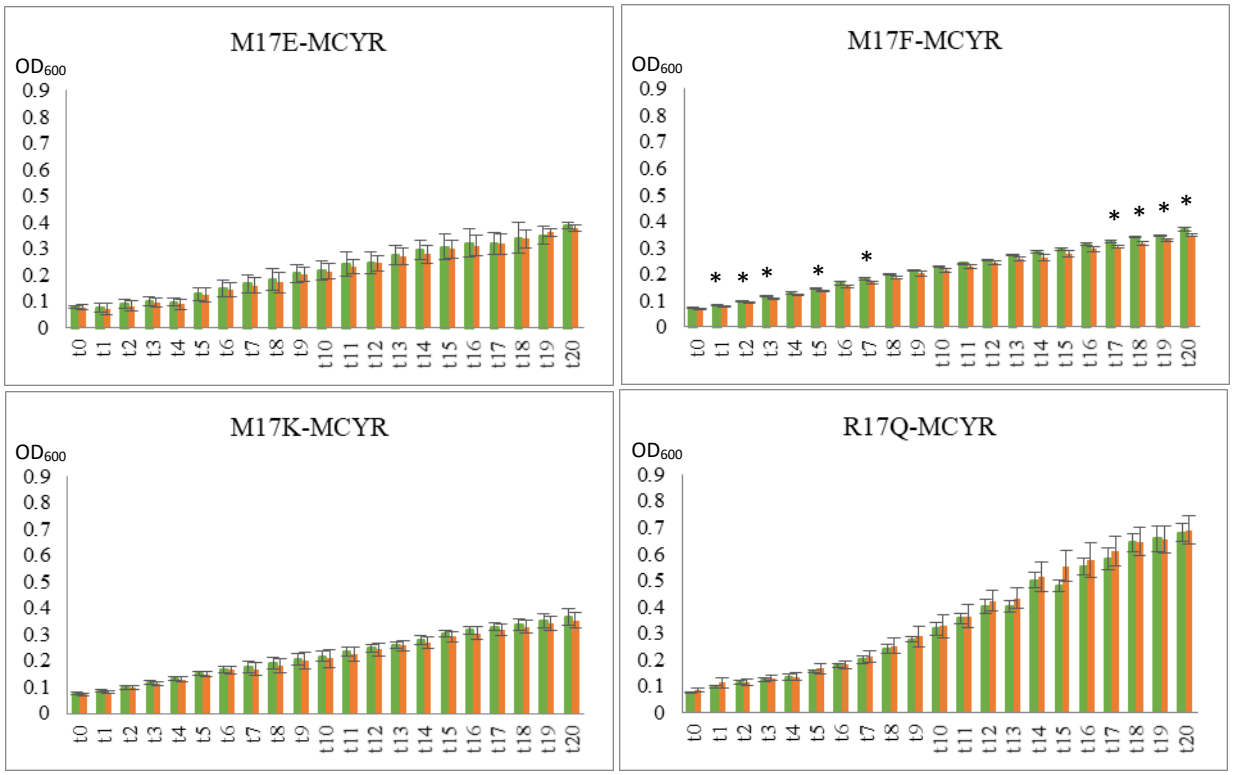


Figure 3.7. Graphs of growth curves from *Flavobacterium* spp. isolates with MCYR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin.

In *Flectobacillus* spp. isolates (figure 3.8; 3.9; 3.10), M17C and M17D, there were no significant differences between the control condition and the stress provoked by any of the microcystins extract, although, in M17C with MCLR extract at 1 nM, M17C appears to have a slightly improved growth (which isn't statistically relevant).

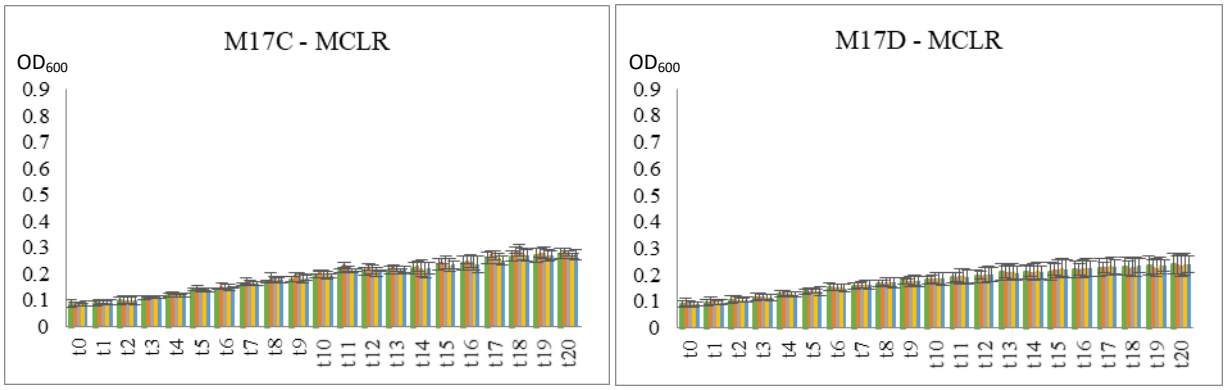


Figure 3.8. Graphs of growth curves from *Flectobacillus* spp. isolates with MCLR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

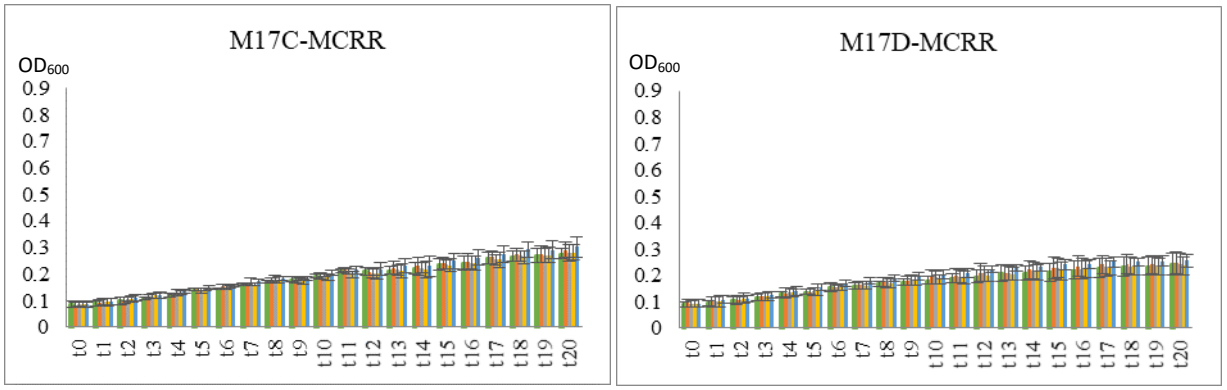


Figure 3.9. Graphs of growth curves from *Flectobacillus* spp. isolates with MCRR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

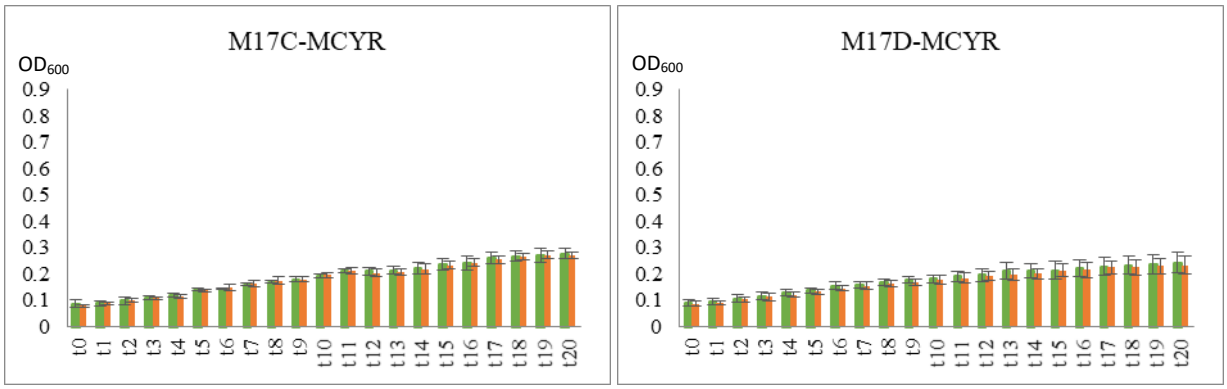


Figure 3.10. Graphs of growth curves from *Flectobacillus* spp. isolates with MCYR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin.

In *Sphingomonas* sp. isolate 594196 (figure 3.11), all growth conditions seem to be affected by the microcystin extracts. However, none is statistically significant, due to biological replicate differences also reflected in the standard deviation, as shown in the graphics.

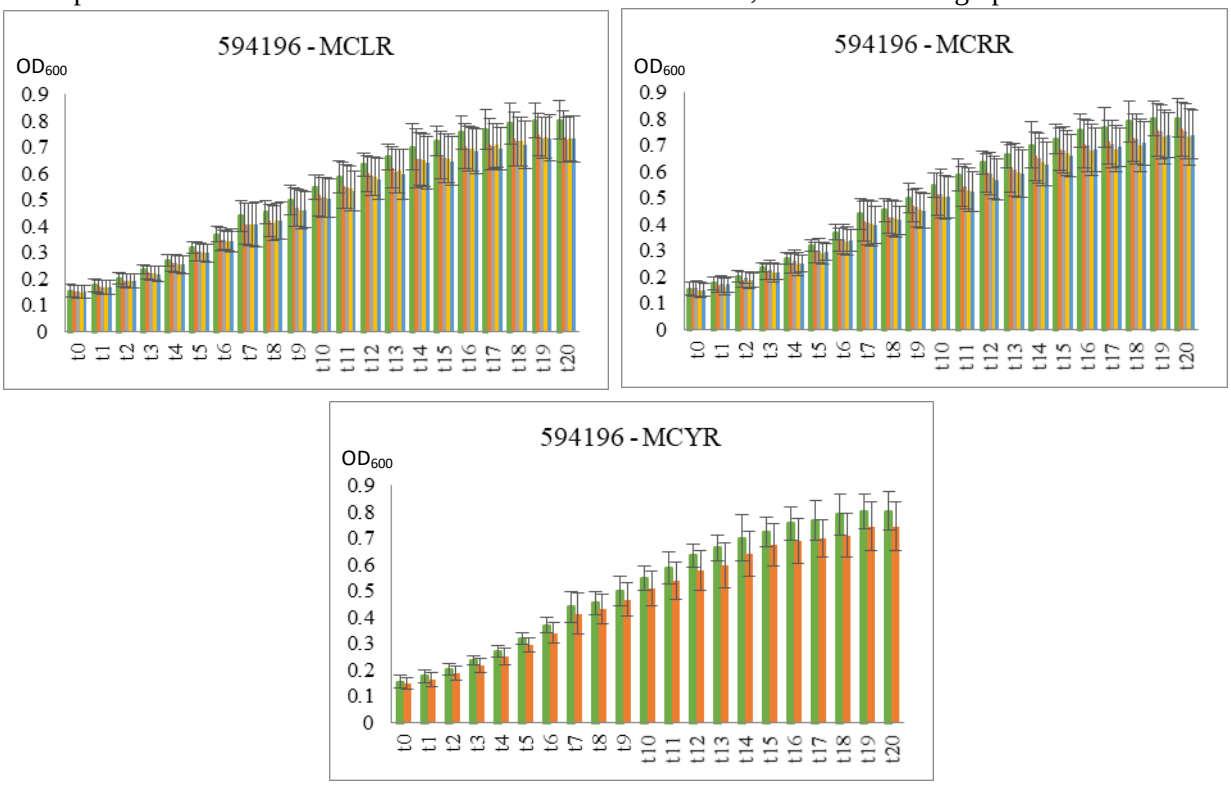


Figure 3.11. Graphs of growth curves from *Sphingomonas* sp. isolate with the different MCs variants over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicate assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

In figure 3.12. it can be observed the graphics representing the growth response to two concentrations of pure MCLR (1 nM and 1µM) in contrast to MCLR extract and control condition.

The stimuli provoked by pure MCLR seems to be more aggressive than MCLR extract, causing a decrease in growth except in isolates M17F and M17M where the response is similar, R17R where a slight increase of growth is observed and in 594196 whose growth was already affected by MCLR extract.

The growth with 1 nM pure MCLR medium doesn't appear to cause effects in growth when compared to the control condition except in isolates 594196, M17E, M17F, M17M, R17T and B3, (and with significantly statistic meaning ($p < 0.05$) for M17F). On the other hand, at 1 µM, pure MCLR seems to affect most of the tested bacteria causing a decrease in growth, these decrease in growth has significant statistic meaning ($p < 0.05$) at M17C (t_4 to t_{20}), M17E (t_{20}), M17K (t_5 to t_{20}) and B3 (t_7 to t_{20}). M17M and R17R appears not to be affected or be slightly affected by 1 µM of pure MCLR.

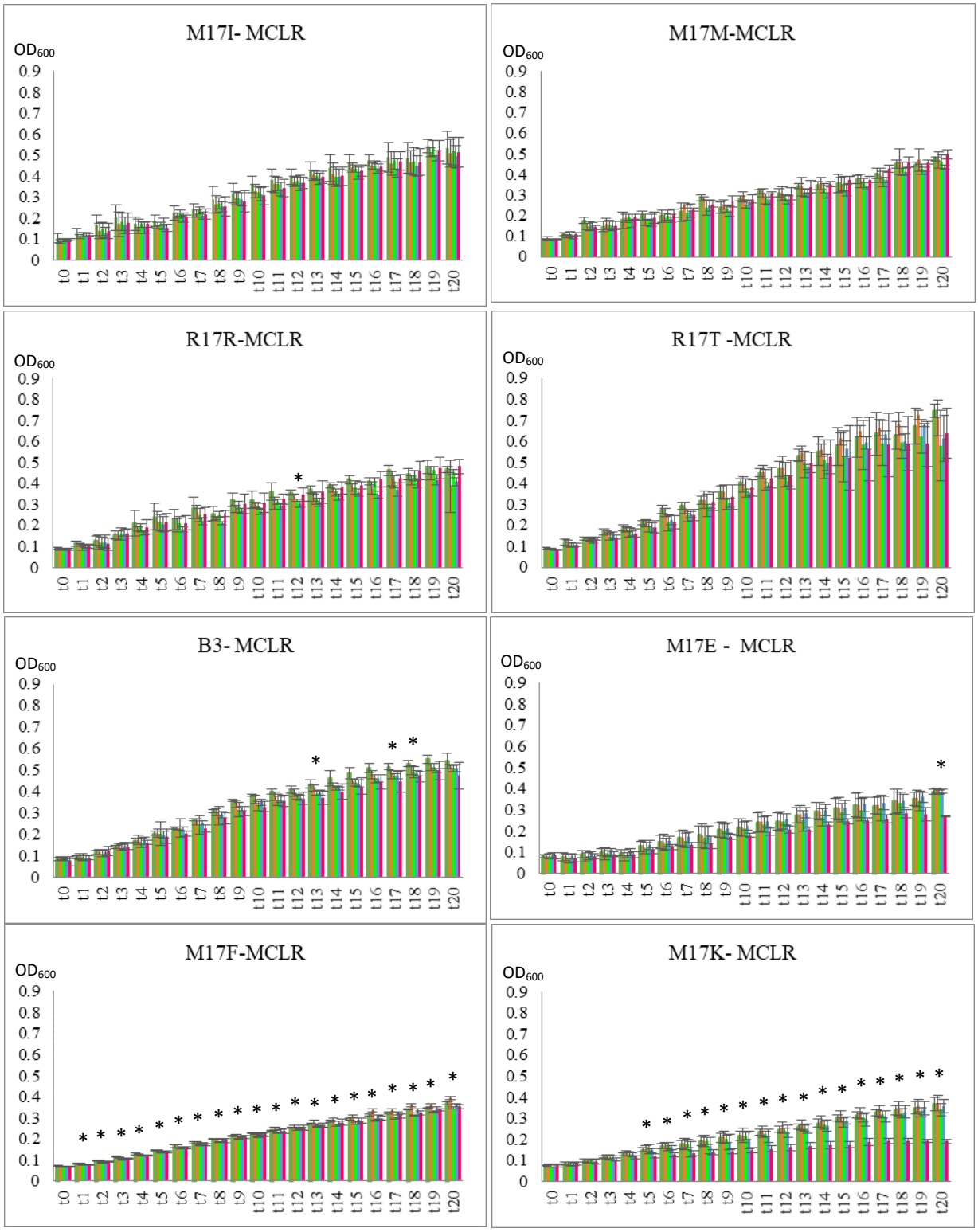


Figure 3.12. Graphs of growth curves from all isolates with both MCLR extract and pure MCLR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicated assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM of MCLR extract, (●) 1 nM of pure MCLR, (●) 1 µM of MCLR extract, (●) 1 µM of pure MCLR.

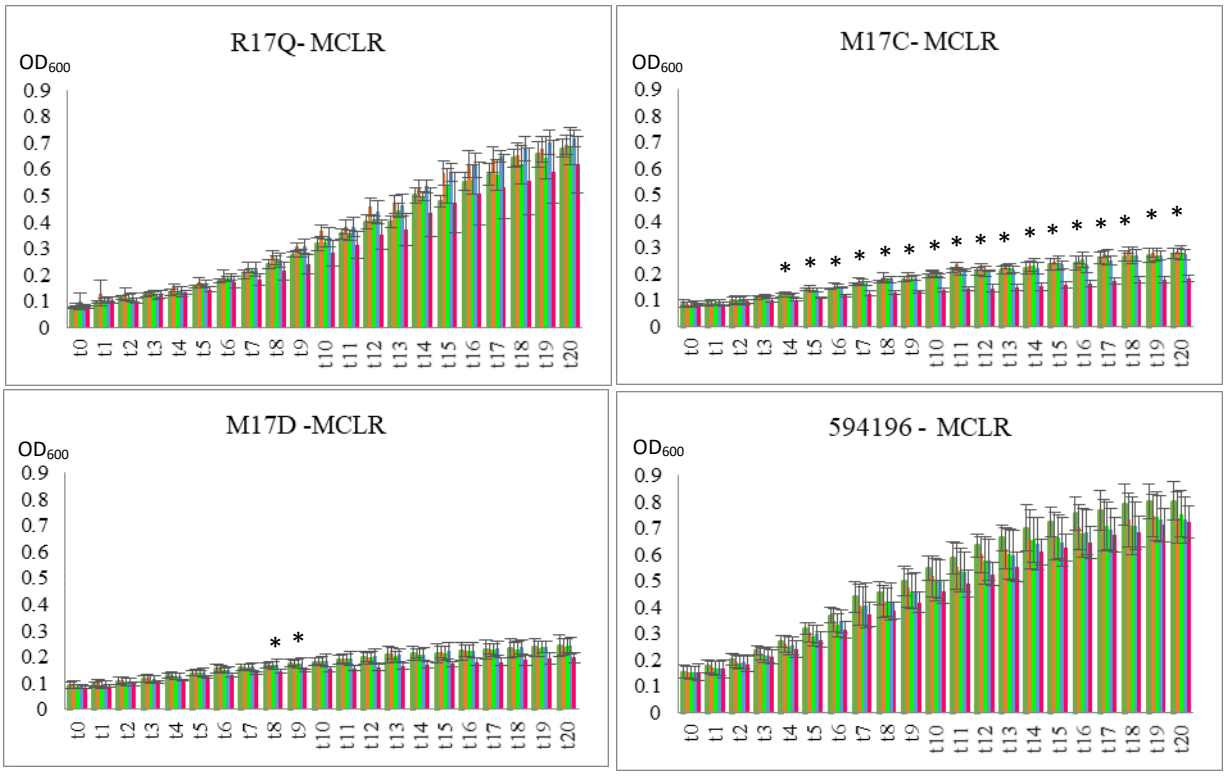


Figure 3.12. (continuation) Graphs of growth curves from all isolates with both MCLR extract and pure MCLR over time (t_0-t_{20}). The values are the mean ± standard deviation of two duplicated assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM of MCLR extract, (●) 1 nM of pure MCLR, (●) 1 µM of MCLR extract, (●) 1 µM of pure MCLR.

In previous studies in ASBE, growth trials were performed with bacteria isolated in 2012 (Miguéns, 2013) from the same reservoir as one in the current study: Albufeira de Magos. Growth trials from isolates of the same genera, isolated both in 2012 and 2017 were placed side by side and compared. Their comparison appears to indicate an adaptation to MCs presence, as bacteria isolated in 2012 seemed more susceptible to MCs presence then the isolate from 2017. In figure 3.13, two graphics from two bacteria, M6 isolated in 2012 (Miguéns, 2013) and M17M isolated in 2017, both exposed to MCLR extract, represent this hypothesis. Both were exposed to the same conditions, however, M6 was more affected by MCs presence than M17M, since M17M appears to have a similar growth pattern both in control condition and when exposed to MCLR.

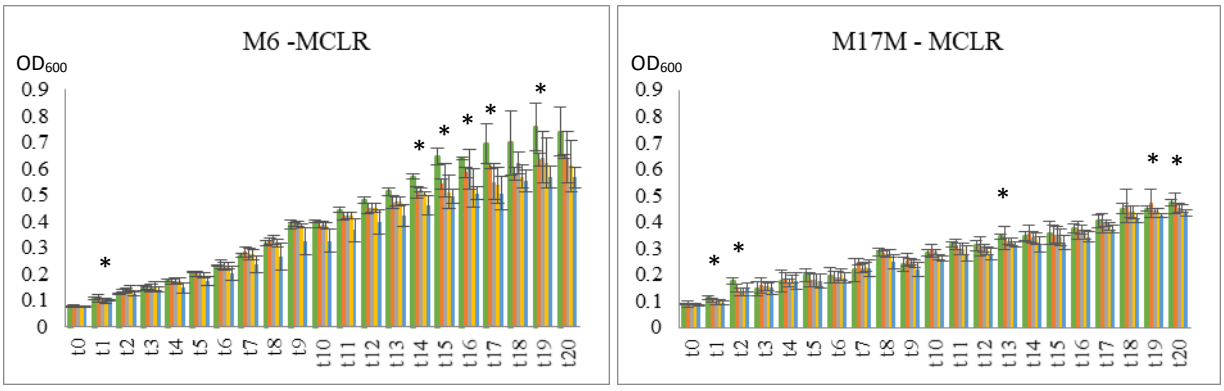


Figure 3.13. Graphs of growth curves from two *Aeromonas* isolates from Magos reservoir with MCLR extract over time (t_0-t_{20}), one isolated in 2012 and one isolated in 2017. The values are the mean ± standard deviation of two duplicated assays. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

The table 3.3 is a summary of growth trials, with growth comparison between MCs and control condition from all isolates tested during this study. In blue, are the growth trials with duplicates, and in black, those without duplicates due to time restrictions or which showed no growth repeatability (graphics in annex). The results in this table, seem to indicate that most bacteria were less susceptible to MCRR than to MCLR and MCYR. As only seven isolates showed a reduction in growth when exposed to MCRR compared with nine and thirteen isolates growth reduction when exposed to MCLR and MCYR, respectively. This difference in toxicity may be related with each variant hydrophobicity.

Table 3.3. Synopsis of data from all isolates growth trial. Growth response (considering results from all tested concentrations) to different microcystin variants when compared to control. (>) growth in stress condition increases compared to control, (=) growth in stress condition is equal to control, (<) growth in stress condition decreases when compared to control. (●) results with two duplicated assays, (●) results without duplicates

		Growth			
		Pure MCLR	**MCLR extract**	**MCRR extract**	**MCYR extract**
Aeromonas	**M17A**	<	<	>	<
	M17B	=	=	>	=
	M17I	<	<	<	<
	M17J	Inconsistent growth between duplicates			
	M17M	=	<	<	<
	M17N	<	=	>	<
	R17R	=	<	<	<
	R17T	<	<	=	<
	B3	<	<	<	<
	M6	<	<	<	<
Acinetobacter	**R17E**	<	>	>	>
Arthrobacter	**R17K**	<	>	>	>
Flavobacterium	**M17E**	<	=	>	<
	M17F	<	=	<	<
	M17G	Inconsistent growth between duplicates			
	M17K	<	=	=	=
	R17Q	<	>	>	=
	M3	<	<	=	<
Flectobacillus	**M17C**	<	=	=	=
	M17D	<	=	=	=
Micrococcus	**R17I**	<	=	=	=
Rahnella	**M17H**	>	>	=	=
Rheinheimera	**M17L**	No growth			
	R17S	No growth			
Shewanella	**B1**	<	=	>	<
Sphingomas	**R17O**	Bacteria formed aggregates that didn't allowed correct absorbance measurements			
	594196	<	<	<	<

3.4. Effects of microcystins on the bacterial antioxidant system

After 12 hours of incubation with each microcystin variant (pure MCLR; MCLR extract and MCRR extract) at a concentration of 10 nM, the CAT activity was measured, as well as, in control cells. In table 3.4 is represented CAT activity (U/mg) for the nine representative isolates chosen to perform this assay.

Except for M17F, M17K and 594196, none of the other isolates showed catalase activity. M17F appears to respond with an increase in CAT activity to the stress provoked by MCRR and with a decrease in CAT both in pure MCLR and MCLR extract, when compared with the control. Furthermore, the CAT activity inhibition was more pronounced with MCLR extract, suggesting that there might be other bioactive compounds in the extract that amplifies the MCLR action. Due to insufficient volume of protein extract to perform the assays both control condition and MCRR extract, CAT activity measurements weren´t tested in M17K. However, M17K showed different activity when exposed to pure MCLR and MCLR extract, with a pattern similar to M17F (another isolate from the same genera) displaying a lower activity when exposed to MCLR extract compared with pure MCLR. Finally, the 594196 isolate shows a decrease in CAT activity when exposed to MCRR extract and pure MCLR compared to the control, on the other hand, no alteration in activity occurs when it is exposed to MCLR extract *vs*. the control.

In table 3.4 the results from previous studies are also presented. These studies support the idea that other enzymes may be linked to H_2O_2 degradation in these heterotrophic bacteria (only six out of 19 isolates were CAT positive). In most CAT positive isolates there was a reduction in CAT activity when isolates were exposed to MCLR extract. Also, in most cases there was an increase in CAT activity when isolates were exposed to MCRR extract.

Table 3.4. Summary of Catalase activity (U/mg) in the present study and from previous studies taken at the DSA-ASBE(INSA) lab.

		Catalase					
		Control	**pure MCLR**	**MCLR extract**	**MCRR extract**	**MCYR extract**	
Aeromonas	**M17I**	0.00	0.00	0.00	0.00	--	This study
	R17R	0.00	0.00	0.00	0.00	--	This study
	B3	0.00	0.00	0.00	0.00	--	This study
Flavobacterium	**M17F**	9.17	6.88	3.06	11.47	--	This study
	M17K	--	13.76	9.17	--	--	This study
	R17Q	0.00	0.00	0.00	0.00	--	This study
	A2	0.00	--	0.00	0.00	0.00	Pinto, 2016
	R1	0.00	--	0.00	0.00	0.00	Pinto, 2016
Flectobacillus	**M17C**	0.00	0.00	0.00	0.00	--	This study
	M17D	0.00	0.00	0.00	0.00	--	This study
Sphingomonas	**594196**	73.39	57.34	73.39	61.93	--	This study
Pseudomonas	**O3**	0.00	--	0.00	0.00	0.00	Pinto, 2016
	R4	22.94	--	9.17	27.52	18.35	Pinto, 2016
Yersinia	**A3**	9.17	--	18.35	22.936	18.35	Pinto, 2016
Bacillus	**M1**	0.00	--	0.00	0.00	0.00	Miguéns, 2013
Raoultella	**P1**	0.00	--	0.00	0.00	0.00	Miguéns, 2013
Shewanella	**P6**	0.00	--	0.00	0.00	0.00	Miguéns, 2013
Vogesella	**R2**	27.52	--	18.35	13.76	13.76	Pinto, 2016
Vogesella	**C4**	0.00	--	0.00	0.00	0.00	Miguéns, 2013

Table 3.5. SOD_t relative activity. Results obtained in the present study and data from previous studies taken at the DSA-ASBE(INSA) lab.

		SOD_t					
		Control	**pure MCLR**	**MCLR extract**	**MCRR extract**	**MCYR extract**	
Aeromonas	**M17I**	0.00	0.00	0.00	0.00	--	This study
	R17R	0.00	0.00	0.00	0.00	--	This study
	B3	0.00	0.00	0.00	0.00	--	This study
Flavobacterium	**M17F**	0.00	0.00	0.00	0.00	--	This study
	M17K	0.00	0.00	0.00	0.00	--	This study
	R17Q	0.00	0.00	0.00	0.00	--	This study
	A2	1.00	--	1.83	-0.83	-0.83	Pinto, 2016
	R1	1.00	--	-1.25	3.25	3.00	Pinto, 2016
Flectobacillus	**M17C**	1.00	1.50	2.00	0.83	--	This study
	M17D	1.00	1.33	0.78	1.00	--	This study
Sphingomonas	**594196**	1.00	0.50	0.42	0.42	--	This study
Pseudomonas	**O3**	1.00	--	1.07	2.29	-1.29	Pinto, 2016
	R4	1.00	--	1.00	-3.50	-1.75	Pinto, 2016
Bacillus	**M1**	1.00	--	1.22	0.96	0.00	Miguéns, 2013
Raoultella	**P1**	1.00	--	0.98	0.97	0.95	Miguéns, 2013
Shewanella	**P6**	1.00	--	1.01	1.01	1.03	Miguéns, 2013
Vogesella	**R2**	1.00	--	0.91	1.64	1.64	Pinto, 2016
Vogesella	**C4**	1.00	--	1.39	3.16	3.19	Miguéns, 2013

After 12 hours of incubation with each microcystin variant (pure MCLR, MCLR extract and MCRR extract) at a concentration of 10 nM, the SOD_t activity inhibition (corresponding to the sum of SOD_1 and SOD_2 contributions) was measured and compared with the control condition, table 3.5. Additionally, SOD_1 and SOD_2 were measured for the three chosen isolates (594196, M17C and M17D), that were selected because they were the only isolates that presented SOD activity and the results are shown in table 3.6.

Observing the results in table 3.5 it can be accessed that only three of the isolates tested from this study displayed SOD_t activity. These isolates are *Flectobacillus* spp. M17C, M17D and *Sphingomonas* sp. 594196. Although from the same genus, M17C and M17D have very different responses to the same MC variants. When exposed to MCLR extract SOD activity duplicates in isolate M17C but reduces in isolate M17D when both are compared to control conditions. When exposed to MCRR extract, M17C has a decrease in SOD activity, on the other hand SOD activity, in M17D, stays the same as in control condition. As M17C and M17D are different strains from *Flectobacillus* spp. there may be some specific strain characteristic or process influencing these results. Also, a bioactive component from MCs extract may be interfering with M17C and M17D differently. Lastly, the 594196 isolate shows a 50% or more decrease in activity in all cases of stress when compared to control.

Table 3.5. also has the content from previous studies (Miguéns, 2013 and Pinto, 2016), that will be discussed further ahead.

To perform the superoxide dismutase assay with the kit tested, a negative control is also necessary. It includes all components except SOD (used in positive control) or cell lysate. This control will establish the maximal increase in absorbance due to superoxide radicals. For that and to start the reaction, xanthine oxidase is added to dH_2O, buffer, xanthine and NBT. The reaction occurs as xanthine is oxidized to $O^{\bullet -}$ that reacts with NBT forming NBT-diformazan. The conversion is followed with absorbance measurements during five min and the rate of conversion is calculated.

Table 3.6. SOD1 and SOD2 relative activity. Results obtained in the present study and data from previous studies taken at the DSA-ASBE (INSA) lab.

		SOD_1 and SOD_2															
		Control			**Pure MCLR**			**MCLR extract**			**MCRR extract**			**MCYR extract**			
		SOD_t	SOD_1	SOD_2	SOD_t	SOD_1	SOD_2	SOD_t	SOD_1	SOD_2	SOD_t	SOD_1	SOD_2	SOD_t	SOD_1	SOD_2	
	M17C	1.00	-1.67	2.67	1.00	-0.11	1.11	1.00	0.67	0.33	1.00	-2.60	3.60				This study
Flectobacillus	**M17D**	1.00	-0.11	1.11	1.00	0.08	0.92	1.00	0.00	1.00	1.00	0.22	0.78				This study
Sphingomonas	**594196**	1.00	0.42	0.58	1.00	-0.50	1.50	1.00	0.00	1.00	1.00	-0.20	1.20				This study
	A2	1.00	0.00	1.00				1.00	0.27	0.73							Pinto, 2016
Flavobacterium	**R1**	1.00	2.75	-1.75							1.00	1.15	-0.15	1.00	1.25	-0.25	Pinto, 2016
Pseudomonas	**R4**	1.00	1.75	-0.75				1.00	-2.00	3.00							Pinto, 2016
Vogesella	**R2**	1.00	0.82	0.18				1.00	0.80	0.20							Pinto, 2016
Yersinia	**A3**										1.00	-0.29	1.29				Pinto, 2016
Bacillus	**M1**	1.00	0.90	0.10				1.00	0.44	0.56	1.00	-0.06	1.06	0.00	0.00	0.00	Miguéns, 2013
Raoultella	**P1**	1.00	0.02	0.98				1.00	0.00	1.00	1.00	0.02	0.98	1.00	0.02	0.98	Miguéns, 2013
Shewanella	**P6**	1.00	0.01	0.99				1.00	0.04	0.96	1.00	0.04	0.96	1.00	0.00	1.00	Miguéns, 2013
Vogesella	**C4**										1.00	-0.02	1.02				Miguéns, 2013

Then the assay is measured, where the only difference is the addition of cell lysate. If the tested isolate has SOD, then SOD activity will convert $O^{\bullet -}$ in H_2O_2, therefore less $O^{\bullet -}$ will be available to react with NBT and the rate of reaction will be slower. Thus, the data obtained with this trial is a % of NBT-diformazan inhibition (figure 3.14) that is then converted in SOD relative activity.

As the values of table 3.6 represent the activity of SOD_1 and SOD_2 in relation to SOD_t (the sum of SOD_1 and SOD_2) these values should be between zero (no activity detected) and one (the activity of SOD equals SOD_t). So, results above one (in blue), results between zero and one (in black) and results below zero (in red) will be referred separately.

The only difference between SOD_t and SOD_2 assays is the addiction of 1% CN^- solution to the cell lysate in SOD_2 assay. CN^- is added to inactivate SOD_1. Then SOD_1 is calculated as the difference between SOD_t and SOD_2. So when SOD_2 activity is above one (more than SOD_t) the values of SOD_1 (in grey) won't be taken into consideration.

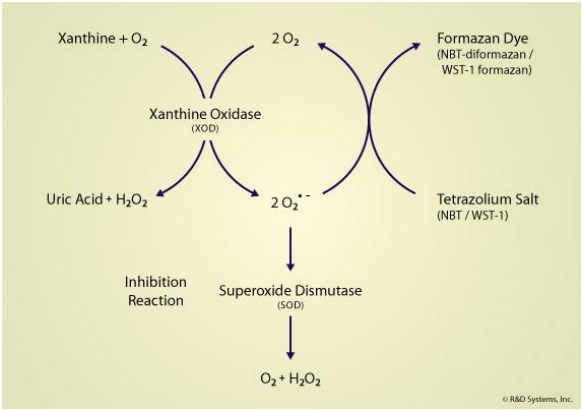


Figure 3.14. SOD role in the inhibition of NBT-diformazan formation. From Superoxide Dismutase Assay Kit, R&S systems®.

In black are assigned the isolates that display SOD_1 and SOD_2 activity. The results from M17C exposed to MCLR extract shows a predominance of SOD_1 activity compared to SOD_2. M17D shows little to no SOD_1 activity when exposed to MCLR (both pure and extract) but when exposed to MCRR there's an increase of SOD_1 activity, still SOD_2 is predominant (almost 4/5 of total activity). In 594196, only control and MCLR extract could be assessed and although in control condition SOD_1 and SOD_2 activity is similar, when exposed to MCLR, 594196 has no SOD_1 activity.

In black are the values of SOD_1 and SOD_2 between zero and one. When exposed to MCLR most SOD_t from isolate M17C was SOD_1. In all three stress conditions SOD_t was almost equivalent to SOD_2 in isolate M17D, with some substantial SOD_1 activity in MCRR condition. In control condition, isolate 594196, SOD_1 activity and SOD_2 activity represented 50% of SOD_t activity each. However, when 594196 was exposed to MCLR extract all SOD_t activity was SOD_2. Comparing results from this and previous studies (Miguéns, 2013; Pinto, 2016) it's observed that in control condition in three bacteria the SOD_t activity matches SOD_2 (A2, P1 and P6) and in two SOD_t activity almost matches SOD_1 (R2 and M1) and in isolate 594196 SOD_t corresponded to similar percentage of SOD_1 and SOD_2 activity. As for SOD activity when isolates were exposed to MC stress, in most isolates SOD_t corresponded mostly to SOD_2 activity (except in isolates M17C, R2 and B1).

The results in red represent results in which, SOD_2 absorbance was higher than negative control absorbance, as referred above, this control should stablish the maximal increase in absorbance. The results in blue show an increase in activity of SOD_2 higher then SOD_t, since SOD_t is the sum of SOD_1 and SOD_2 these results are contradictory and other processes related to antioxidative stress or assay performance related may be in action. These unexpected results will be discussed further ahead.

4. Discussion

4.1. Isolates characterization

From previous studies (Miguéns, 2013; Pinto, 2016) it was accessed that *Aeromonas* spp. and *Flavobacterium* spp. seemed to be especially resistant to microcystins, this point needed further research and elucidation. Because of this, yellow and mucous (potential *Flavobacterium* spp.) and white and mucous (potential *Aeromonas* spp.) bacteria were selected, obtaining a total of eleven white isolates and nine yellow isolates. Moreover, also two pink isolates (one of which revealed to carry the *mlr* cluster) were also recovered.

After morphological characterization and phylogenetical positioning, their identification was confirmed. It was accessed that, eight out of twelve white isolates were *Aeromonas* spp. and five out of nine yellow isolates were *Flavobacterium* spp.

In addition to the thirteen isolates already described as A*eromonas* and *Flavobacterium* spp. other nine isolates were selected. Morphological characterization and phylogenetical positioning revealed these isolates were distributed into seven different genera, namely *Acinetobacter* sp. (R17E), *Arthrobacter* sp. (R17K), *Flectobacillus* spp. (M17C and M17D), *Micrococcus* sp. (R17I), *Rahnella* sp. (M17H), *Rheinhemera* sp. (M17L and R17S) and *Sphingomonas* sp. (R17O).

Comparing the bacteria isolated in this study to the study from 2009 (Berg *et al*.), that was focused in the heterotrophic bacteria associated with cyanobacteria, we can conclude that bacteria isolated in the present study agree with what has been found previously in habitats where both heterotrophic bacteria and cyanobacteria co-habit.

In order to identify these bacteria, 16S DNA sequences were amplified using the 16S PCR primer pair 16S_8F and 16S_1492R. Although, the amplification of 16S gene displayed the expected 1500 bp length in all isolates, no usable forward sequence was achieved leading to the assumption that primer 16S_8F fits the purpose of 16S gene amplification but is unable to work as a sequencing primer. Still sequences with 700-750 nucleotide with good resolution were obtained (using primer 16S_1492R) thus allowing the construction of a phylogenetical tree.

It should be noted that, *Flavobacterium* isolate C3 may be seen in the figure 3.1 as phylogenetically closer to *Aeromonas* spp. but it's identification as a *Flavobacterim* has been confirmed in previous studies (Miguéns and Valério, 2015).

It was verified that the phylogenetical tree obtained using gene 16S sequences mostly allowed the identification of the genera to each isolate belongs. For instance, it is impossible to access if M17A is an *Aeromonas veronii* or an *Aeromonas caviae* since *Aeromonas* species, aren't differentiated in the referred tree. Given that gene 16S is the most widespread and conserved gene, also being the one with the more sequences in databases, it was the one chosen to perform the molecular identification of the isolates. However, in other to fully identify these isolates at species level further work would be needed, for instance using a set of genes that allowed the differentiation between species from the same genera or a polyphasic approach integrating not only phylogenetic data but also phenotypic and chemotaxonomic data.

4.2. *mlr* genes

The search for *mlr* genes brought different interesting results. Whereas, it was identified an isolate which possesses all four *mlr* genes: M17C, no other isolate had *mlr* genes amplified. M17C is a *Flectobacillus* sp. from class Cytophagia. Up until now, full *mlr* gene cluster was only identified in class Alphaproteobacteria (Kormas *et al*, 2013; Rastogi *et al*, 2014; Li *et al*, 2016), mostly in order Sphingomonodales and recently, one isolate from order Rhizobiales (Zhu *et al*, 2016). As *mlr* genes from M17C showed high similarity with *Novosphingobium* sp. isolate

THN-1 (Jiang *et al*, 2011) the possibility of gene transfer is high. However, as the search for full *mlr* cluster has been mainly centered in order Sphingomonodales, the extent of other bacteria with this gene cluster is still unknown.

Finding an *mlr*+ gene cluster allowed further comparison in growth and antioxidant system response to MCs induced stress between *mlr*+ and *mlr*- isolates (discussed further ahead).

Other observation was that in most cases unspecific amplifications were obtained, leading to conclusion that the *mlr* primers used in this study are redundant. However, four different pairs of primers were used (one for each *mlr* gene), and they show different degrees of redundancy. With *mlrC* primer pair, few unspecific amplifications have been obtained (data not shown). On the other hand, *mlrB* and *mlrD* primers both amplified many different genes (table 3.2). And *mlrA* primers, although not amplifying as many different genes as those with *mlrB* and *mlrD*, it also showed some unspecific amplifications (table 3.2). Since these *mlr* primers were designed using only gene sequences from *Sphingomonas* sp. (as these genes had only been previously detected in *Sphingomonas* spp. bacteria) the similarity with further genes in other bacteria wasn't so far determined. In this thesis, several bacteria from different genera were tested, so it can be expected that these primers could amplify other genes besides the ones of interest. Since most of the nonspecifically amplified genes had a similar size as the target genes, it became difficult to discriminate the fragments only performing a DNA electrophoresis. In this study, they were differentiated only when sequencing the amplified PCR products.

The primers used in this study were proposed in 2003 (Saito *et al*) and 2007 (Ho *et al*), since then several studies (Harada *et al*, 2004; Valéria *et al*, 2006; Jiang *et al*,2011; Zhu *et al*, 2016; Fugimoto *et al*, 2017) were made and data bases have now a greater number of *mlr* sequences. Therefore, to overcome the problems observed, a new set of degenerated primes based in sequences from different bacteria could be a good alternative to find more specific primers.

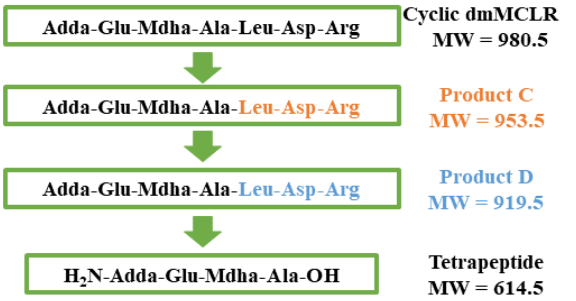


Figure 4.1. biodegradation pathway suggested by Dziga *et al* (2017). The products C and D are cyclic molecules, whereas the tetrapeptide is linear molecule. Colours indicate the regions of biotransformation. Adapted from Dziga *et al*, 2017.

We suggest a new set of degenerated *mlrA* primers obtained using 22 *mlrA* sequences from NCBI data base and the one from the isolate M17C, all from ten different genera, the primers are: forward primer 5'-TGCGCTATGGGKCAGATCCVST-3' and reverse primer 5'-CGCGACYTGCCVRCRMTGT-3'with a GC % of 45 and 52, respectively, an approximate melting temperature of 66.5 ˚C, and generating a fragment with 204 bp. Their self-dimer and cross-dimer analysis was checked (https://www.eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/).

Recent studies on *mlr* cluster, have been focusing on *mlrA* gene, as this gene has a key role in breaking the MCs aromatic ring (as mentioned before). This pair of primers with low redundancy would a possible key to rapidly identify MCs degrading bacteria which use this pathway.

In 2017, Dziga *et al*, proposed a new biodegradation pathway to degrade MCs. Different from the already known Mlr pathway, the linearization of MCs molecules didn't occur in the Arg-Adda bound as shown in figure 4.1. However, as crude samples (lake water) containing naturally occurring microorganisms were used (after MC absence confirmation), some questions are raised. Thus, as it is not known yet which enzymes or chemical components could be in these waters, there's no way to know if exterior factors were influencing MC-degradation. And, since a

consortium of bacteria was analyzed, how many species where involved? Was this pathway specific to a species? Or a small set of species?

Although these and other questions are still unanswered, this is the first described alternative pathway to MC degradation. With the increase and more intense blooms caused by climate change, understanding how MC biodegradation works has become more important, granting this study major importance.

In some studies, the attention has turned to probiotic bacteria as an alternative to naturally occurring bacteria from freshwater, as it has been shown that some probiotic bacteria like *Lactobacillus rhamnosus* (Nybom *et al*, 2008), could degrade MCs. The advantage of such bacteria would be, the fact that these microorganisms have been considered safe by the European Food Safety Authority (EFSA), making them a worthy prospect (EFSA, 2010).

Also, some of them have been shown to have a xenobiotic metabolism (being able to remove environmental contaminants, such as heavy metal and mycotoxins) (Halltunem *et al*, 1998). Bacteria with xenobiotic metabolism may reveal to be an important alternative, as studies suggest that these bacteria may play an important role in MC-degradation (Berg *et al*, 2009; Mou *et al*, 2013; Lezcano *et al*, 2017).

4.3. Bacterial growth

Performing the characterization of several isolates from *Aeromonas* spp. and *Flavobacterium* spp. was a major objective, as previous studies (Miguéns 2013, Pinto 2016) indicate that their growth wasn't particularly affected by MCs.

In this study, all *Flavobacterium* spp. and *Aeromonas* spp. were able to grow in all the stress conditions tested. On the other hand, other bacteria also tested in this study were also able to grow quite well in the presence of MCs, some even outgrowth control conditions (table 3.2).

This takes us back to the initial question, when it comes to bacteria and microcystins. Why most bacteria aren't significantly affected by microcystins (at least at concentrations naturally occurring in freshwaters, which were the ones tested in the studies carried out at DSA-ASBE)?

Previous studies demonstrate that the *mlr* cluster may not be the only way to counteract the effects MCs caused in living cells (Nybom *et al*, 2008; Manage *et al*, 2009; Mou *et al*, 2013;Wang *et al, 2017*). This study supports these previous findings.

The two pink isolates chosen, belong to the same genera being both *Flectobacillus* spp., one *mlr*+, M17C and one *mlr*-, M17D. Although they didn't grow easily in nutrient broth medium, their isolation and characterization allowed further comparison of these two bacteria from same genera, one with and one without *mlr* genes. Comparing both growth results, it demonstrated that something else must be surpassing the bacterial response to MCs induced stress, since M17C and M17D have a similar growth response.

The use of pure MCLR revealed interesting results. When observing the growth graphic of bacteria in the presence of MCLR extract and pure MCLR, it was observed that the response to pure MCLR in contrast to MCLR extract presented a decrease in growth. These results lead to the conclusion that something else present in the MCs extract may be counter-balancing the microcystin effect as the MCs extract (used many times in MCs studies due to the elevated price of pure MCs) contains not only microcystin but also other bioactive compounds that may be produced by cyanobacteria.

Comparing the growth graphics both from *Flavobacterium* spp. and *Aeromonas* spp. it's possible to see that in most cases *Flavobacterium* spp. appears to be less affected by MCs in the medium then *Aeromonas* spp., as their growth is more similar to control condition, thus suggesting a better adaptation to MCs presence.

Another observation made in this study was that *Flavobacterium* spp. and *Aeromonas* spp. isolated from Albufeira de Magos in 2012 appear to be more susceptible to MC when compared to *Flavobacterium* spp. and *Aeromonas* spp. isolates from 2017 (example in figure 3.13). Thus, comparing results from 2012 and 2017, seems to suggest that these bacteria are suffering an adaptation making them less susceptible to MCs, maybe due to the fact that toxic cyanobacteria blooms are frequent in this reservoir. To confirm this hypothesis, further studies on this matter would be necessary, *e.g*: (1) determine that the isolates tested are indeed the same strain; (2) further analyze isolates from 2012 (Miguéns, 2012), isolated in the same reservoir and compare with the ones from 2017; (3) isolating more bacteria from this reservoir through the years to screen possible adaptations; (4) compare bacteria in other reservoirs and lakes to understand if the same is occurring, for instance, in Roxo, Monte da Barca or Patudos, bacteria from this places were also isolated in 2012 (Miguéns, 2013) and blooms of cyanobacteria are frequent, making them perfect to further test the hypothesis.

Some bacteria from other species namely, *Rahnella* sp. isolate M17H and *Micrococcus* sp. isolate R17I (table 3.3) also seem interesting to carry on further studies, as their growth rates are not affected by MCs or even increase (also in medium with addition of pure MCLR). It would be interesting to understand if such growth adaptation is related with the isolates, as a strain characteristic or is more related to the species itself. However, to carry on these further studies it would be necessary to perform the assays with more isolates from same species or genera.

The summary of data from all isolates growth trial presented in table 3.3 seem to indicate a different interaction between bacteria and the different variants of MC. One of the reasons may related to the fact that the MCs variants selected have different hydrophobicities. MCRR is hydrophilic while MCLR and MCYR are more hydrophobic. This difference in hydrophobicity is relevant to growth response since more hydrophobic MC have been suggested to be more cell permeable than the more hydrophilic ones. Thus, compared with MCRR, MCLR and MCYR might more easily penetrate the membrane, being more available for uptake and transportation and consequentially be more toxic to these bacteria.

The results gathered in this study, raise questions, and to shade some light in some of them, it would be interesting to follow one of two possible pathways. One, to identify if degradation of the MC molecules is occurring when these isolates are grown in contact with it (for instance using HPLC or other analytical techniques), allowing us to understand if these isolates are part of MCs degradation on their habitat. Alternatively, with the possibility of complete genome sequencing, it would be interesting to directly search genes related to xenobiotic metabolism, as such genes have been already linked with MC-degrading activity.

4.4. Antioxidant system activity

Search for CAT activity showed that only three bacteria, M17F, M17K and 594196, possessed this enzyme. As only three bacteria were CAT positive and showed different results between each other no stress induced pattern could be identified.

Analyzing the results obtained in this study in comparison with those obtained previously by Pinto (2016) and Miguéns (2013), same conclusions can be drawn: (1) Most of the studied bacteria have no CAT activity in these three studies, a total of 19 isolates were tested so far, but CAT activity was detected in only six of them. (2) Most isolates presented a reduction in activity when they were exposed to MCLR extract (except for A3) and most isolates had an increase in CAT activity when exposed to MCRR extract, fact possibly related with MC hydrophobicity as referred above (3) *Sphingomonas* sp., isolate 594196, also appears to have a completely different response to MCs variants exposure, compared to the other isolates.

To prove these observations a classic catalase test should be performed and if there were still uncertainties more tests with these and other isolates (from the same genus) should be made to clarify this question.

The results in table 3.5 show that only three of nine studied bacteria have SOD activity, namely, M17C, M17D and 594196. If comparing the MC effect in growth and the MCs effect in SOD activity it becomes clear that 594196, a bacteria that showed a decrease in growth in the presence of MCs, also had its SOD_t activity reduced to half or less when exposed to MCs. On the other hand, M17C and M17D, growth wasn't severely affected by MCs, as only had a reduced activity with MCLR extract (M17D) or MCRR extract (M17C) and never reduced more than 25%. Their different response to each MC variant may be related with strain characteristics as the SOD_t activity of each bacteria had a different response to each variant.

We also tested pure MCLR, to understand if there could be other components in the MC purified extracts that could be influencing this trial, however they were inconclusive as only three bacteria with SOD activity have been tested with exposure to pure MC, and these three bacteria have very different responses to both MCLR extract and pure MCLR. In *Sphingomonas* sp. isolate 594196, SOD_t activity doesn't seem to be affected by bioactive components from MC extract as the SOD_t activity was similar both when the isolate was exposed to MC extract and pure MC. The activity in each *Flectobacillus* spp. isolate was very different. Exposure to pure MCLR lead to a decrease in SOD_t activity in M17C and an increase in SOD_t activity in M17D, compared to MCLR extract. However, as referred before, since these two bacteria are two different strains, it can't be assessed that this difference is related bioactive components present in the MCs extract which may interact differently with each strain, or if this difference is more related to the strains characteristics and specificities that may interfere with the antioxidant system.

Comparing results from this study with those from Miguéns (2013) and Pinto (2016) shows that there's no pattern in SOD_t activity in isolates exposed to different MCs. In some cases, such as *Vogesella* sp. isolate C4, there is an increase in SOD_t activity for all stress conditions, in others, no alterations in activity are detected as in *Shewanella* sp., isolate P6 or *Raoultella* sp. P1. In other cases, the same bacteria showed different SOD_t activity when exposed to different MC, like *Flectobacillus* sp. M17D or *Bacillus* sp. B1. We may thus conclude that this difference in activity isn't a species characteristic, since both *Flectobacillus* spp. isolates have completely different SOD_t activity response to different MC exposure, and particularly represented by *Flavobacterium* spp. isolates, as some isolates had SOD enzymes (SOD_t activity detected) and some didn't. Thus, these results support the idea that bacterial response to MCs is a strain characteristic. As the antioxidative enzyme system is a major component of the cell, these results lead to the conclusion that other mechanisms may be in action in degrading $O_2^{\bullet -}$.

The results in black (table 3.6) don't allow many conclusions. Comparison of the three isolates tested was only possible in stress condition with MCLR extract (as it is the only condition were results are between zero and one). In this specific case both M17D and 594196 isolate have a predominance of SOD_2 activity compared to SOD_1 the same doesn't go for M17C, which has a predominance of SOD_1 activity.

Comparing this and previous studies (Miguéns, 2012 and Pinto, 2016), it's observed that: (1) in control conditions, SOD_t activity either matched SOD_1 (two isolates) or SOD_2 (three isolates), (2) when isolates were exposed to MCLR or MCRR or MCYR extract, in most cases SOD_2 represented the highest percentage of SOD_t.

To understand what may be happening with the results of SOD_2 (values in blue from table 3.6) one must have in mind that the samples were composed of total protein extracts from the tested isolates. This means that other enzymes were present in the extract, and some of these

enzymes may influence the results, as CAT or peroxidases or other enzymes involved in the antioxidant system may also be present.

Other important point is demonstrated by figure 3.14 where the procedure to measure SOD activity is illustrated. The SOD activity measurement is based in the conversion of $O_2^{\bullet-}$ and NBT into NBT-diformazan. This means that the negative control defines the maximum activity measured, when all $O_2^{\bullet-}$ is converted into NBT-diformazan. Then, during the assay, SOD will reduce $O_2^{\bullet-}$ in H_2O_2 and O_2 and less NBT-diformazan will be produced causing a decrease in absorbance compared with the negative control (formula to determine de inhibition of NBT-diformazan - Equation 4.1.).

$$\frac{(\Delta A_{550}\ /\ min)_{Negative\ control} - (\Delta A_{550}\ /\ min)_{test}}{(\Delta A_{550}\ /\ min)_{Negative\ control}}\ X\ 100 = \%\ inhibition\ of\ NBT\text{-}diformazan$$

Equation 4.1. Determination of the % inhibition for the test samples.

The difference in procedure between SOD_t trial and SOD_2 trial is the previous addiction of CN^- to inactivate SOD_1.

Now, having all these aspects in mind, three hypotheses have been identified to justify the increase in SOD_2 above the SOD_t: (1) The existence of another protein in the extract that can convert $O_2^{\bullet-}$ in H_2O_2 and somehow CN^- addiction promotes its activation. (2) The catalase present in the extract (for instance isolate 594196 is CAT positive) degrades H_2O_2 leaving SOD_2 free to convert more $O_2^{\bullet-}$. Or, (3) a repressor is inactivated by CN^-, thus activating an enzyme that promulgates the pathway $O_2^{\bullet-} \rightarrow H_2O_2$.

To understand if the addition of CN^- alone could affect the results (with alterations in the absorbance), a trial identical to the negative control was performed, in which CN^- was added as a sample and tested. This test showed that the absorbance of the negative control and CN^- sample was different. That said and although the difference between this two isn't enough to justify the difference between SOD_2 and SOD_t observed in all the assays, this trial shows that the addiction of CN^- itself also affects the activity measurements, leading to erroneous SOD_2 activity measurements. To overcome such problem a negative control where CN is also added should be used when testing for SOD_2 and SOD_1.

The results in red represent (table 3.6) results in which, SOD_2 absorbance was higher than negative control absorbance, this means that more NBT-diformazan was being produced, for that to happen we suggest three hypotheses (1) as these bacteria were exposed to stress induced factors the protein extract may contain additional $O^{\bullet-}$, leading to an increase in production of NBT-diformazan (2) xanthine oxidase or other enzyme capable of xanthine conversion to $O^{\bullet-}$ was present in the protein extract. However, both hypotheses are unlikely as $O^{\bullet-}$ would start NBT-diformazan formation really early in the trial, before zero base was measured, and absorbance measurements wouldn't stabilize. (3) A xanthine-like metabolite was present in these bacteria and may also convert $O^{\bullet-}$, leading to an increase of NBT-diformazan formation.

As many of SOD positive bacteria ended up being CAT negative, other pathways should be involved in degrading H_2O_2, such as peroxidases. Understanding the way that CN^- may affect these enzymes would be important in order to improve this kit performance, as it seems unreliable for a confidant determination of SOD_1 and SOD_2 detection in bacteria.

5. Conclusion

Several conclusions are retrieved from this study. The growth trials support the hypothesis from previous studies (Miguéns, 2013; Pinto, 2016) that most heterotrophic bacteria are little to not affected by MCs exposure. Moreover, it is strongly suggested that their behavior (growth assays and antioxidant enzymes response) when exposed to these toxins is a strain characteristic. Also, both bacteria from *Aeromonas* spp. and *Flavobacterium* spp. seem to be quite resistant to microcystins as suspected, but *Flavobacterium* spp. seem to be the most well adapted.

The different effects observed with the three MCs variants tested and pure MCLR may be related to (1) hydrophobicity of the variants; (2) composition of the MCs purified extract.

Flectobacillus sp. M17C and M17D strains have different genetic and physiological features, but present similar growth responses to MCs, reinforcing the hypothesis that other mechanisms are involved in counteracting MCs effects.

Although further studies are in need to confirm this hypothesis, growth trials with isolates from 2012 (Miguéns, 2013) and from this study (2017) indicate that bacteria from Albufeira de Magos have been adapting to the continuous MCs exposure.

A new *mlr*+ bacteria was identified: *Flectobacillus* sp. M17C.

The study of antioxidative enzyme system response to MCs leads to the several conclusions. In CAT activity trial: (1) most bacteria tested must have another pathway (such as peroxidases) to degrade H_2O_2. (2) Cells exposure to MCLR leads to a reduction in CAT activity. (3) Cells exposure to MCRR mainly leads to an increase in CAT activity. In SOD trials: (1) The results support the idea that SOD activity response to MCs is a strain characteristic. (2) Other mechanisms may be in action in degrading $O_2^{\bullet-}$. (3) In control conditions, SOD_t activity either matched SOD_1 or SOD_2, (4) when isolates were exposed to MCLR or MCRR or MCYR extract, in most cases SOD_2 represented the highest percentage of SOD_t.

In the future, it would be interesting to fully identify the isolates from this study at species level. Also, it would be interesting to test if the bacteria from this study are degrading MCs, as they have grown when exposed to these toxins. On the other hand, with total genomic sequences from bacteria of interest, for instance some *Flavobacterium* spp. or *Aeromonas* spp., the search of xenobiotic related genes would be an interesting approach. If these genes were detected, their involvement in MCs degradation could be tested.

Another interesting approach for the future would be to understand if bacterial adaptation to MC was occurring in Albufeira de Magos, and if confirmed possibly extend the search to other reservoirs. Also, as bacteria from other species, *Rahnella* sp. and *Micrococcus* spp. revealed a good growth rate when exposed to MCs, it would be interesting to understand if bacteria from these species are also particularly well adapted to MC presence.

6. References

1. Berg, K.A., Lyra, C., Sivonen, K., Paulin, L., Suomalainen, S., Tuomi, P., Rapala, J., 2009. High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. ISME journal. 3, 314-325.
2. Blom, J.F., Robinson, J.A., Jüttnera, F., 2001. High grazer toxicity of [D-Asp3,(E)-Dhb7]microcystin-RR of Planktothrix rubescens as compared to different microcystins. Toxicon. 39, 1923-1932.
3. Bourne, D.G., Riddles, P., Jones,G. J. Smith,W., Blakeley, R.L., 2001. Characterisation of a gene cluster involved in bacterial degradation of the cyanobacterial toxin microcystin LR. Environmental Toxicology. 16 (6), 523–534.
4. Cabeen, M.T., Jacobs-Wagner C., 2005. Bacterial cell shape. Nature Reviews Microbiology. 3, 601–610.
5. Carmel-Harel, O., and Storz, G., 2000. Roles of the Glutathione- and Thioredoxin-Dependent Reduction Systems in the *Escherichia Coli* and *Saccharomyces Cerevisiae* Responses to Oxidative Stress. Annual Review of Microbiology. 54, 439-461.
6. Chaves, S. (2005) Diversidade de procariotas sulfato-redutores e desnitrificantes em amostras ambientais. Tese de doutoramento. Universidade de Lisboa, Lisboa, Portugal.
7. Chen, X., Yang, X., Yang, L., Xiao, B., Wu, X., Wang, J., Wan, H., 2010. An effective pathway for the removal of microcystin LF via anoxic biodegradation in lake sediments. Water Res. 44 (6),1884.
8. Christoffersen, K., Lyck, S., Winding, A., 2002. Microbial activity and bacterial community structure during degradation of microcystins. 27, 125-136.
9. Churro, C., Dias, E., Valério, E., 2012. Risk Assessment of Cyanobacteria and Cyanotoxins, the Particularities and Challenges of *Planktothrix* spp. Monitoring, in Luo, Y., Novel Approaches and Their Applications in Risk Assessment. IntechOpen, DOI: 10.5772/37910.
10. Ding, W., Ong, X.N., 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. FEMS Microbiology Letters. 220, 1-7.
11. Duy, T.N., Lam, P.K., Shaw, G.R., Connell, D.W., 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. Rev. Environ. 163, 113-85.
12. Dziga, D., Maksylewicz, A. Maroszek, M., Budzyńska, A., Napiorkowska-Krzebietke, A., Toporowska, M., Grabowska, M., Kozak, A., Rosińska, J., Meriluoto, J., 2017. The biodegradation of microcystins in temperate freshwater bodies with previous cyanobacterial history. Ecotoxicology and Environmental Safety. 145, 420-430.
13. EFSA, European Food Safety Authority. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2010 update, published 2014 December), 2010.
14. Fujimoto, N., Ohno, N., Tanaka, K., Narahara, I., Ohnishi, A., Suzuki, M., Iwami, N., Mizuochi, M., Inamori, Y., 2007. Degradation of the Cyanobacterial Hepatotoxin Microcystin by Bacteria Isolated from a Monoxenic Culture of the Flagellate Monas guttula. Japanese Journal of Water Treatment Biology. 43, 99-111. DOI 10.2521/jswtb.43.99.
15. Giaramida, L., Manage, P.M., Edwards, C., Singh, B.K., Lawton, L.A., 2013. Bacterial communities' response to microcystins exposure and nutrient availability: Linking degradation capacity to community structure. International Biodeterioration & Biodegradation. 84, 111-117.

16. Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.
17. Ho, L., Hoefel, D., Saint, C.P., Newcombe, G., 2007. Isolation and identification of a novel microcystin-degrading bacterium from a biological sand filter. Water Research. 41, 4685-4695.
18. Ho, L., Tang, T., Monis, P.T., Hoefel, D., 2012. Biodegradation of multiple cyanobacterial metabolites in drinking water supplies. Chemosphere. 87 (10), 1149-154.
19. Halttunen, T., Salminen, S., Tahvonen, R., 2007. Rapid removal of lead and cadmium from water by specific lactic acid bacteria. International Journal of Food Microbiology. 114, 30-35.
20. Harada, K., Imanishi, S., Kato, H., Mizuno, M., Ito, E., Tsuji, K., 2004. Isolation of Adda from microcystin-LR by microbial degradation. Toxicon. 44 (1), 107-109.
21. Imanishi, S., Kato, H., Mizuno, M., Tsuji, K., Harada, K., 2005. Bacterial Degradation of Microcystins and Nodularin. Chem. Res. Toxicol. 18, 591-598.
22. Jiang, Y., Shao, J., Wu, X., Xu, Y., Li, R., 2011. Active and silent members in the *mlr* gene cluster of a microcystins degrading bacterium isolated from Lake Taihu, China. FEMS Microbiol Lett. 322, 108–114.
23. Kim, H.P., Lee, J.S., Hah, Y.C., Roe, J.H., 1994. Characterization of the major catalase from *Streptomyces coelicor* ATCC 10147. Microbiology. 140, 3391-3397.
24. Kormas, K.A., Lymperopoulou, D.S., 2013. Cyanobacterial toxin degrading bacteria: Who are they?. BioMed Research International. Volume 2013, Article ID 463894, 12 pages.
25. Lezcano, M.A., Morón-López, J., Agha, R., López-Heras, I., Nozal, L., Quesada, A., El-Shehawy, R., 2016. Presence or absence of *mlr* genes and nutrient concentrations co-determine the microcystin biodegradation efficiency of a natural bacterial community. Toxins. 8, 318.
26. Lezcano, M.Á., Velázquez, D., Quesada, A., El-Shehawy, R., 2017. Diversity and temporal shifts of the bacterial community associated with a toxic cyanobacterial bloom: An interplay between microcystins producers and degraders. Water Research. 125, 52-61.
27. Li, H., Xie, P., Zhang, D., Chen, J., 2009. The first study on the effects of microcystin-RR on gene expression profiles of antioxidant enzymes and heat shock protein-70 in *Synechocystis* sp. PCC6803. Toxicon. 53, 595–601.
28. Li, J., Li, R., Li, J., 2017. Current research scenario for microcystins biodegradation – A review on fundamental knowledge, application prospects and challenges. Science of the Total Environment. 595, 615–632.
29. MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., Codd, G.A., 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett. 264, 187-192.
30. Manage P.M., Edwards C., Singh B.K., Lawton L.A., 2009. Isolation and Identification of Novel Microcystin-Degrading Bacteria. Appl Environ Microb. 75, 6924–6928.
31. Miguéns, Diana, 2013. Impact of microcystins on the growth and antioxidant system of aquatic bacteria. Faculty of Science of the University of Lisbon (FCUL). 58 pages. Master's degree in Applied Microbiology. FCUL 2013.
32. Miguéns, D., Valério, E., 2015. The impact of some microcystins on the growth of heterotrophic bacteria from Portuguese freshwater reservoirs. Limnetica. 34 (1), 215-226.
33. Morón-López, J., Nieto-Reyes, L., El-Shehawy, R., 2017. Assessment of the influence of key abiotic factors on the alternative microcystin degradation pathway(s) (*mlr*−): A detailed comparison with the *mlr* route (*mlr*+). Science of the Total Environment. 599–600, 1945–1953.

34. Mou, X., Lu, X., Jacob, J., Sun, S., Heath, R., 2013. Metagenomic identification of bacterioplankton taxa and pathways involved in microcystin degradation in Lake Erie. PLoS One. 8, Issue 4, e61890.
35. Neumann, A.C., Wang, X., Niessner, R., and Knopp, D., 2016. Determination of microcystin-LR in surface water by a magnetic bead-based colorimetric immunoassay using antibody-conjugated gold nanoparticles. Anal. Methods. 8, 57-63.
36. Nybom S.M.K., Salminen S.J., Meriluoto J.A.O., 2012. Specific strains of probiotic bacteria are efficient in removal of several different cyanobacterial toxins from solution. Toxicon. 52, 214–220.
37. Pearson, L., Mihali, T., Moffitt, M., Kellmann, R., Neilan, B., 2012. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Mar. Drugs. 8(5), 1650–1680.
38. Pflugmacher, S., 2004. Promotion of oxidative stress in the aquatic macrophyte *Ceratophyllum demersum* during biotransformation of the cyanobacterial toxin microcystin-LR. Aquat Toxicol.70(3), 169-78.
39. Pinto, André, 2016. Explorando a razão de bactérias do meio aquático não serem afetadas por microcistinas: Sistema antioxidante VS. degradação. Faculty of Science of the University of Lisbon (FCUL). 38 pages. Master's degree in Human and Environmental Biology. FCUL 2016.
40. Pratheepa, V.K., Silva, M., Vasconcelos, V., 2014. Viscera-associated bacterial diversity among intertidal gastropods from northern-Atlantic coast of Portugal. Current Microbiology. 68 (2), 140-148.
41. Radha, P., 2010. Antioxidant responses evoked in vitro and in vivo by Bacopa monnieri leaf extracts. Ph.D. Thesis. Avinashilingam Deemed University for Women, Coimbatore, India.
42. Rastogi, R.P., Sinha, R.P., Incharoensakdi, A., 2014. The cyanotoxin-microcystins: Current overview. Rev. Environ. Sci. Bio/Technol. 13, 215–249.
43. Saito, T., Okano, K., Park, H.D., Itayama, T., Inamori, Y., Neilan, B.A, Burns, B.P., Sugiura, N., 2003. Detection and sequencing of the microcystin LR-degrading gene, *mlrA*, from new bacteria isolated from Japanese lakes. FEMS Microbiology Letters. 229, 271-276
44. Sivonen, K., Jones, G. 1999. Cyanobacterial toxins. In: I. Chorus, J.Bartram (Eds.) Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management, E & FN Spon, London, pp.41-111.
45. Shimizu, K., Maseda, H., Okano, K., Itayama, T., Kawauchi, Y., Chen, R., Utsumi, M., Zhang, Z., Sugiura, N., 2011. How microcystin-degrading bacteria express microcystin degradation activity. Lakes & Reservoirs: Research and Management.16, 169–178.
46. Smith, M., and Doolittle, R., (1992). A comparison of Evolutionary rates of two major kinds of Superoxide Dismutase. J Mol Evol. 34 (2), 175-184.
47. Tamura, K., Stecher, G., and Kumar, S. (1993). MEGA6: Molecular Evolutionary Genetics Analysis Version 7.0 [Computer software] Retrieved from https://www.megasoftware.net/
48. Turkseven, S., Kruger, A., Mingone, C.J., Kaminski, P., Inaba, M., Rodella, L.F., Ikehara, S., Wolin, M.S., Abraham N.G., 2005. Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes. Am J Physiol Heart Circ Physiol. 289, H701–H707.
49. Valeria, A.M., Ricardo, E.J., Stephan, P., Alberto W.D., 2006. Degradation of Microcystin-RR by *Sphingomonas* sp. CBA4 isolated from San Roque reservoir (Córdoba - Argentina). Biodegradation. 17 (5), 447-455.

50. Valério, E., Chambel, L., Paulino, S., Faria, N., Pereira. P., Tenreiro, R., 2010. Multiplex PCR for Detection of Microcystins-Producing Cyanobacteria from Freshwater Samples. Environmental Toxicology. 25 (3), 251-260. DOI 1y0.1002/tox.201502.
51. Vassilakaki, M., Pflugmacher, S., 2008. Oxidative stress response of *Synechocystis* sp. (PCC 6803) due to exposure to microcystin-LR and cell-free cyanobacterial crude extract containing microcystin-LR. Journal of applied Phycology. 20 (3), 219-225.
52. Walls, J., Wyatt, K.H., Doll, J.C., Rubenstein, E.M., Rober, A.R., 2018. Hot and toxic: Temperature regulates microcystin release from cyanobacteria. Science of the Total Environment. 610–611, 786–795.
53. Wang, Q., Xie, P., Chen, J., Liang, G., 2008. Distribution of microcystins in various organs (heart, liver, intestine. Gonad, brain, kidney and lung) of wistar rat via intravenous injection. Toxicon. 52, 721-727.
54. Wang, H., Zhang, H., Yan, H., Zhang, X., Liu, X., Yin, C., Xu, Q., Lv, L., 2017. Biodegradation of microcystins: A review. International Journal of Molecular Sciences. 18, 10.
55. Wiegand, C., Pflugmacher, S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicology and Applied Pharmacology. 203 (3), 201-218.
56. Yang, C.Y., Wang, W.B., Li, D.H., Liu, Y.D., 2008. Growth and Antioxidant System of *Escherichia coli* in response to Microcystin-RR. B Environ Contam and Tox. 81, 427-431.
57. Yang, F., Zhou, Y., Yin, L., Zhu, G., Liang, G., Pu, Y., 2013. Microcystin-Degrading Activity of an Indigenous Bacterial Strain *Stenotrophomonas acidaminiphila* MCLTH2 Isolated from Lake Taihu. Plos One. 9 (1), e86216. DOI 10.1371/journal.pone.0086216.
58. Zanchett, G., Oliveira-Filho, E.C., 2013. Cyanobacteria and Cyanotoxins: From Impacts on Aquatic Ecosystems and Human Health to Anticarcinogenic Effects. Toxins. 5, 1896-1917.
59. Zhou, L., Yu, H., Chen, K., 2002. Relationship between microcystin in drinking water and colorectal cancer. Biomed Environ Sci. 15, 166-171.
60. Zhua, X., Shen, Y., Chen, X., Hub, Y.O.O., Xiang, H., Tao, J., Ling, Y., 2016. Biodegradation mechanism of microcystin-LR by a novel isolate of *Rhizobium* sp. TH and the evolutionary origin of the *mlrA* gene. International Biodeterioration & Biodegradation. 115, 17-25.

7. Annexes

7.1. Growth trial graphics from other isolates

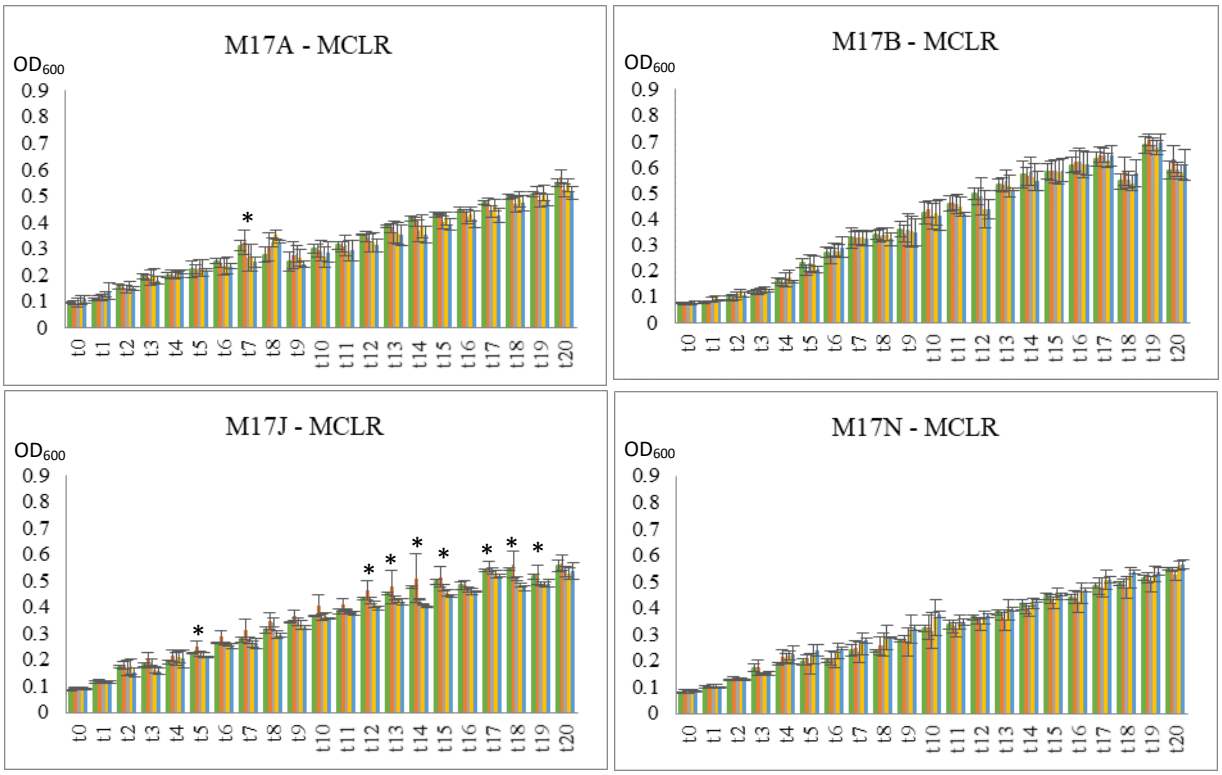


Figure 7.1. Graphs of growth curves from *Aeromonas* spp. isolates with MCLR over time (t_0-t_{20}). The values are the mean ± standard deviation from three replicates. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

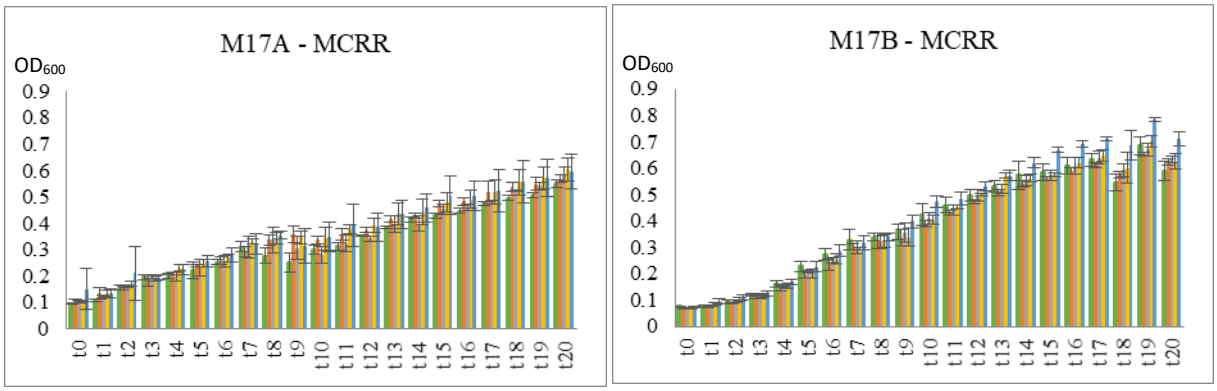


Figure 7.2. Graphs of growth curves from *Aeromonas* spp. isolates with MCRR over time (t_0-t_{20}). The values are the mean ± standard deviation from three replicates. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

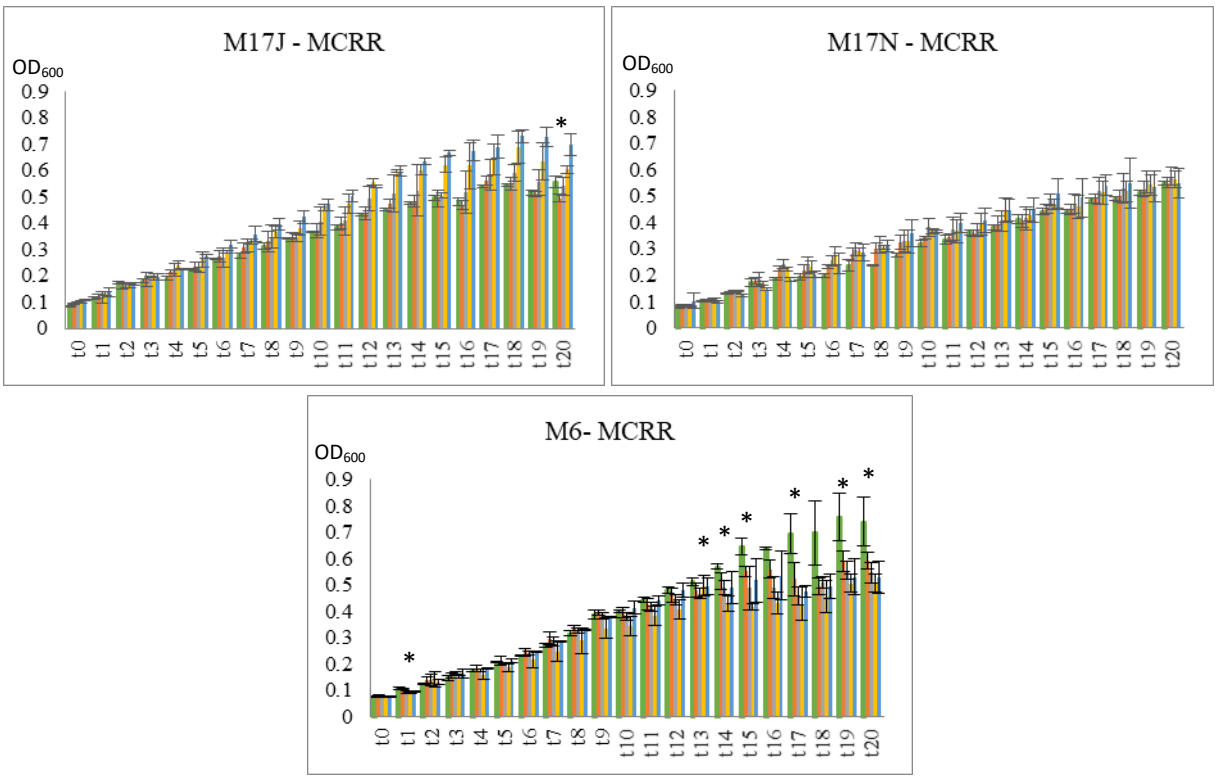


Figure 7.2. (continuation) Graphs of growth curves from *Aeromonas* spp. isolates with MCRR over time (t_0-t_{20}). The values are the mean ± standard deviation from three replicates. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

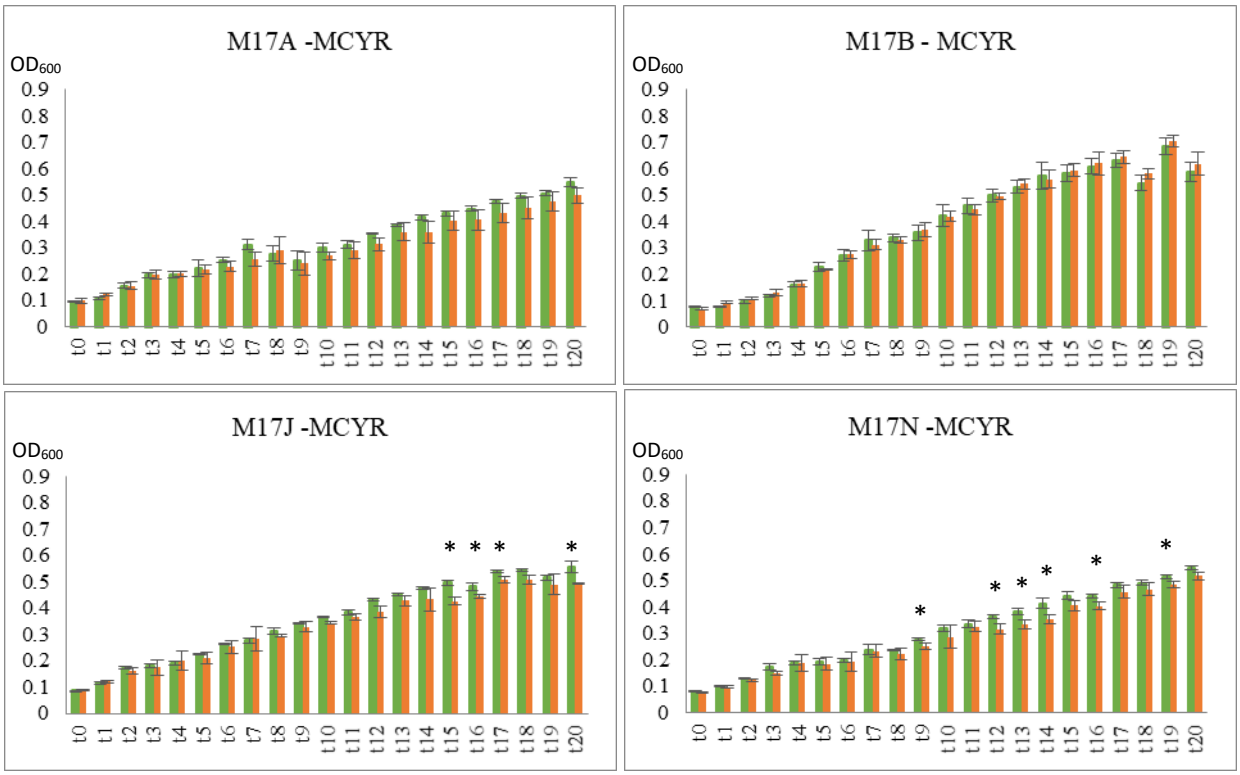


Figure 7.3. Graphs of growth curves from *Aeromonas* spp. isolates with MCYR over time (t_0-t_{20}). The values are the mean ± standard deviation from three replicates. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin.

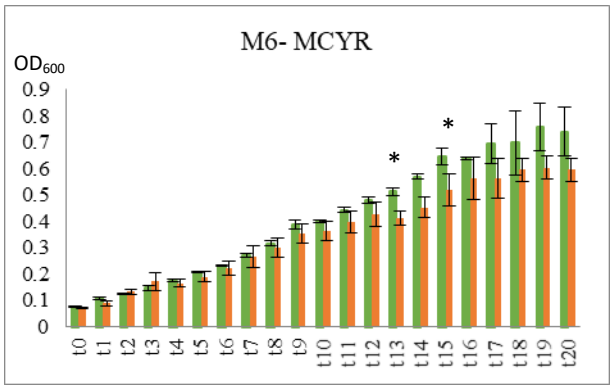


Figure 7.3. (continuation) Graphs of growth curves from *Aeromonas* spp. isolates with MCYR over time (t_0-t_{20}). The values are the mean ± standard deviation from three replicates. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin.

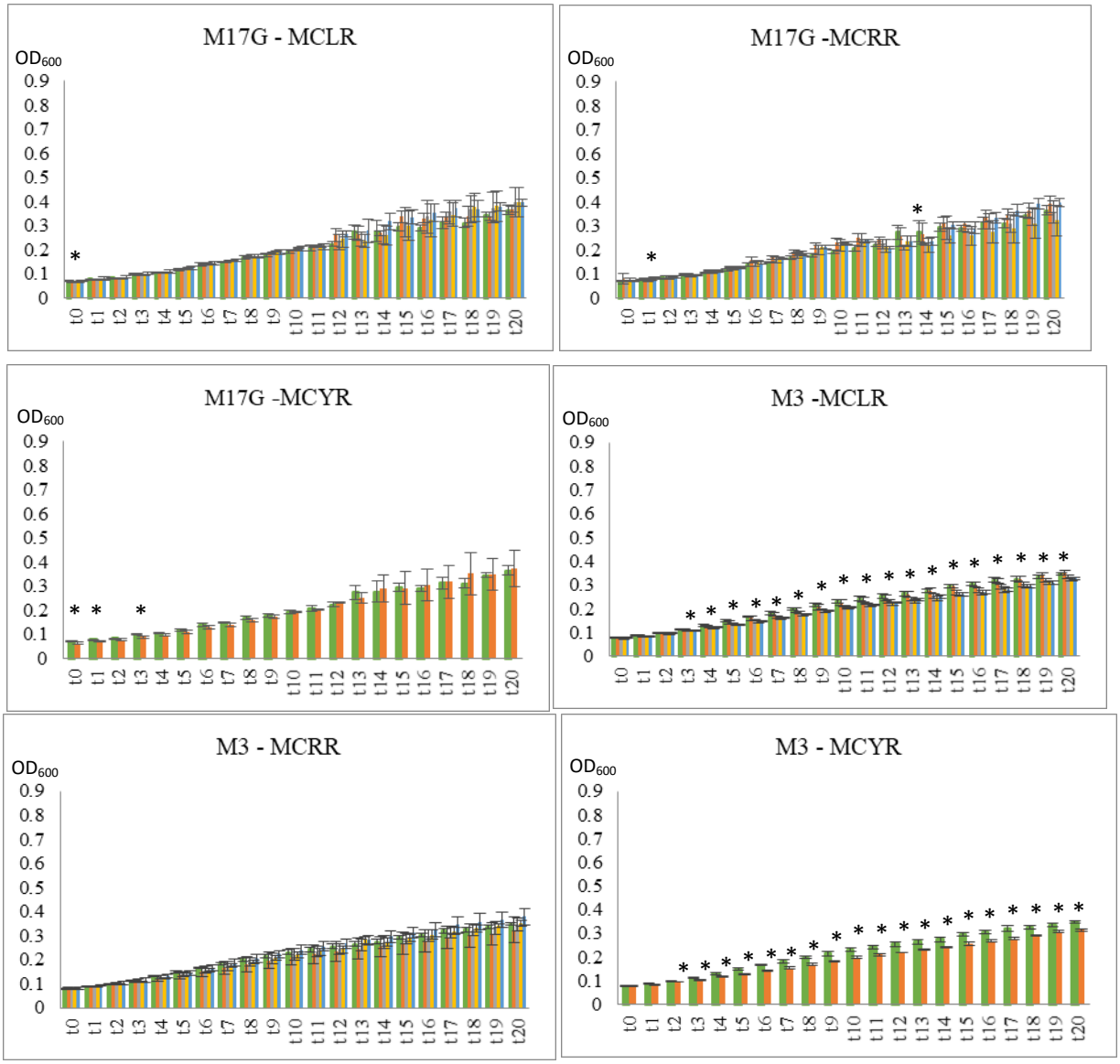


Figure 7.4. Graphs of growth curves from *Flavobacterium* sp. isolate with the different MCs variants over time (t_0-t_{20}). The values are the mean ± standard deviation from three replicates. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

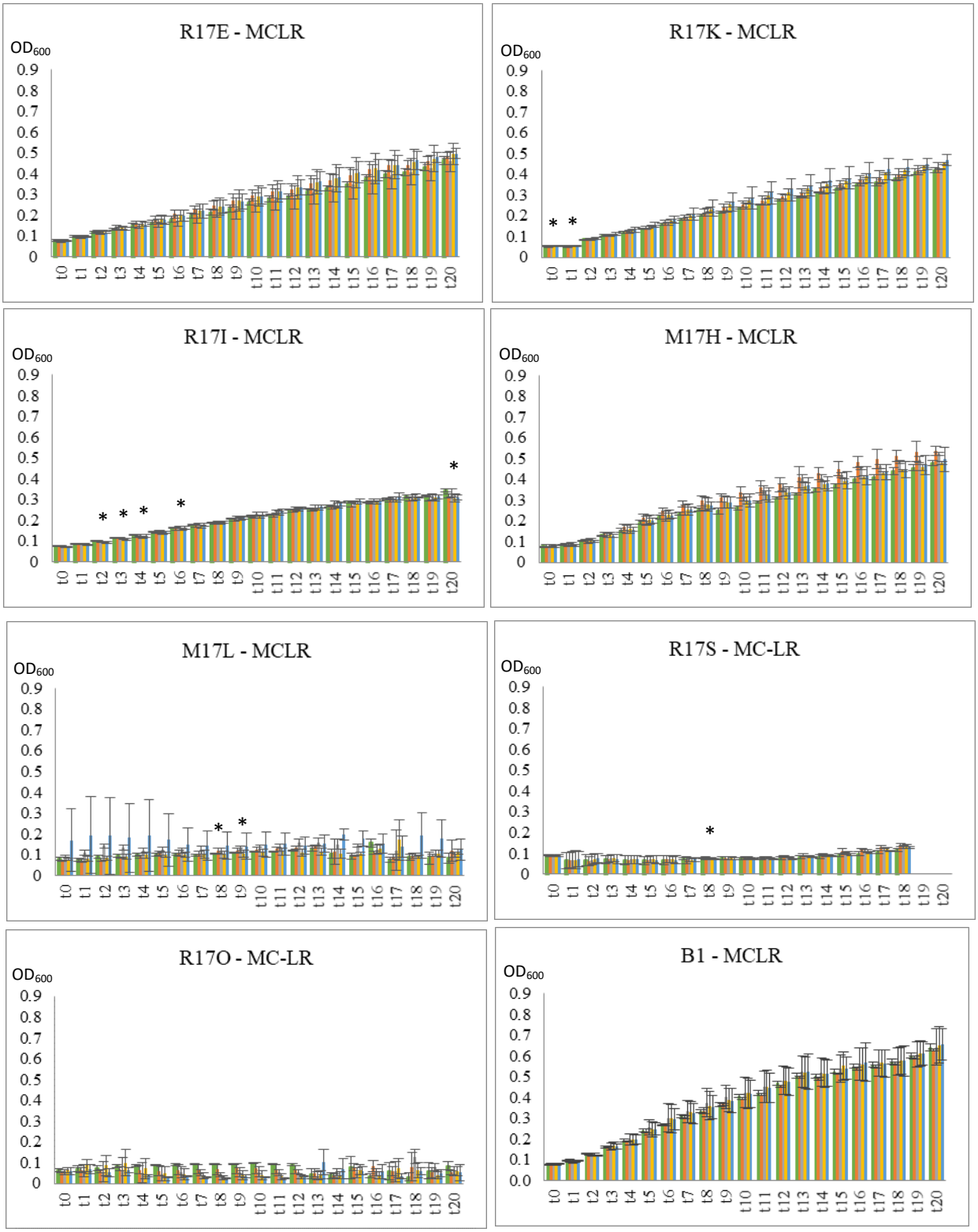


Figure 7.5. Graphs of growth curves from all other bacterial isolates with MCLR over time (t_0-t_{20}). The values are the mean ± standard deviation from three replicates. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystin, (●) 10 nM microcystin, (●) 100 nM microcystin, (●) 1 µM microcystin.

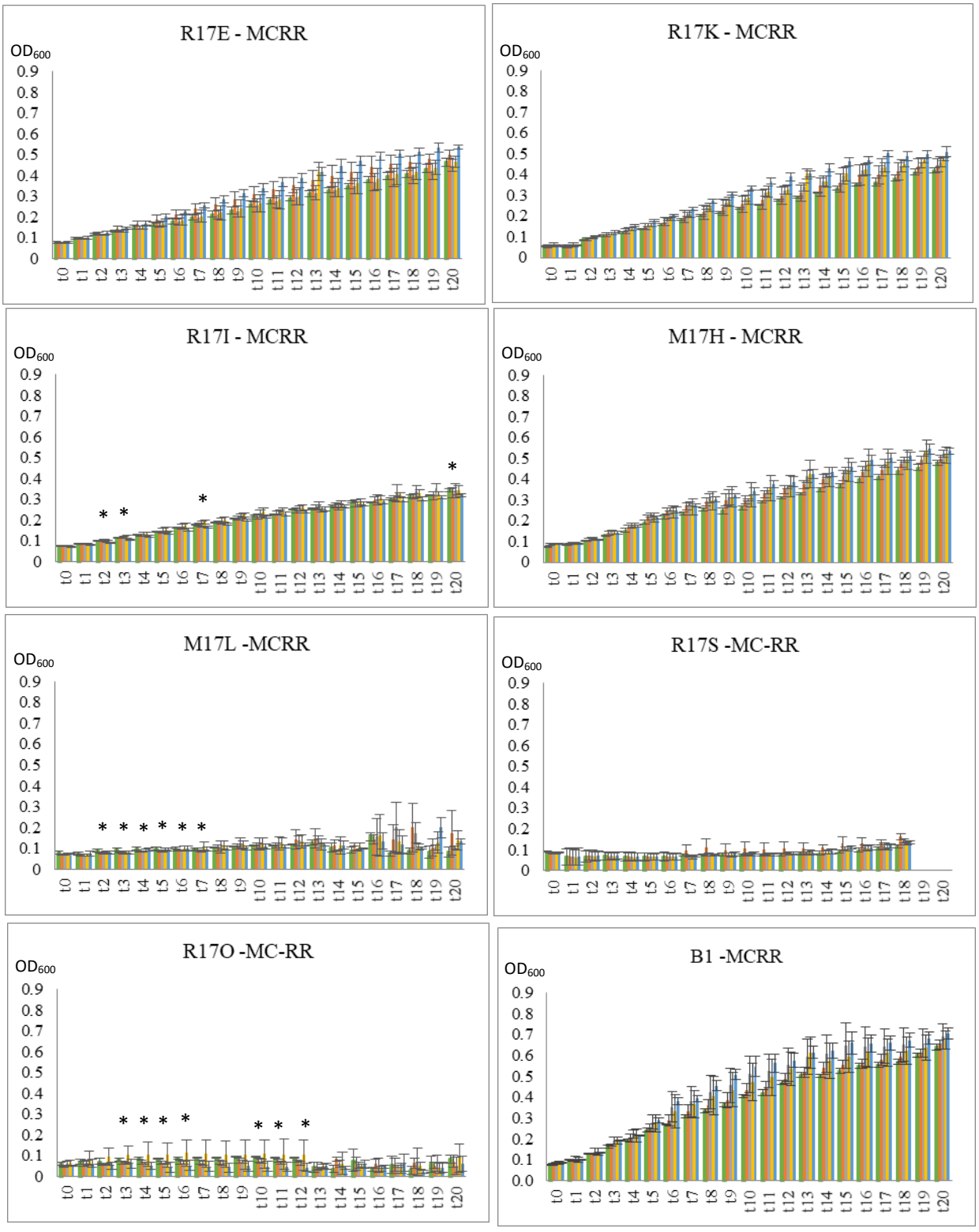


Figure 7.6. Graphs of growth curves from all other bacterial isolates with MCRR over time (t_0-t_{20}). The values are the mean ± standard deviation from three biological samples. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystins exposure cells, (●) 10 nM microcystins exposure cells, (●) 100 nM microcystins exposure cells, (●) 1 µM microcystins exposure cells.

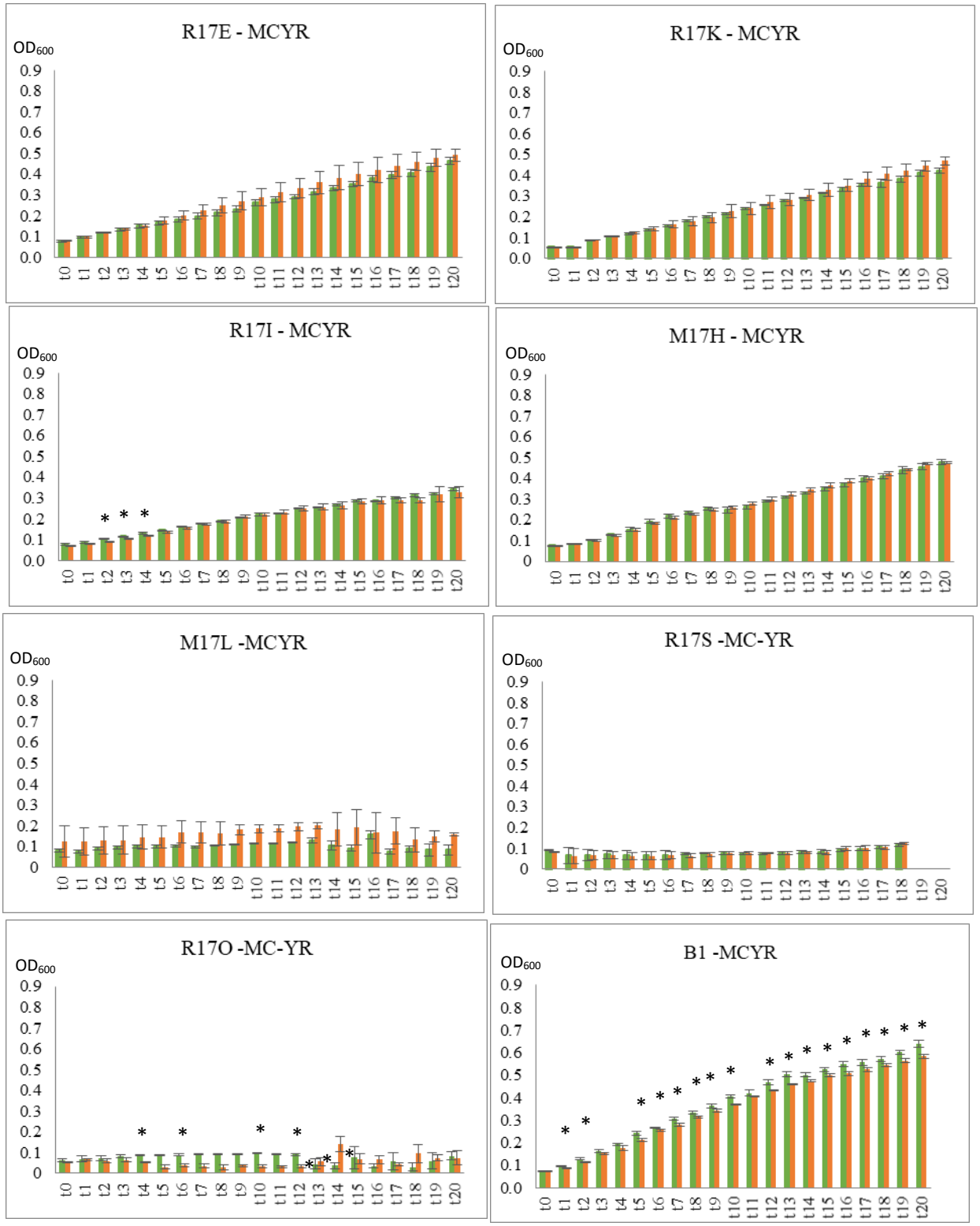


Figure 7.7. Graphs of growth curves from all other bacterial isolates with MCYR over time (t_0-t_{20}). The values are the mean ± standard deviation from three biological samples. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM microcystins exposure cells.

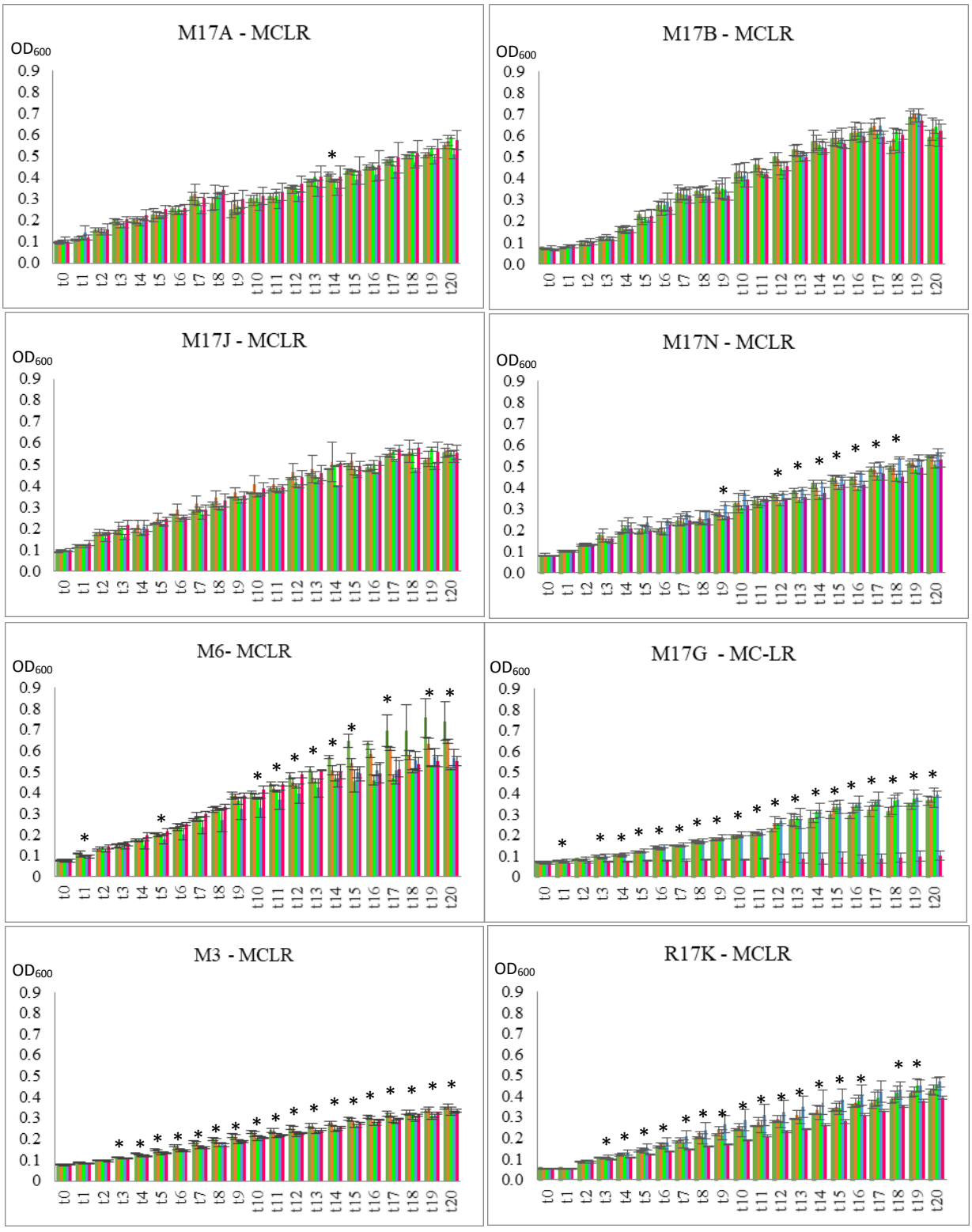


Figure 7.8. Graphs of growth curves from all remaining isolates with both MCLR extract and pure MCLR over time (t_0-t_{20}). The values are the mean ± standard deviation of three replicates. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM of MCLR extract, (●) 1 nM of pure MCLR, (●) 1 µM of MCLR extract, (●) 1 µM of pure MCLR.

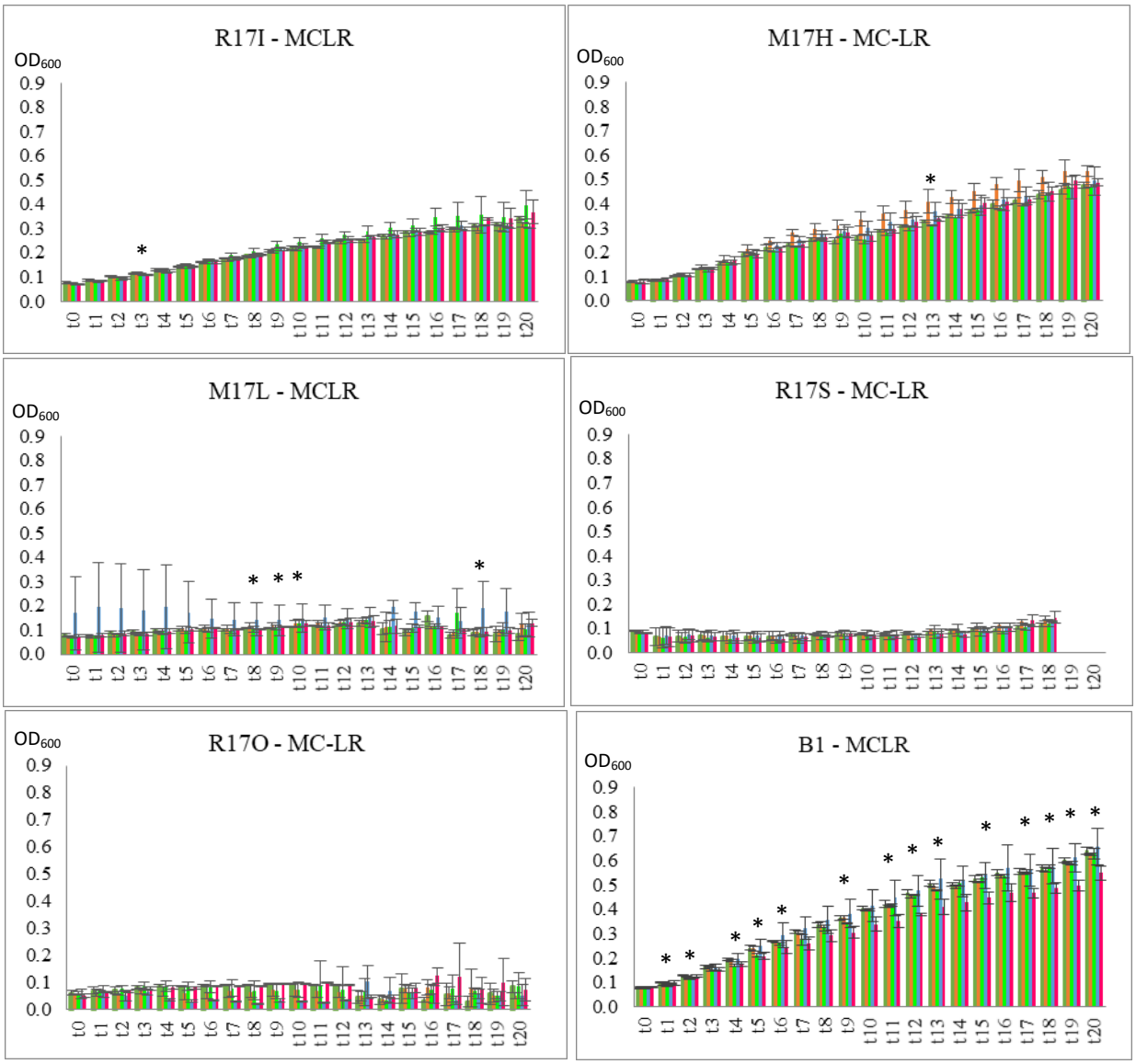


Figure 7.8. (continuation) Graphs of growth curves from all remaining isolates with both MCLR extract and pure MCLR over time (t_0-t_{20}). The values are the mean ± standard deviation of three replicates. (*) indicates a significant difference from the control ($p < 0.05$). (●) Control bacterial group, (●) 1 nM of MCLR extract, (●) 1 nM of pure MCLR, (●) 1 µM of MCLR extract, (●) 1 µM of pure MCLR.