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Effects of benzene and several pharmaceuticals on the growth and microcystin production in *Microcystis aeruginosa* PCC 7806

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ABSTRACT

Effects of benzene and several pharmaceuticals on the growth and microcystin production in *Microcystis aeruginosa* PCC 7806

Currently, the presence of several pharmaceuticals and other organic compounds called 'emerging' contaminants has increased in water bodies. These compounds do not need to persist in the environment to cause negative effects because they are continuously introduced. Hence, assessing the effects of these compounds on aquatic ecosystems is essential. Therefore, the aim of the present study was to analyse the effects of several emerging contaminants (ibuprofen, atenolol, diclofenac and paracetamol) and benzene on *Microcystis aeruginosa* PCC 7806 growth and toxicity. For this purpose, the growth and intracellular microcystin concentration in *M. aeruginosa* PCC 7806 were measured in presence of the four emerging contaminants and benzene. The growth rate was estimated by chlorophyll *a* concentration, and no relevant changes were found. Changes in the expression of the operon *mcy* were examined by semi-quantitative RT-PCR. Intracellular microcystin production was determined using a MicroCystest® kit, and no changes were observed compared to control cells. Thus, the tested concentrations of the contaminants analysed in this study do not have a significant effect on microcystin production in this strain under laboratory conditions.

Key words: Microcystin-LR, emerging contaminants, *mcyD*.

RESUMEN

Efecto del benceno y algunos fármacos en el crecimiento y producción de microcistina en* Microcystis aeruginosa *PCC 7806

El objetivo del presente estudio ha sido analizar el efecto del benceno y de algunos de los contaminantes emergentes más abundantes como el ibuprofeno, atenolol, diclofenac y paracetamol sobre el crecimiento de una cianobacteria toxica, Microcystis aeruginosa *PCC7806, y, su producción de microcistina. Con este propósito, se determinó el crecimiento y la concentración de microcistina intracelular en* M. aeruginosa *cuando ésta se encontraba en presencia de los fármacos y de uno de los solventes orgánicos más comunes en nuestros ríos. El crecimiento fue estimado mediante la determinación de la cantidad de clorofila* a*, la cual no presentó cambios apreciables en las condiciones ensayadas. Los cambios de expresión del operon* mcy *se estudiaron a través del análisis del gen* mcyD *mediante RT-PCR semicuantitativa. La producción de microcistina intracelular fue determinada mediante MicroCystest®sin mostrarse cambios relevantes en comparación con las células control. Se concluye que los contaminantes analizados en este estudio no tienen un efecto significativo en la producción de microcistina, al menos, en los experimentos realizados en el laboratorio, en esta cepa, y con las dosis utilizadas.*

Palabras clave: *Microcistina-LR, contaminantes emergentes,* mcyD.

INTRODUCTION

The progress of society often causes changes in the environment. In river basins, many human activities that developed during the Industrial Revolution, continue to reduce the river flow and to cause influxes of many contaminants, including nutrients from untreated water, inorganic and organic fertilisers and industrial waste. These changes primarily affect phytoplankton, biofilms and bacteria, which are responsible for water quality (Vitousek *et al.*, 1997). For decades, efforts have been focused on identifying toxic, persistent and bioaccumulative chemical contaminants. Over 100 000 hazardous substances have been registered by the European Union (Fuerhacker, 2007). Nonetheless, the development of more accurate analytical methods has permitted the detection of new hazardous contaminants in water bodies; these contaminants are called emerging contaminants. Pharmaceutical compounds are causing greater concern due to their high levels of production and consumption, along with insufficient degradation in wastewater treatment plants. Pharmaceuticals are continuously introduced into the ecosystem; thus, these contaminants do not have to persist in the ecosystem to produce negative effects on the environment (Hartmann *et.al.*, 1998; Jones *et al.*, 2001; Petrovic *et al.*, 2004; Arslan-Alaton & Caglayan, 2006). Analgesics/anti-inflammatory drugs such as ibuprofen and diclofenac, carbamazepine and β-blockers are among the most commonly used medical prescriptions (Petrovic *et al.*, 2005; Hernando *et al.*, 2006). The project AquaTerra has estimated that three tons of pharmaceuticals per year are discharged into our rivers (Jones *et al.*, 2001; Gros *et al.*, 2007). In many cases, the consequences of the presence of these pharmaceuticals in the environment are not clear; in other cases, the risk is evident. For example, diclofenac affects mammalian kidneys; this drug has also been associated with the disappearance of the white-backed vultures in India and Pakistan (Fent *et al.*, 2006). Fent and colleges also observed that exposure to propranolol affects zooplankton (Fent *et al.*, 2006).

Cyanobacteria are prokaryotes with high morphological complexity and physiological diversity. These organisms have an outstanding capacity to adapt to a wide range of environments and to survive in extreme or highly degraded environments. Certain cyanobacterial species can produce a broad range of bioactive secondary metabolites called cyanotoxins that are potentially toxic to eukaryotic organisms (Carmichael *et al.*, 2001; Codd *et al.,* 2005). The human pressures exerted on water resources, in combination with climate change, promote accelerated eutrophication of water bodies. This process results in an increase in blooms, particularly cyanobacterial blooms, which are frequently toxic. A growing number of blooms dominated by toxic cyanobacteria may result in severe health and environmental problems. The toxins produced by cyanobacteria are primarily hepatotoxic and neurotoxic. *Microcystis aeruginosa* frequently forms blooms, and microcystin is the most common toxin produced by these cyanobacteria. Microcystin is synthesised in a mixed polyketide synthase/nonribosomal peptide synthetase system called microcystin synthetase (*mcy* operon), which is also involved in tailoring and transporting microcystin. Tillett and co-workers identified and sequenced the *mcy* operon, which is a 55-kb sequence that consists of ten open reading frames that are bidirectional transcribed from a central 732-bp intergenic region between *mcyA* and *mcyD* (Tillett *et al.*, 2000). Microcystin synthesis is an inducible

Table 1. Oligonucleotides that were used as primers in the semi-quantitative RT-PCR analyses. *Oligonucleótidos utilizados como cebadores en el análisis de RT-PCR semicuantitativa.*

Primers	Sequence 5′ → 3′	Length	Tm (°C)
R16S dir	CAAGTCGAACGGGAATCT TC	20	47
R16S rev	CTCAAGTACCGTCAGAACTTC	21	
mcyD dir	GAGCATTAAGGGCTAAATCG	20	45
mcyD rev	CTTGGTTGCTTCATCAACTC	20	

event that is thought to be controlled by many environmental and nutritional factors. Two of these factors are iron availability and the C:N balance, which regulate the *mcy* operon through two master transcriptional regulators, Fur (ferric uptake regulator) and NtcA (Martin-Luna *et al.*, 2006; Kuniyoshi *et al.*, 2011).

Currently, how environmental variables influence cyanotoxin production remains unknown. Thus, monitoring and assessing the effects of benzene and of some of the most used pharmaceuticals on microcystin production is of interest. Paracetamol, atenolol, diclofenac and ibuprofen are pharmaceuticals with high levels of production and consumption. The concern is whether the presence of those pharmaceuticals and of one of the more common organic solvents, benzene, which is found in our rivers, might cause an increase in toxin synthesis. Because *M. aeruginosa* PCC 7806 produces a high amount of microcystin per biomass, this cyanobacterium was used to examine the effects of several emerging contaminants and benzene on its toxicity.

MATERIALS AND METHODS

Growth conditions

The axenic strain *M. aeruginosa* PCC 7806, which was provided by the Pasteur Culture Collection (Paris, France), was used in this study. Cells were grown under batch conditions in BG11 media with 2 mM of $NaNO_3$ and continuous agitation at 25 °C. The cyanobacteria were grown with a light intensity of 40 μmol of photons m^{-2} s^{-1}; the light intensity was measured using a Quantum Sensor photometer (Skye Instruments, SKP 200). The cultures were started with equal aliquots of 0.3 OD (600 nm), and stress treatments were applied when the cells reached the exponential growth phase (0.6-0.7 OD). Experiments were performed in 250 mL Erlenmeyer flasks with continuous agitation. Control flasks were maintained under the same conditions as stressed flask. The following concentrations of pharmaceuticals and benzene were added to the cultures at exponential phase: 200 μg/L ibuprofen; 20 μg/L atenolol; 20 μg/L diclofenac; 27 μg/L and 166 μg/L paracetamol; and 50 μg/L and 100 μg/L benzene. All the pharmaceuticals are water soluble at these concentrations. Aliquots were collected at four days after the addition of the different compounds and then compared with non-stressed cells.

Analytical methods and microcystin quantification

Chlorophyll *a* concentrations were determined spectrophotometrically by measuring the absorbance at 650 nm according to the procedure described by Mackinney (Mackinney, 1941). The pigment was extracted from 1 mL samples using 80% methanol with stirring.

Aliquots (5 mL) were collected, centrifuged for 5 min at 4000 rpm, and frozen at −20 °C for microcystin determination. Total intracellular microcystin was determined from 4 extractions, which were performed using 1 mL of 80% methanol, 0.1% trifluoroacetic acid (TFA), and 0.1% Tween. For each extraction, the samples were stirred for 15 min and then centrifuged at 4000 g for 10 min. Next, the extracts were pooled and quantified using a MicroCystest® kit (Zeulab). Statistical analyses of all the data were performed using Student's test ($p < 0.05$).

Sampling and RNA isolation

In total, 25 mL of each culture was harvested by centrifugation at 4000 g for 4 min at 4 °C. The manipulation was performed carefully to avoid altering the RNA. Each cell pellet was suspended in 600 μL of 50 mM Tris-HCl (pH 8), 100 mM EDTA and 130 μL of chloroform and incubated on ice for 3 min to eliminate external RNases. After removing the buffer by centrifugation at 13 000 g for 5 min at 4 °C, the cell pellets were frozen in liquid nitrogen and kept at −80 °C until RNA isolation was achieved.

RNA was extracted using TRIzol (Invitrogen) and chloroform extractions according to the manufacturer's instructions. In the last extraction, RNA was collected from the aqueous layer and precipitated in isopropanol and liquid

nitrogen. Then, the RNA pellet was washed twice with 75% ethanol.

Reverse transcription (cDNA synthesis)

Total RNA was treated with 40 units of DNase I (Pharmacia) to eliminate the DNA in the samples. The digestions were incubated at 37 °C for 1 h in a volume of 100 μL using a buffer containing 4 μL of 1 M Tris-HCl (pH 7.5) and 0.6 μL of 1 M $MgCl_2$ in DEPC-H_2O. After digestion, the enzyme was inactivated by phenol acid:chloroform extraction, and RNA was precipitated with absolute ethanol. The result of the digestion was assessed by PCR using primers targeting the16S rRNA gene.

RNA integrity was verified using a 1% agarose gel, and the concentration was determined by measuring the absorbance at 260 nm using a NanoVue (GE Healthcare).

A reverse transcriptase enzyme kit was used for the reverse transcription reaction. In total, 1 μg of total RNA was diluted with annealing buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA, and 150 mM KCl) and mixed with 150 ng of random hexamer primers (Invitrogen Corp.) in a final volume of 10 μL. The mixture was heated at 85 °C for 10 min and then incubated at 50 °C for 1 h. RNA was reverse transcribed using 200 U of SuperScript™ (Gibco BRL) in the presence of 2 μL of deoxyribonucleoside triphosphate mixture (2.5 mM each one), 2 μL of dithiothreitol (100 mM) and 4 μL of the 5x buffer, adjusting with DEPC-H_2O to reach a final volume of 20 μL. Then, the mixture was incubated at 47 °C for 1 h and finally heated at 75 °C for 15 min.

RT-PCR analysis of gene expression

Semi-quantitative RT-PCR analyses were performed with the obtained cDNA samples. 16S r*RNA* (*rrs*) was used as the housekeeping gene, and specific primer sets were designed to amplify both the studied genes and the endogenous reference gene (Table 1). *rrs* was used to normalise the possible variation in cDNA concentration as described previously (González *et al.*, 2010). The exponential phase of the amplification was estimated by measuring the amount of PCR products after different numbers of cycles. That number of cycles was utilised for gene normalisation.

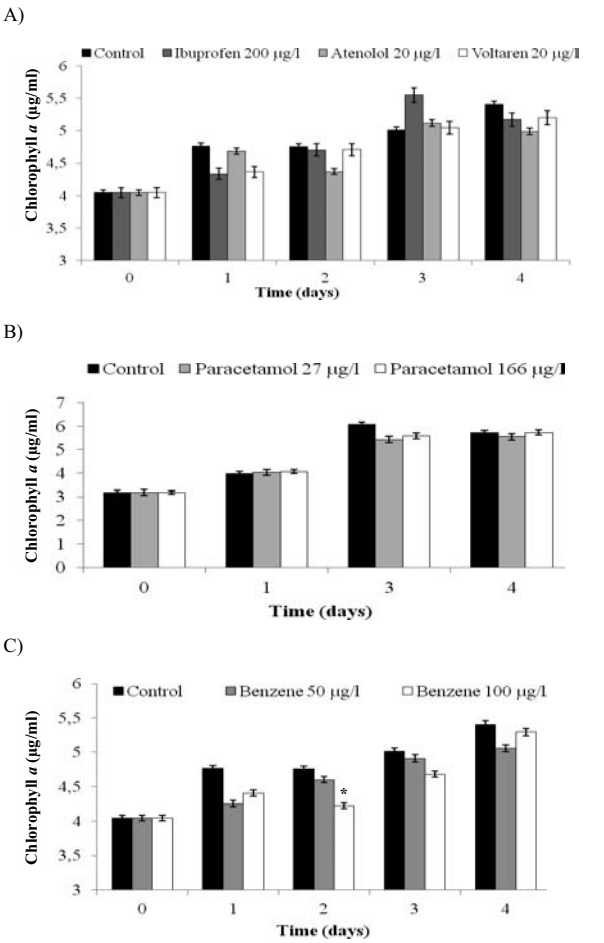

Figure 1. *M. aeruginosa* PCC 7806 growth in the presence of different drugs at their maximum concentrations found in water bodies. The response was estimated using changes in chlorophyll *a* concentrations. Panel A: Non-stressed cells and ibuprofen (200 μg/L)-, atenolol (20 μg/L)- and diclofenac (20 μg/L)-treated cells. Panel B: Non-stressed cells and paracetamol (27 μg/L and 166 μg/L)-treated cells. Panel C: Non-stressed cells and benzene (50 μg/L and 100 μg/L)-treated cells. *A significant decrease (p<0.05) was observed with 100 μg/L of benzene after two days of treatment. *Curva de crecimiento de* Microcystis aeruginosa *PCC7806 en presencia de diferentes fármacos a la concentración maxima disuelta encontrada en aguas. La respuesta fue analizada mediante variaciones de clorofila a. Panel A: células control, células tratadas con ibuprofeno 200 μg/l, atenolol 20 μg/l and diclofenac 20 μg/l. Panel B: células control, células tratadas con paracetmol a las dos concentraciones estudiadas: 27 μg/l y 166 μg/l. Panel C: células control, células tratadas con benceno a las dos concentraciones estudiadas: 50 μg/l and 100 μg/l. *Descenso significativo (p<0.05) en el segundo día tras el tratamiento con 100 μg/l de benceno.*

PCR-amplified DNA fragments were separated by 1% agarose gel electrophoresis stained with ethidium bromide and analysed using a Gel Doc 2000 Image Analyser (Bio-Rad).

RESULTS

Effects of benzene, paracetamol, ibuprofen, atenolol, and diclofenac on *M. aeruginosa* PCC7806 growth

The concentrations of emerging contaminants used in this study were in the upper range or moderately higher (10-20 times) than the concentrations found in some Spanish rivers according to the literature (Barceló & López, 2007; Gros *et al.*, 2007). The concentration of paracetamol in surface water has a wide range, from 0.1 ng/L to 2.4 μg/L (Osorio *et al.* 2012). Ibuprofen and diclofenac are the most concentrated anti-inflammatory drugs, with concentrations up to the mg/L range (Osorio *et al.* 2012). Atenolol contamination can reach values of approximately 0.5 mg/L. Figure 1 shows the changes in chlorophyll *a* concentrations when cells were grown in the presence of the different contaminants. Panel A shows the small changes in cell growth that were observed in the presence of ibuprofen, atenolol and diclofenac (Voltaren). Panel B reflects the growth observed when two doses of paracetamol were used; no differences in cell growth were observed between the stressed cells and the control cells. Panel C indicates changes in cell growth in the presence of benzene.

Benzene is a well-known, volatile carcinogen that is one of the twenty chemical products with major production. The benzene concentrations studied, 50 μg/L and 100 μg/L, were chosen based on levels under specific pollution episodes described in the literature (Hedgecott & Lewis, 1997; Fernández *et al*., 2013). However, some industrial effluents, for instance, leachates from dumpsites of an old lindane factory, can reach considerably higher amounts, in the mg/L range (Fernández *et al*., 2013). When *M. aeruginosa* cells were exposed to benzene, their growth was slightly affected during the early days and was significant (Student's test with $p < 0.05$) on the 2nd day of treatment with 100 μg/L benzene. However, after 4 days, *M. aeruginosa* recovered and reached the same density as that of control cells (Fig. 1C) most likely due to the high volatility of benzene. *Microcystis* growth curves for at least 3 different samples were analysed; the results are shown in Figure 1.

Effects of benzene, paracetamol, ibuprofen, atenolol, and diclofenac on *mcyD* expression levels

The extracted total RNA was reverse transcribed, and RT-PCR was performed with cDNA using the *rrs* gene (16S) as the housekeeping gene. Changes in mRNA levels after 4 days of exposure are shown in Figure 2. The *mcyD* gene encodes a polyketide synthase that is responsible for incorporating the Adda group, which is involved in the toxicity of the molecule (Nishizawa *et al.*, 2000; Tillett *et al.*, 2000). This gene is the first gene that is transcribed and, therefore, is essential for microcystin synthesis. *mcyD* expression was slightly induced in cells treated with 50 μg/L diclofenac and benzene compared with that in control cells (Fig. 2A). Nevertheless, *mcyD* expression decreased in cells grown in the presence of 100 μg/L ibuprofen and benzene (Fig. 2A). In the case of paracetamol, we collected aliquots of cells treated with the highest concentration of paracetamol (166 μg/L) for RNA reverse transcription. When we analysed the gene expression levels in the paracetamol-treated cells, we observed an extreme decrease in transcription levels compared with those of control cells (Fig. 2B).

Microcystin-LR content in treated *M. aeruginosa* cells

The levels of Microcystin-LR and the minority D-Aps[3] MC-LR, which are the only two variants found in this strain (Wiedner *et al.*, 2003), were measured using a MicroCystest® kit. This test is based on phosphatase 2A inhibition. The results, which are expressed as total microcystin-LR equivalents, are represented in Figure 3. The

obtained results did not show significant changes (Student's test with $p < 0.05$) in microcystin levels in cells treated with pharmaceuticals. The total microcystin content was slightly decreased in ibuprofen- and Voltaren-treated cells, while the microcystin content in atenolol-treated cells was similar to non-treated cells. Both concentrations of paracetamol showed slightly decreased microcystin content versus control cells.

Figure 3C shows the level of microcystin measured in benzene-treated cells. No significant change ($p < 0.05$) was observed. However, the microcystin contents at both benzene concentrations were slightly lower than control cells after 4 days.

DISCUSSION

In recent years, significant amounts of substances that have gone unnoticed thus far have been detected. For instance, the Ebro River was studied during years within the framework of the European project AquaTerra (Integrated modelling of the river-sediment-soil-groundwater system). Data from other fluvial systems indicated specific episodes of high levels of emerging contaminants (Hedgecott & Lewis, 1997; Jurado *et al.,* 2012; Osorio *et al.,* 2012). Wastewater treatment stations were unable to eliminate many of these emerging contaminants; thus, these contaminants flowed into the rivers. Approximately three tons of drugs per year were discharged into the Ebro River; the four pharmaceuticals used in this work were among these drugs (Gros *et al.*, 2007).

Some anti-inflammatory drugs employed in this study, i.e., diclofenac and ibuprofen, are ubiquitous in the affluent of every wastewater treatment plant (EDARs) (Barceló & López, 2007; Gros *et al.*, 2007). Other drugs, including β-blockers such as atenolol represent a high percentage of the total contaminants in all EDARs. In addition to wastewaters, atenolol is among the compounds that represent more than 50% of the total load of the river (Fuerhacker, 2007). Some studies have examined the effect of antibiotics on cyanobacteria; sensitivity to antibiotics has been reported (Holten-Lützhøft *et al.*, 1999; Halling-Sørensen *et al.*, 2000). Notably, the results presented in this work may be influenced by changes in the real concentration exposure. Additionally, our work was performed under laboratory conditions, eliminating the complexity of natural ecosystems. Therefore, with the caution of a laboratory study in a single strain, the results obtained with the chosen concentrations of the pharmaceuticals tested indicated that exposure to these drugs does not significantly affect the

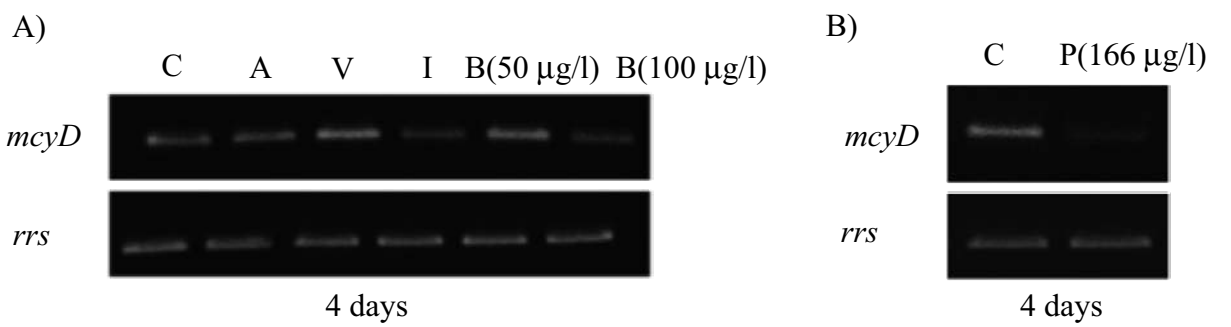

Figure 2. Changes in *mcyD* expression in *M. aeruginosa* PCC 7806 cells treated with drugs and benzene. *mcyD* expression was analysed by semi-quantitative RT–PCR. The housekeeping gene *rrs* was used as a control. Determinations for each gene were performed in the exponential phase of PCR. Panel A: *mcyD* expression in atenolol (A)-, Voltaren (V)-, ibuprofen (I)- and benzene (B)-treated cells and in non-treated cells (C). Panel B: *mcyD* expression in paracetamol-treated cells (P) compared to that in control cells (C). *Cambios en la expression de mcyD en células de* Microcystis aeruginosa *PCC 7806 tratadas con los distintos fármacos y benceno. La expresión fue estudiada mediante RT-PCR semicuantitativa. Se utilizó el gen* rrs *como gen de referencia. La determinación de cada gen se llevó a cabo en la fase exponencial de la PCR. Panel A: expresión de* mcyD *en células control (C) y células tratadas con atenolol (A), voltaren (V), ibuprofeno (I) y benceno (B). Panel B: expresión de* mcyD *en células control y células tratadas con paracetamol (P).*

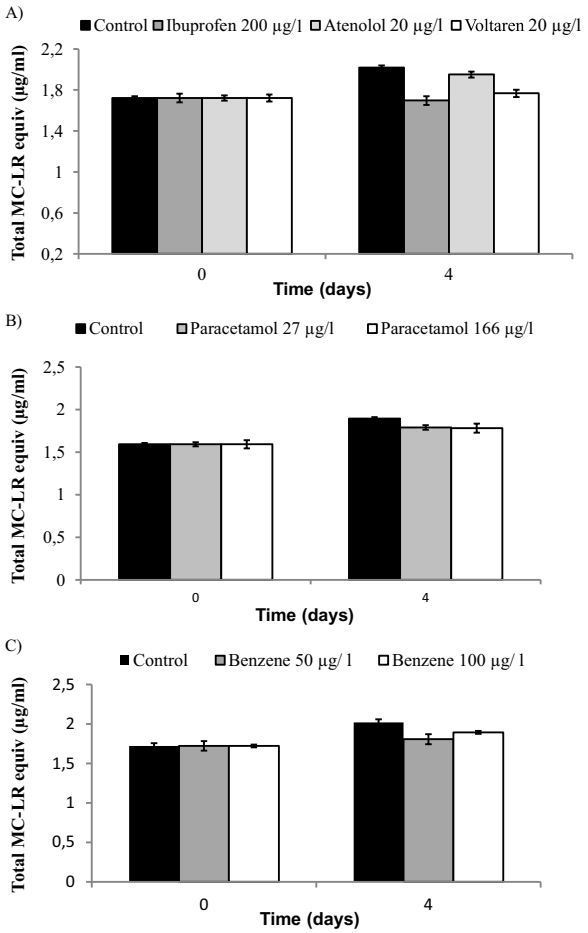

Figure 3. Intracellular microcystin content, which was determined as microcystin-LR equivalents, in stressed cells and in control cells. Panel A: Non-treated cells, ibuprofen (200 µg/L)-, atenolol (20 µg/L)- and Voltaren (20 µg/L)-treated cells. Panel B: Non-treated cells and paracetamol-(27 µg/L and 166 µg/L)-treated cells. Panel C: Non-treated cells and benzene (50 µg/L and 100 µg/L)-treated cells. *Cantidad de microcistina intracelular expresada en equivalentes de Microcistina-LR presente en las células control y en las células tratadas. Panel A: células control, células tratadas con ibuprofeno 200 µg/l, atenolol 20 µg/l and diclofenac 20 µg/l. Panel B: células control, células tratadas con paracetamol a las dos concentraciones estudiadas: 27 µg/l y 166 µg/l. Panel C: células control, células tratadas con benceno a las dos concentraciones estudiadas: 50 µg/l and 100 µg/l.*

growth of *M. aeruginosa* PCC 7806 under these conditions.

Although we observed a slight decrease in cell growth with some of the treatments, no increase in microcystin production was observed. Figures 2 and 3 show that the observed changes in transcriptional levels did not correlate with microcystin-LR content. We observed a slight reduction in the total amount of toxin in cells treated with ibuprofen and diclofenac. Nonetheless, only ibuprofen lowered gene expression. Intracellular microcystin contents did not exhibit appreciable changes when the cells were treated with benzene; these results are consistent with *mcyD* expression.

The most remarkable finding was that paracetamol treatment had a slight effect on the growth of *Microcystis* and was accompanied by an important reduction in *mcyD* transcription. However, the analysis of the total microcystin content in these cells indicated that a slightly lower amount of toxin was synthesised in these cells than that in control cells.

In summary, no significant changes in growth and microcystin levels were detected in *M. aeruginosa* treated with overdoses of ibuprofen, atenolol, Voltaren, paracetamol and benzene compared with amounts described in natural waters.

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