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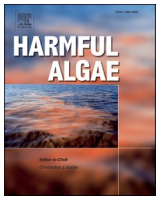

Experimental evidence for the selection of salinity-tolerant ecotypes of the *Microcystis aeruginosa* complex

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ABSTRACT

Microcystis aeruginosa complex (MAC) generate harmful blooms in multiple aquatic ecosystems, from freshwater to estuaries. The intra-complex genetic diversity and its phenotypic plasticity emerge as the main hypothesis of ecological success of the group in eutrophic ecosystems. This intra-complex diversity is composed of ecotypes, phylogenetically closely related organisms differing in their ecological preferences that could be considered species. Under the hypothesis of a rapid selection of ecotypes caused by salinity, here, we experimentally evaluated the role of salinity (0, 5, 10 and 25 ppt) in the ecotype community configuration and toxicity of MAC. We assessed the response to different salinities by analyzing ecotype composition (*mcyJ* based genotyping) combined with *mcyJ* amplicon sequencing for ecotypes identification, MC-producing cells abundance and their toxin-production activity (*mcy* gene copies and transcripts abundance, and MC variants concentration), and machine learning methods for data analysis. We found that, although the abundance of MC-producing cells, the *mcyE* transcription and MC concentration were negatively affected by increasing salinity, the treatments at 10 and 25 ppt selected MAC ecotypes showing different profiles of MC variants. The results indicate that when freshwater MAC species are transported to brackish waters, the salt-induced stress (salinities between 5 and 10 ppt) selects salt-tolerant ecotypes, generating a shift in the MC variants produced. At higher salinities (25 ppt), the selected ecotypes maintain their abundance but MC production decreases. The existence of MC-producing ecotypes adapted to different salinity conditions is relevant in determining the success of MAC organisms in a wide range of ecosystems and poses a risk to estuarine health, justifying further research.

1. Introduction

Anthropogenic activities and global climate change increases the intensity and frequency of toxic cyanobacterial blooms in freshwater bodies. Eutrophication, precipitation, and seawater intrusion are the most important forcing in freshwater bodies and estuaries (Nielsen et al., 2003; Verspagen et al., 2006; Loáiciga et al., 2012; White et al., 2017; Vu et al., 2018). The *Microcystis aeruginosa* complex (MAC) includes the most common and widespread blooming cyanobacteria in freshwater ecosystems worldwide (Adloff et al., 2018; González-Piana et al., 2017; Haakonsson et al., 2017; Lehman et al., 2022) and could synthesize >279 variants of the cyanotoxin microcystin (MC) (Bouaïcha et al., 2019). Field studies have shown that species of MAC could be transferred along a freshwater-marine continuum where they could survive and proliferated at high salinities (Kruk et al., 2021; 2017; Martínez de la Escalera et al., 2017; Preece et al., 2017). Due to eutrophication, extreme precipitation and drought events, MAC blooms are becoming more frequent in brackish waters (Trung et al., 2022). MAC organisms are described as one of the freshwater cyanobacteria with the highest salinity tolerances (Verspagen et al., 2006) and it has been observed that *Microcystis* spp. has variable salinity tolerance threshold from 4 ppt (Chen et al., 2015), 10 ppt (Kruk et al., 2017; Tonk et al., 2007; Verspagen et al., 2006) to 35 ppt (Kruk et al., 2021; 2017; Martínez de la Escalera et al., 2017), suggesting a large variability that could be

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attributed either to intraspecific diversity or to the presence of more species than those described by morphology. This high variability may explain the presence of blooms in brackish waters.

The biosynthesis of MC (cyclic heptapeptide) is non-ribosomal, and it is mediated by an enzyme complex (Fischbach and Walsh, 2006) encoded by the *mcy* genetic cluster (55 kb) that contains 10 genes (*mcyA-J*) (Tillett et al., 2000). MAC present different genotypes, which may contain the complete, partial, or no *mcy* cluster (Kaebernick and Neilan, 2001; Yancey et al., 2022). The transcription of the *mcy* genes starts in a bidirectional promoter located between *mcyA* and *mcyD* genes (Tillett et al., 2000) (Kaebernick et al., 2002). Several studies have analysed which environmental variables influence the expression of some *mcy* genes and MC synthesis. Among the environmental variables identified are light intensity (Kaebernick et al., 2000), temperature (Martin et al., 2020), as well as salinity (conductivity) (Chen et al., 2015; Martínez de la Escalera et al., 2023) and turbidity (Martínez de la Escalera et al., 2023). MC production by MAC organisms decreases when salinity exceeds 10 ppt (Chen et al., 2015; Tonk et al., 2007). However, the regulation and synthesis of MC, as well as its ecological role, are complex and not yet fully understood (Neilan et al., 2013; Omidi et al., 2018).

Different experimental approaches have reported that MC-producing MAC organisms exposed to brackish waters can have competitive advantages and lower MC production than in freshwater environments, even decreasing when salinity exceeds 10 ppt (Robson and Hamilton, 2003; Tonk et al., 2007). Also, metabolomic analysis showed that the tolerance to estuarine salinity was strain-specific and involved compatible solute production (des Aulnois et al., 2019). These authors showed that under a 16.9 ppt salinity MAC reduced the growth and MC production rates. These findings imply that the ability of MC-producing MAC organisms to proliferate at estuarine salinities would be linked to the genomic potential to produce different compatible solutes according to their environmental adaptations (des Aulnois et al., 2019; Sandrini et al., 2015; Tanabe et al., 2019, 2018). The synthesis and accumulation of compatible solutes (low molecular weight osmoprotectant) is an important mechanism that gives bacteria the ability to tolerate high salinities, avoiding osmotic pressure difference and dehydration (Le Rudulier et al., 1984; Roberts, 2005). Since most experiments addressing the effect of salinity are based on isolated strains of *Microcystis,* which are generally single celled (Chen et al., 2015; Tonk et al., 2007), the impact of salinity on the dynamics and diversity of naturally occurring MC-producing MAC is poorly understood.

In previous studies, we found that MC-producing MAC community is composed of very closely related populations that are ecologically distinct (ecotypes), each specialized to a specific arrangement of environmental variables. Particularly, in a large subtropical riverine to estuarine ecosystem (the río Uruguay River-Río de la Plata estuary, South America) we distinguished six groups of *mcyJ* genotypes that were associated with different combinations of water temperature, conductivity, and turbidity (Martínez de la Escalera et al., 2022). We proposed that each *mcyJ* variant associated with a defined environmental condition (water temperature, conductivity, and turbidity) is an ecotype whose relative abundances vary according to their fitness in the local environment. This diversity of ecotypes would explain the success of MC-producing MAC in such a wide array of environmental conditions.

Understanding how MAC populations respond to increased salinity, in MC production, and activity level is crucial for the management and prediction of MC-producing blooms. Here, we experimentally evaluated the effect of salinity over the abundance and diversity of MC-producing MAC populations, the expression of *mcy* genes, and the concentration of MC variants. Thus, we exposed a natural, freshwater MAC community to different salinities to emulate a salinity gradient from a freshwater reservoir to the outer end of an estuary. Our working hypothesis was that the freshwater MAC community includes multiple ecotypes with different fitness relative to salinity, closely related to the synthesis of MC variants. We expected compositional changes in MAC in response to salinity, with an effect on growth, transcription, and genetic diversity, as well as the selection of different MC-producing ecotypes which produce different MC variants.

2. Materials and methods

2.1. Samples, culture conditions and experimental design

Laboratory experiments were conducted with water samples from Salto Grande reservoir (31°11′S, 57°52′W) a large (area = 783 ha) and deep (z~20 m) human-made eutrophic reservoir that frequently present MAC blooms (Kruk et al., 2017; Martínez de la Escalera et al., 2017; O'Farrell et al., 2012). MAC organisms (inoculum) were subsurface sampled with sterile plastic 20 liters carboys in summer (December) during a bloom (Kruk et al., 2017; Martínez de la Escalera et al., 2023, 2017). A volume of approximately 2 litres of the samples was transported to the lab and acclimated for 24 h at 21 °C under a light intensity of 30 μmol ms^{-1}.

We generated four salinity levels 0, 5, 10, 25 ppt attained with the addition of artificial sea salt mixture (Sera – Marin, France) to simulate the freshwater (0 ppt) – estuary (5 and 10 ppt) – ocean (25 ppt) salinity gradient. Salinity concentrations were selected based on knowledge about the ability of MAC species to grow between 0 and 6 ppt salinities, to tolerate salinities of up to 10 ppt (Tonk et al., 2007) and cell lysis at salinity larger than 10 ppt (Orr et al., 2004). The experiments were performed in 500 ml Schott flasks using a final volume of 300 ml in temperature and light (30 μmol ms^{-1} daylight fluorescent tubes with a 16:8 hs light:dark photoperiod) controlled incubating chambers at 21 °C. The position of the flasks in the incubating chamber were randomly changed each day. All treatments started with an inoculum of ca. 1×10^4 organisms ml^{-1} (organism refers to colonies), similar values to those found in a bloom (Kruk et al., 2017), to generate a scum on the top of the flasks. To minimize the differences in community composition, the initial inoculum of each bottle was generated by taking three random samples from the acclimated natural bloom sample. All treatments were run in triplicate with a total duration of 10 days. The bottles were gently mixed manually twice a day. Samples for toxic genotype quantification, gene expression and toxic genotype diversity were taken at initial time (T0 = inoculum), T1 = 10 min, T2 = 4hs, T3 = 18hs, T4 = 4 days, T7 = 7 days and T10 (final time) = 10 days (Table 1).

2.2. Nucleic acid extraction

For DNA extraction, 1 ml of the scum of each treatment replicate was collected in sterile tube, which were immediately frozen at −20 °C until processing. Procedures for nucleic acid extraction were based on the methodology described in Deus et al., 2020 (Álvarez et al., 2020). Briefly, cell lysis was performed using ceramic beads (2 mm in diameter) and extraction buffer with 1 % CTAB. The scum sample was placed together with the beads and the extraction buffer in sterile 2 ml tubes and were homogenized for 40 s at 6 ms^{-1} in the Fast-Prep 24 equipment

Table 1
Activities carried out, samples taken, and analyses performed during the experiment.

Activity	Time						
	0	10 min	4 h	18 hours	4 days	7 days	10 days
Inoculum preparation.	x						
Inoculation and start of experiment.		x					
Quantification of *mcyE*-harboring cells.		x	x	x	x	x	x
Quantification of *mcyE* gene transcripts.	x	x	x	x	x	x	x
MC quantification.		x	x	x		x	
MAC ecotypes and *mcyJ* genotypes diversity.		x					x

(MP Biomedicals). The DNA was subsequently extracted using chloroform:isoamylalcohol (24:1) three times and then precipitated with isopropanol (0.6 v/v) for one hour at room temperature (~20) and washed with cold ethanol 70 % v/v. For RNA extraction, one millilitre of the scum of each treatment replicate was used to RNA extraction. Samples were immediately processed with Pure Link RNA kit (Life Technologies) for RNA isolation, which was then applied to retro-transcription to produce cDNA using High-Capacity RNA to cDNA kit (Invitrogen). The concentration and purity of DNA and RNA was determined by micro-volume spectrophotometer (Nanodrop), DNA and cDNA samples were stored at −20 °C.

2.3. Quantitative PCR (qPCR)

Two microlitres of DNA extracts from each sample (ca. 20 ng DNA) were applied to the Power SYBR Green PCR (Invitrogen) with a final reaction volume of 20 µl. Cycling conditions were 15 min at 95 °C and 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 60 °C, including a last melting step from 65 to 95 °C, at increases of 1 °C each 4 s. A 96 FLX Touch TM thermal cycler (Bio-Rad) was used. To quantify the total abundance of *mcyE* gene (DNA) and *mcyE* transcripts (cDNA) in the water samples curves were achieved using five serial dilutions from 1/10 to 1/100,000 of the cloned gene and applied to qPCR in the same PCR plate where the samples were assayed. Samples were run in triplicate. The *mcyE* gene copies was used as a proxy of MC-producing MAC organisms, while the *mcyE* transcript abundance was used as indicator of MC synthesis by the MC-producing MAC organisms.

2.4. Microcystin quantification

Samples containing *Microcystis* were held for 72 h at −20 °C to release the intracellular toxins from cyanobacteria cells. After thawing, the samples were passed through a Polyethersulfone (PES) 0.45 µm syringe filter to extract the MC toxins. Four calibration curves were carried out using five levels with Abraxis™ MC Standard solution for each MC variants: MC-LR, MC-RR, MC-YR and MC-WR. Liquid chromatography was performed based on EPA Method 544. Chromatographic separation was performed using the Thermo Scientific™ Vanquish™Core LC system equipped with a Thermo Scientific™ Accucore™ C18 LC column (2.6 × 100 mm, 2.6 µm) maintained at 30 °C. The mobile phase was Water (0.05 formic acid + 0.05 TFA)-Acetonitrile. The injection volume was 5 µL. Each MC variant was separated and identified by comparing the acquired mass spectrum and retention time to the reference spectrum and retention time for calibration standard acquired under the same LC-MS/ MS conditions. MC-LR, MC-RR, MC-YR and MC-WR were detected on a Thermo Scientific™ TSQ Fortis™ triple quadrupole mass spectrometer equipped with a Heated Electrospray Ionization source (HESI). Each MC concentration was determined by calibration curve comparison.

2.5. Genotyping of MC-producing MAC organisms

The genotype of MC-producing MAC population was characterized at the final of each treatment an approach based on high resolution melting analysis (HRMA) of *mcyJ* amplicons and multivariate Classification and Regression Trees (*m*CART) was applied (Martínez de la Escalera et al., 2022). DNA samples having a concentration of ~ 30 ng/µl of DNA each were applied to the qPCR reactions using the MeltDoctor HRMA kit (Invitrogen), the TouchTM Real-Time PCR Detection System (BIO RAD) thermal cycler and the software Bio-Rad Precision Melt Analysis. The melting curves were normalized to the same level of fluorescence (RFU) using the pre and post melting regions. Normalized RFU values were used as response variables (output variables) for *m*CART and salinity values as explanatory variables (input variables).

2.6. Analysis of MC-producing MAC diversity

To link the *mcyJ* ecotypes detected by HRMA-*m*CART with their specific sequence, amplicon sequencing of *mcyJ* gene was performed at the beginning (T0) and at the end of the experiment (Tf = 10 days) for all treatments. The *mcyJ* gene, which is 930 bp long, was amplified by PCR using the following specific primers generating a 300 bp amplicon (Kim et al., 2010). The adapters and barcodes required for sequencing in the S5 Ion Torrent (Life Technology) are available at the IIBCE sequencing facility. The reads were analysed with the statistical program R using the package {DADA2} (Erickson et al., 2019). Adapter sequences, barcodes, primers and the first 15 bp were removed from the reads. Then, the reads were filtered based on sequence quality (quality score > 25) and amplicon size (300 bp), and chimeras were removed. Sequences were assigned to ASV (Amplicon Sequence Variant) at the level of single-nucleotide differences. Those ASV whose abundance was less than or equal to five in the total number of samples were eliminated. Sequences were submitted to the nucleotide archive GenBank with the BioProject ID PRJNA1150617.

2.7. Data analysis

Spearman's bivariate correlation (r_s) was used to evaluate the relationship between MAC variables: $\log_{10}$ abundance of *mcyE* gene copies (copies ml-1), $\log_{10}$ abundance of *mcyE* gene transcripts (copies ml^{-1}), MC-producing MAC diversity and concentration of MC variants. Means of linear and Log linear models using likelihood ratio test (LRT) were used to determine whether treatment and incubation time affected the abundance of MC-producing MAC organisms (*mcyE* gene), the abundance of *mcyE* gene transcripts, as well as the production of the different MC variants. In all cases, treatment and incubation times were used as categorical explanatory variables. The models were compared based on Akaike's information criteria (AIC) (Liddle et al., 2009) and the model with the lowest AIC value was selected.

*m*CART was applied to describe the relationship between the different MC-producing MAC organisms genotypes and salinity (Martínez de la Escalera et al., 2022). Salinity values of each treatment were used as explanatory variables. The melting curves obtained for the *mcyJ* gene at the beginning and at the end of each treatment were normalised to the melting region (75 - 82 °C) for applying the *m*CART.

To analyse the diversity of MC-producing MAC, ASV richness (number of ASV per sample), Shannon diversity and equitability indexes were calculated. Differences in richness and diversity indexes between treatments were evaluated by LRT method. Differential abundance analysis was performed using log fold change (LFC), using the base 2 logarithm of the ratio between two abundance values: $LFC = \log_2(A/B)$, where A and B represent the abundance of an ASV under two different conditions. $LFC \geq 1$ or ≤ 1 and a p-value of 0.05 were set as the criteria for identifying statistically significant changes in ASVs abundance.

All analyses were carried out in the statistical software R version 4.0.1 (R Core Team, 2019) and the packages: {vegan}, {rpart}, {fda}, {DESeq}, {nlme}, and {PMCMR} (Anders, 2012; Oksanen et al., 2015; Pinheiro et al., 2017; Pohlert and Pohlert, 2018; Ramsay et al., 2014; Therneau et al., 2023).

3. Results

3.1. Temporal variation of MC-producing MAC organisms' abundance (mcyE gene)

The abundance of MC-producing MAC organisms increased over time to saturation in a similar pattern in all treatments except for the 25-salinity treatment, where the abundance of MC-producing MAC organisms decreased during the first 18 h of incubation and then increased to values similar to the remaining treatments (Fig. 1). It was observed that the temporal dynamics of the organisms included a stabilization stage

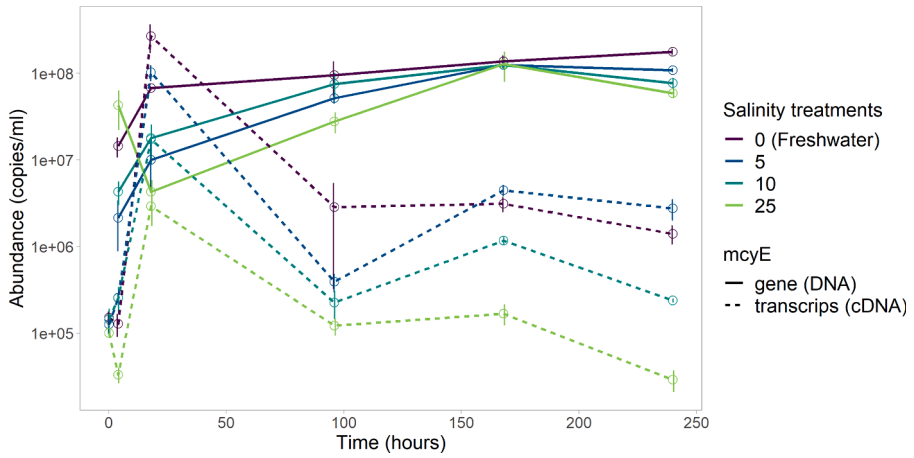

Fig. 1. Temporal variability (hours) of the abundance of toxic genotypes (*mcyE* gene ADN copies/ml, solid lines) and the abundance of *mcyE* transcripts (*mcyE* transcripts cDNA copies/ml, dash line) for each salinity treatments: 0 (Freshwater), 5, 10 and 25. Medians, maximum and minimum are shown. Y-axis in log10.

(during the first 18 h), a growth stage (from 18 h to 7 days) and a saturation stage (from day 7 to the end) (Fig. 1). During the growth phase (from 18 h to 7 days) a significant increase in the abundance of MC-producing MAC organisms was detected in all treatments (LR = 5.50 to 10.62, $p < 0.05$). A negative effect of salinity in MC-producing MAC abundance at the end of the trial (day = 10) was observed, with lower abundance at the larger salinities (10 and 25: LR = 10.23 and 11.25 respectively, $p < 0.05$).

3.2. Temporal variation of mcy transcription (mcyE gene transcripts abundance)

Transcript abundance behaved differently from *mcyE* gene abundance, reaching higher values at the beginning, and decreasing towards the end of incubation. A negative effect of salinity 10 and 25 was observed compared to the control treatment (LR = 5.75 and 6.61 respectively, $p < 0.05$). During the first hours of incubation, the abundance of *mcyE* gene transcripts increased in the freshwater control and, in 5 and 10 salinity treatments, while decreased in the 25-salinity treatment. After that, the abundance of *mcyE* transcripts increased both in the freshwater control and in all salinity treatments, reaching a maximum after 18hs of incubation. At the end of the experiment (10 days, 240 hs), higher transcript abundances were observed at salinity 0 and 5, and lower transcript abundances at 10 and 25 (Fig. 1). The abundance of *mcyE* gene transcripts was not associated with *mcyE* gene abundance ($r_s = 0.20$, $p > 0.05$).

3.3. Microcystin (MC) production

The four MC variants (LR, YR, RR and WR) were detected in all treatments. Also, the sum of concentration of all MC variants was positively associated with *mcyE* gene expression levels ($r_s = 0.48$, $p < 0.05$).

The MC-LR presented the highest concentration values across all treatments (range: 23.57 - 5648.03 µg/L) and the variant showing the lowest concentration range was MC-YR (range: 0.98 - 76.68 µg/L), while the MC-RR and MC-WR variants showed intermediate ranges (2.97 to 1119.06 µg/L and 0.61 to 214.18 µg/L, respectively; Fig. 2). The MC variants YR, RR and LR correlated positively and significantly with each other (r_s = between 0.64 and 0.93, $p < 0.05$).

The MC variants significantly differed in the final time between freshwater and 10 and 25 treatments (LR = 12.28 and 4.21 respectively, $p < 0.05$). In the salinity 10 treatment, the concentration of MC-RR increased and MC-WR decreased in relation to the freshwater treatment (LR = 13.03 and 7.42 respectively, $p < 0.05$). Concentration of MC-YR and MC-LR variants decreased in the salinity 25 treatment compared to the freshwater treatment (LR = 5.56 and 10.31 respectively, $p < 0.05$).

3.4. Selection of MC-producing MAC ecotypes

The *m*CART presented four-leaf whose error was significantly lower than the one calculated by random permutation (LR = 8.32, $p < 0.05$). The first threshold value for salinity selected by the model was 2.5, then

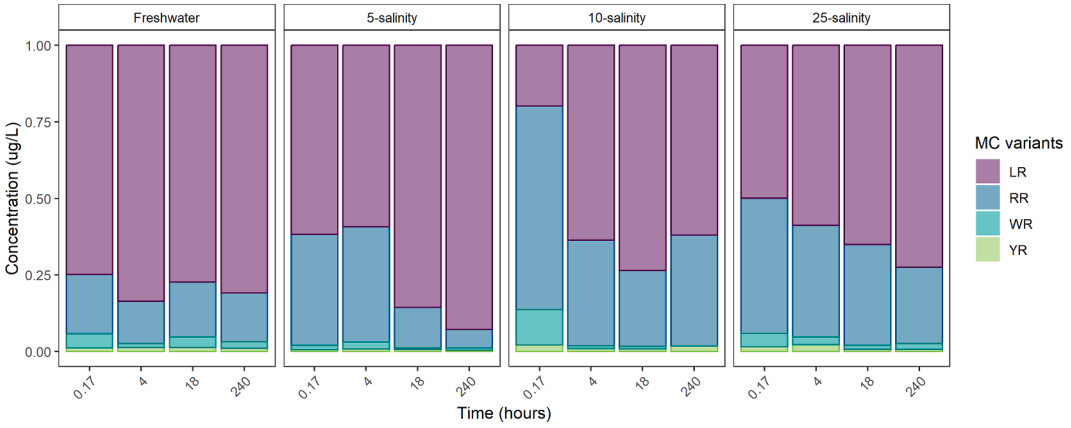

Fig. 2. Temporal variability (hours) of microcystins (MC) variants concentration (µg/L) normalized to 0–1: LR, RR, WR and YR, for each treatment: Freshwater (0-salinity), 5-salinity, 10-salinity and 25-salinity.

7.5 and finally a value of 17.5 was selected (Fig. 3). This analysis showed that the ecotype composition of MC-producing MAC in the control freshwater treatment did not change during the experiment. On the other hand, MC-producing MAC ecotypes community clearly differed between the different salinity treatments at the end of the experiment. Based on the *mcyJ* HRMA and *m*CART analyses, three different ecotypes of MC-producing MAC adapted to different salinities dominated each treatment (Fig. 3).

3.5. MC-producing MAC community composition

A total of 72 ASVs of the *mcyJ* gene affiliated to MC-producing MAC were detected. At the beginning of the experiment ASV richness, Shannon and evenness index did not show significant differences between freshwater control and salinity treatments (richness: mean = 14, SD = 1.7; Shannon: mean = 0.80, SD = 0.074; evenness: mean = 0.30, SD = 0.031) (LRT, $p > 0{,}05$). At the end of the experiment, the Shannon diversity in the salinity 10 treatment presented the highest value (mean = 1.08, SD = 0.10) (LR = 3.71 to 5.17, $p < 0{,}05$). Based on the phylogenetic tree constructed from the 72 *mcyJ* ASVs, five clusters could be differentiated (Fig. 4A).

Only one ASV (ASV2) was present in all treatments, which showed 98.84 % Blast identity with *mcyJ* sequences of an uncultured *Microcystis* from Lake Taihu, China (Accession number KC435508.1). Eleven out of 72 ASVs showed significant differences in their relative abundance between treatments ($\log_2$FC = −12.87 to 30, $p < 0.05$; Figure 4B). Besides, several ASV were found to be selected by salinity. Six ASVs (ASV1, ASV4, ASV7, ASV11, ASV24 and ASV34) increased their abundance in the salt treatments (5, 10 and 25 salinity) (Figure 4B). ASV35 was detected in high abundance only in salinity-10 treatment, while another ASV28 increased its abundance in high salinity treatments (10 and 25 salinity) (Figure 4B). Two ASVs (ASV10 and ASV16), which were present in the freshwater inoculum, significantly decreased their abundance in freshwater and salinity 5 at the end of the experiment, while became undetectable at higher salinities (Figure 4B). The ASV25 showed a general significant increase, suggesting that they could be broad range ecotypes. From the phylogenetic tree calculated using the ASV sequences showing differential abundance between treatments, we found two clear sequence clusters of *mcyJ* genotypes belonging to salinity-tolerant ecotypes (Figure 4C). The first one includes ASV1, ASV4, ASV7 and ASV34, and the second salinity-tolerant cluster includes sequences from ecotypes that appeared in freshwater and all salinities except 10 (ASV24, ASV25 and ASV35).

Summarizing, at the end of the experiment different ASV (corresponding to different *mcyJ* genotypes) were selected in the different treatments, strongly indicating that these sequences correspond to the different ecotypes found by HRMA-*m*CART.

4. Discussion

In this work, we found that the MAC freshwater community is composed of rich diversity of microcystin-producing ecotypes with the potential to survive and grow under different salinity conditions. These salinity-tolerant ecotypes were also identified by *mcyJ* amplicon sequencing and showed patterns of microcystin production that were different from their freshwater counterparts. Interestingly, the MAC community response observed was related to the speed of the processes involved. Physiological changes (e.g., *mcy* gene expression) of the organisms occurred within hours to a few days of exposing the communities to the environmental changes, while community composition changes occurred more slowly over a few to several days.

This is consistent with the observation of the transfer of cyanobacteria from freshwater ecosystem to marine waters (Kruk et al., 2021; Preece et al., 2017; Reignier et al., 2023). Freshwater cyanobacterial blooms in brackish waters have been increasing in recent years as a result of anthropogenic activities (Kruk et al., 2021; O'Neil et al., 2012; Paerl et al., 2018; Rigosi et al., 2014). *Microcystis* is one of the most frequent cyanobacteria to form blooms in both freshwater and brackish eutrophic ecosystems (Otten et al., 2017). In previous studies we detected species of *Microcystis* genus in the Uruguay river – Río de la Plata estuarine gradient, even at salinities as higher as 32 (Kruk et al., 2021). Moreover, in the same system we detected different populations

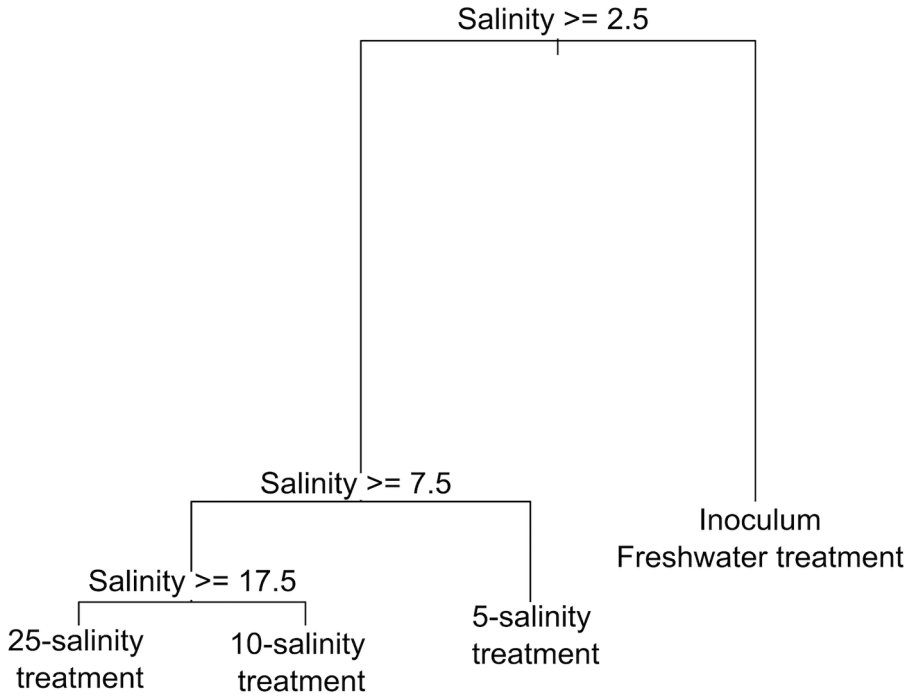

Fig. 3. Ecotypes defined by the multivariate regression tree (*m*CART). Multivariate regression tree showing the salinity values explaining the profile diversity of toxic genotypes. Salinity threshold values are shown at each node. At the end of each branch, the end of the experiment (Tf = 10 days) of each treatment (Freshwater 0-salinity, 5-salinity, 10-salinity and 25-salinity) and the beginning (T0) inoculum are shown.

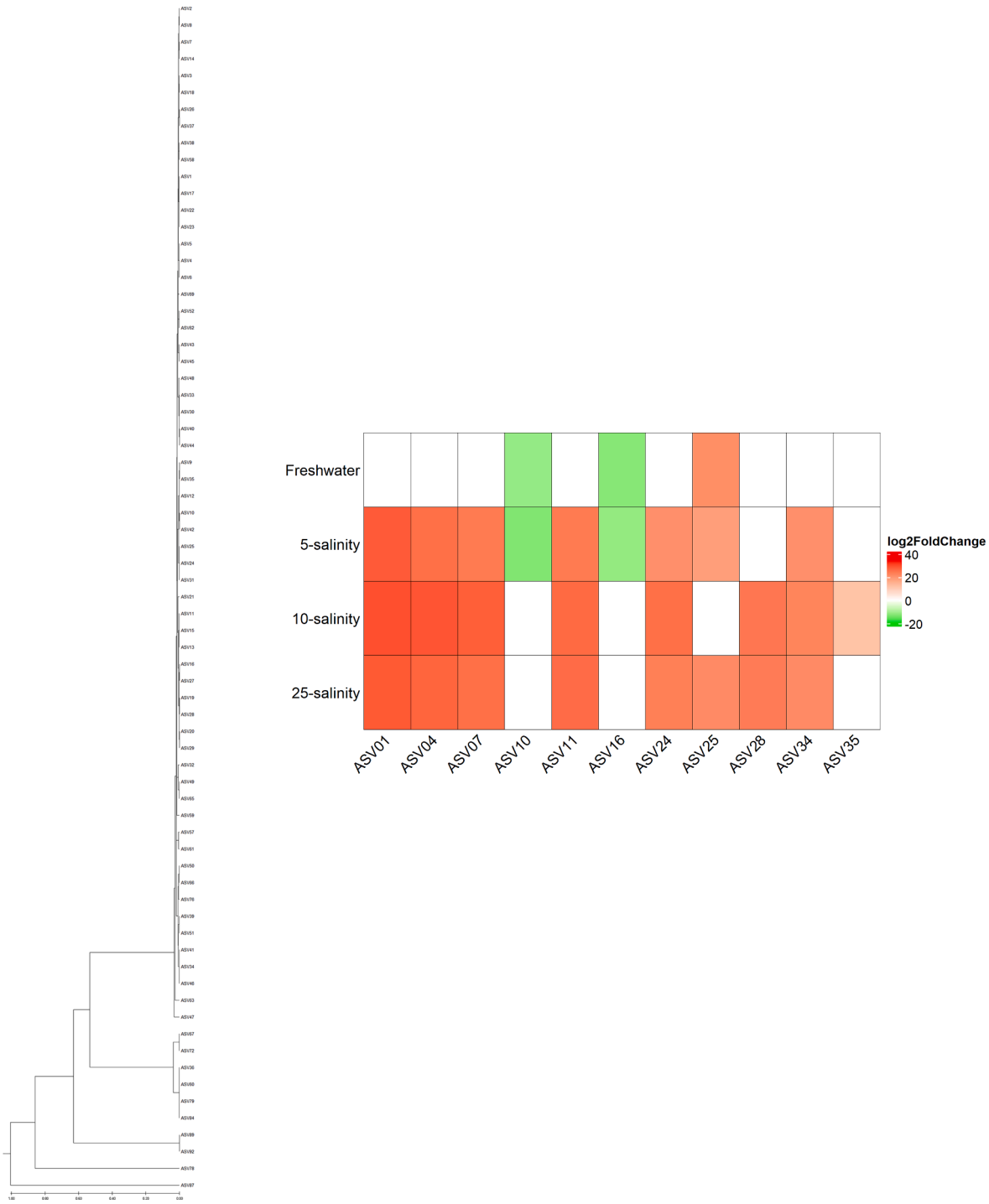

Fig. 4. : *mcyJ* ASVs showing significant differences in abundance between treatments. In A) UPGMA phylogenetic tree of the 72 *mcyJ* ASV. B) heatmap showing differential ASVs abundance between the inoculum (T0) and the end of the experiment (Tf = 10 days) for each treatment (Freshwater 0-salinity, 5-salinity, 10-salinity and 25-salinity). These 11 ASVs showed significant differences between treatments ($log_2FC = -12.87$ to 30, $p < 0.05$). Values of $log_2FoldChange > 0$ means that these ASV increase its abundance significantly compared to the inoculum (T0). Values of $log_2FoldChange < 0$ means that these ASV decrease its abundance significantly compared to the inoculum (T0). C) UPGMA phylogenetic tree based on *mcyJ* ASV sequences showing the sequences belonging to salinity-tolerant ecotypes (dark blue), those that were only abundant in the initial freshwater inoculum (white) and a wide salinity range ecotype increasing at all treatments except 10-salinity (orange).

of microcystin-producing *Microcystis* differing in their environmental preferences of temperature, salinity, and turbidity (Martínez de la Escalera et al., 2022).

4.1. Effect of salinity on the abundance of MAC toxic genotypes

Present results support previous observation of positive growth rates until salinities of 10, then a stagnant period and a decrease in growth and abundance above 30. After 18 h of exposure to salt, the treatments

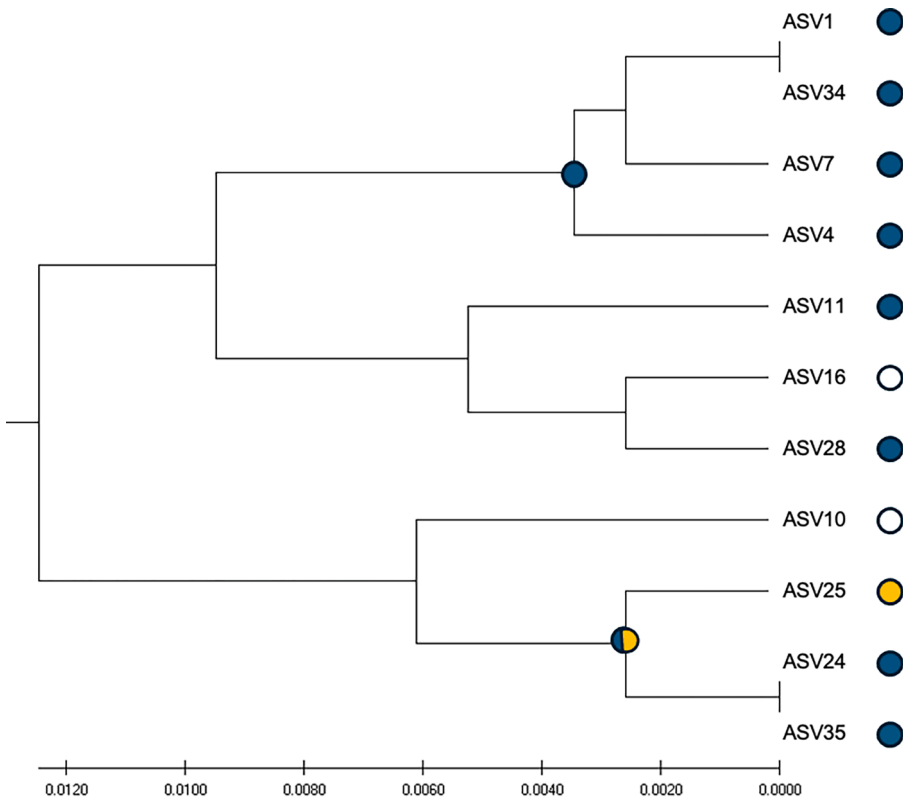

Fig. 4. (*continued*).

above 10 in salinity affected negatively the abundance of MC-producing MAC organisms. Ross et al. (2019) reported positive growth rates at salinities 3 and 7 of the *M. aeruginosa* strain (LB 2385) (Ross et al., 2019). However, at salinity 10 cell abundance remained constant for 11 days of incubation (Ross et al., 2019). On the other hand, Qiu et al. (2022) using *M. aeruginosa* strain FACHB-905 evaluated the effect of different salinity levels, between 4 and 15 ppt, and found that at lower salinities (4 and 7 ppt), the growth rate increases significantly compared with the control (1 ppt) (Qiu et al., 2022). At 10 ppt salinity cell density increases after seven days of incubation, and at 15 ppt salinity the growth rate decreased. These results indicated that low salinities promote the growth of some *M. aeruginosa* strains and that they can tolerate and adapt to salinity 10. However, it has been proposed that at higher salinity (~15 ppt) *M. aeruginosa* strains could not survived (Qiu et al., 2022). Present results not only showed that MC-producing MAC organisms can survive at higher salinity concentrations (25), but also that they can grow under these conditions, being the unique study we are aware of evaluating MAC growth under this condition in laboratory experiments.

Previous reports suggesting decrease above salinity 20 can be attributed to the fact that we used naturally occurring, colonial MAC, while most of the reported experiments were generally carried out on cultivated strains that are usually single-celled organisms (Des Aulnois et al., 2020; Qiu et al., 2022; Ross et al., 2019; Tonk et al., 2007). In natural environments, *Microcystis* spp. organisms are found forming colonies with their cells embedded in a mucilage having a diverse microbiota (Komárek J., 2002; Piccini et al., 2024; Reignier et al., 2023) we termed the holobiont, which provides protection to the cells from the external environment (Liu et al., 2019). Thus, results from single-cell cultures could lead to an underestimation of the ability these organisms to tolerate higher salinities in a natural environment, where they are protected by a thick mucilage (Kruk et al., 2021; Piccini et al., 2024; Reignier et al., 2023).

In field studies, Robson & Hamilton (2003) found that the maximum growth rate (μmax = 1.2 days^{-1}) of *Microcystis* occurs at salinity 4 and above salinity 25 the growth rate becomes negative (Robson and Hamilton, 2003). Orr et al. (2004), experimenting with samples obtained from a *Microcystis* bloom, observed that the bloom showed tolerance up to 10 salinity and a half-life of 0.33 days (μ = −2.1 days^{-1}) at 23 salinity (Orr et al., 2004). These authors conclude that the *Microcystis* population used for the experiment was not a homogeneous monoculture, but a mixture of genotypes with different salinity tolerance and toxicity (Orr et al., 2004). As in the present work, these studies used natural *Microcystis* communities composed of a mixture of genotypes, able to tolerate salinities close to 25, a higher value than reported previous studies. This implies that populations with a MC-producing genotype capable of tolerating estuarine salinities for several days are feasible in nature.

4.2. *Effect of salinity on the transcription*

The expression of *mcyE* genes involved in microcystin synthesis affected physiological functions of the organism. Few studies have evaluated the effect of salinity on *mcy* gene expression. Since the concentration levels of salinity evaluated in this work differ greatly with literature studies, and that the literature experiments are carried out with isolates, therefore the results reported here are not concordant in some cases. Chen et al. (2015) evaluated *mcyD* gene expression in *Microcystis* cultures treated with different NaCl concentrations (from 0 to 0.53 salinity) during 12 days of incubation (Chen et al., 2015). As a result, they found that both NaCl treatment and incubation time negatively affected the gene expression. On the other hand, Ross et al. (2019) conducted experiments with cultures of a *M. aeruginosa* strain exposed to different salinities: 0, 3, 7 and 10 for 11 days (Ross et al., 2019). Among other variables, they quantified the level of *mcyD* gene expression at different times, which was not significantly affected by either treatment or incubation time, however after seven days of incubation they detected expression levels close to zero in all treatments. In this sense, salinity

may inhibit MC synthesis, or *mcy* gene activation may be related to other environmental conditions such as elevated temperatures or nitrogen concentration (Kramer, 2018; Ross et al., 2019).

The study by Martin-Luna et al. (2015) evaluated the effect of two salinities (6 and 9) on *mcyD* gene expression at different incubation times and found that at both salinities, expression decreased after four hours compared to the control treatment (Martín-Luna et al., 2015). Then at 48 and 72 h of incubation, *mcyD* gene expression increased, the authors attribute this increase to the acclimatization of the culture after osmotic stress caused by salinity (Martín-Luna et al., 2015). In the present study, an increase in *mcyE* gene expression was observed at 18 h incubation in all treatments including the freshwater control, a possible explanation for this phenomenon could be due to the acclimatization of the MC-producing MAC community to the experimental environment, where MC synthesis could play a role (Schatz et al., 2007). After 18 h of incubation, *mcyE* transcription decreased in all treatments, which has been reported previously (Martín-Luna et al., 2015; Yancey et al., 2022). In this sense, the expression of the *mcyE* gene decreased but was never inhibited, so the possibility of MC synthesis cannot be ruled out.

4.3. Effect of salinity on the production of MC variants

There are few studies assessing the effect of salinity on the concentration of different MC variants and in general only total MC are quantified or only the MC-LR variant is determined (Chen et al., 2015; des Aulnois et al., 2019; Martín-Luna et al., 2015). At salinities between 4 and 7, it has been observed that the MC concentration per cell remains constant after 11 days of incubation, therefore, the total MC increases due to cell growth (Qiu et al., 2022). Also, a negative effect of salinity on the concentration of microcystin (MC total and MC-LR) has been found when salinity exceeds values of 9 - 10 (Martín-Luna et al., 2015; Qiu et al., 2022). In another study using cultures of a *Microcystis aeruginosa* strain exposed to three salinities (3, 7 and 10), it was observed that at 10 a significant increase in the extracellular concentration of MC was induced (Ross et al., 2019). In this context, the authors suggest the existence of a salinity threshold (~10) above which MC would be released into the environment. The same authors found that extracellular MC concentration correlated negatively with cell abundance, confirming the hypothesis of MC release due to cell lysis (Ross et al., 2019). The results from present study are in agreement with the positive correlation between intra and extracellular toxin levels previously reported and the decreases of MC concentration with increasing salinity.

The peak of variants of MC production at salinity of 10 could be due to changes in the MC-producing MAC community composition, favouring the populations that produce different variants of MC. The concept of chemotype to classify *Microcystis* populations based on their oligopeptide synthesis profile, which includes MC variants (Agha et al., 2014; Welker et al., 2007) can be useful. Lezcano et al. (2019) studied the relationship between MC variants and *mcyB* gene variability using strains and colonies isolated of *Microcystis* from different environments (Lezcano et al., 2019). They observed a low correlation between *mcyB* genotypes and chemotypes, indicating that the structural diversity of MC was not only explained by the genetic variability of *mcyB* gene (Kurmayer e 417 t al., 2002; Lezcano et al., 2019), and suggested that environmental conditions determine the diversity of MC variants produced, as well as post-transcriptional modifications (Agha et al., 2014; Lezcano et al., 2019). On the other hand, Mikalsen et al. (2003) found a strong correlation between MC variants and the genetic variability of *mcyB1* module, resulting from recombination between modules, such as between *mcyB1* with *mcyC* (Mikalsen et al., 2003). Our results showed that the MC variants production pattern is related to the selection of salinity-resistant populations of MC-producing MAC, which could be also associated to the occurrence of recombination between *mcy* modules induced by salinity. Osmotic stress could induce the expression of other *mcy* genes generating genetic variants and therefore the synthesis of different variants of MC (Des Aulnois et al., 2020; des Aulnois et al., 2019).

4.4. Effects of salinity on MAC ecotype selection

Estuarine salinities result in changes in the MC-producing MAC community, inducing growth or dominance of ecotypes adapted to different salinities. Specifically, 10 and 25-salinity treatments induced more similarity changes in MAC ecotypes than to 5-salinity and freshwater treatments. Likewise, and in agreement with previous studies in the same ecosystem presence of different MAC ecotypes were found in brackish water with salinity ranges from 17 to 30 (Kruk et al., 2021; Martínez de la Escalera et al., 2017).

To gain further knowledge of the ecotypes defined by the HRMA-*m*CART method, each ecotype was sequenced and the composition of ASVs was obtained. In this sense, it was determined how diverse the ecotypes are according to the composition of ASVs, whether there is dominance or mixture of ASVs. A decrease in the ASV diversity (Shannon index) was found at salinities of 10 and 25. Moreover, some ASVs prefer salinities higher than 0 in which they can grow and increase their relative abundance according to salinity tolerance. These results indicate that the previously defined ecotypes are composed of a mixture of ASVs that change their abundance according to salinity. Likewise, these ecotypes adapted to the different salinity conditions produce different MC profiles. Ecotypes of freshwater and 5-salinity present the same MC profiles, dominated by the MC-LR variant. Ecotype found at 10-salinity produced more MC-RR and MC-YR variants, while ecotype found at 25-salinity is characterized by synthesizing more MC-RR variant. The findings indicate that there would be MC-producing MAC populations capable of tolerating estuarine conditions for at least 10 days and that they are different ecotypes from those found in freshwater.

An important mechanism that gives bacteria the ability to tolerate high salinities is the accumulation of low molecular weight osmoprotectant molecules called compatible solutes (Le Rudulier et al., 1984). Through the accumulation of compatible solutes, the water potential inside the cells decreases and thus they can avoid the osmotic pressure difference and dehydration (Roberts, 2005). In cyanobacteria, the synthesis and accumulation of various osmoprotective solutes, mainly sucrose and α-glucosylglycerol, has been reported (Hagemann, 2011). However, within the genus *Microcystis* genes involved in sucrose synthesis have been detected so far in only three strains (des Aulnois et al., 2019; Sandrini et al., 2015; Tanabe et al., 2019, 2018). In this regard, Tanabe et al. (2018) have shown that these strains have acquired the genes for sucrose synthesis through recent horizontal gene transfer events (Tanabe et al., 2018). Melero-Jimenez et al. (2019) propose that adaptation of *Microcystis* spp. to increased salinity could also occur by spontaneous mutations (Melero-Jiménez et al., 2019). Furthermore, des Aulnois et al. (2019) reported differences in salinity tolerance between two strains of *Microcystis aeruginosa* (overexpression of polyunsaturated lipids, trehalose, sucrose, glycine betaine and proline) in response to an increase in salinity, suggesting intraspecific variability (des Aulnois et al., 2019). The evidence suggests that, in the present experiment, the shift from a freshwater community to conditions of higher osmotic pressure (salinities 10 and 25) would have induced changes in community structure. These changes could be due either to the increased growth of MAC populations carrying genes responsible for osmoprotective metabolites synthesis that allowed them to survive and show some activity during the time of the experiment, or due to the proliferation of populations carrying spontaneous mutations. In addition, the mucilage surrounding MAC colonies, which sustain a rich and diverse microbiome (Croci et al., 2025), has been shown to increases its thickness in high salinity conditions (Sampognaro et al., 2020). The presence of a thick mucilage densely populated by bacteria would buffer the impact of changes in salinity by promoting a salinity gradient and avoiding abrupt exposure of the cells to salt (Seymour et al., 2017).

The freshwater MAC community is composed of ecotypes with different fitness. When they are confronted with different salinity

conditions, the dominant ecotypes change by those having the ability to synthesise compatible solutes. This salinity-tolerant ecotypes show different profiles of microcystin variants. Our conceptual model could explain why MAC, considered freshwater organisms, can develop and generate toxicity in estuarine environments where salinity reaches values of 25. Further investigation should be carried out to know how this change in MC variants synthesis can affect human and ecosystem health.

CRediT authorship contribution statement

Gabriela Martínez de la Escalera: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Angel M. Segura:** Writing – review & editing, Methodology, Conceptualization. **Carla Kruk:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Carolina González:** Methodology. **Claudia Piccini:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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