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ABSTRACT
Recent reports predict increases in harmful cyanobacteria in water systems worldwide due to climatic and environmental changes, which would compromise water quality and public health. Among abiotic changes, higher salinities are expected to promote the growth of certain harmful species, such as *Planktothrix agardhii*, which forms blooms in brackish waters. Since *P. agardhii* is a common producer of cyanotoxin, we investigated the growth and tolerance of this species when exposed *in vitro* to a range of salinities, while assessing variations in its microcystin diversity and production in batch cultures during a time-frame experiment spanning 18 days. The study revealed salt acclimation of the brackish *P. agardhii*, which continued to produce microcystins in salty cultures, while maintaining its growth capacity at low to medium NaCl (ranging from 0 to 7.5 g l^{-1}). With higher concentrations (10 to 15 g l^{-1}) significantly less growth occurred, corresponding to the shortening of cyanobacterial filaments, which nevertheless maintained their metabolic functions, as revealed by the high intensity of chlorophyll auto-fluorescence and persistent microcystin production. These findings showed that moderate to high salt levels do not inhibit microcystin production by *P. agardhii*, at least for several weeks. This raises questions concerning the persistence of harmful cyanobacteria in shallow water systems more exposed to evaporation and consequently to an increase in salinity in the future, as predicted by various climate models.

INTRODUCTION

The massive occurrence and proliferation of cyanobacteria worldwide are serious issues, as their bloom-forming ability impairs water quality (Twoney *et al.* 2002) in several ways, e.g. by increasing turbidity, reducing biodiversity, and eventual anoxia of the water column. In addition, some common species can produce various toxic metabolites, such as hepatotoxins and/or neurotoxins (Chorus & Bartram 1999). The most frequently found toxic metabolites in waterbodies, including brackish areas (Sivonen & Jones 1999), are hepatotoxic microcystins (MCs), which can affect all living organisms from ciliates to fish (Combes *et al.* 2013; Ressom *et al.* 1994) and threaten human health (Chorus & Bartram 1999; Pouria *et al.* 1998).

MCs are cyclic heptapeptides that strongly (and irreversibly) inhibit serine-threonine protein phosphatases type 1 and 2A (Pearson *et al.* 2010), leading to cell disruption and even death (Djediat *et al.* 2011). MCs have many structural variations, depending on the L-amino acid at positions 2 and 4, respectively, from the overall MC architecture; to date, over 200 MC variants have been identified (Spoof & Catherine 2017) with different cytotoxic potentials depending on the MC variant tested (Shimizu *et al.* 2014). While studies concerning biosynthesis and chemical processes involving MCs

are ongoing, the forces underlying toxin production, i.e. the ecological and biological functions of MCs for the producing cells, remain elusive and mostly contradictory (Babica *et al.* 2006). Various hypotheses for the possible roles of MCs have been proposed, including allelopathic effects (Leao *et al.* 2009), grazer defences, and adaptation for light harvesting (Kaebernick & Neilan 2001). Recent findings suggest involvement in intracellular processes and primary metabolism (Zilliges *et al.* 2011) while excluding an essential role for growth (Hesse & Kohl 2001). Moreover, one of the most challenging questions is how the environment influences changes in MC concentration during cyanobacterial blooms. Indeed, a better understanding of environmental factors triggering and/or involving variations in MC production and changes in the composition of toxic vs non-toxic cells is very much required to help predict potential health hazards.

Numerous studies have shown that certain environmental parameters may influence MC production in toxic cells, including (1) the prevalence of toxic clones vs non-toxic ones (Briand *et al.* 2005) during unfavourable conditions (Kurmayer *et al.* 2004), (2) increases in the level of MCs in toxic cells (Sivonen & Jones 1999), and (3) changes in MC variant composition (Pearson *et al.* 2010; Tonk *et al.* 2005).

Among the possible causal factors are nutrient concentration (Downing *et al.* 2005), temperature and light (Wiedner *et al.* 2003), iron abundance (Sevilla *et al.* 2008), and pH (Song *et al.* 1998). Much less is known regarding many other abiotic parameters such as hydrologic variability, water bioavailability and salinity oscillations (N'Dong *et al.* 2014). Besides, the results are largely inconsistent, as many factors, both abiotic and biotic, may act in synergy and affect the physiological state of the cells at different levels (Davis *et al.* 2009).

Recent reports tend to predict that climatic change will exacerbate the dominance of harmful cyanobacteria in aquatic ecosystems worldwide (Carey *et al.* 2012; Paerl & Paul 2012). Indeed, eutrophication exacerbated by human activity coupled with environmental changes, such as rising temperature and enhanced stratification of the water column, should trigger and increase the frequency, biomass, and duration of harmful cyanobacterial proliferation of specific species in waterbodies (Fastner *et al.* 1999; Hagemann 2011; Paerl & Otten 2013). With regards to global warming, different climate models forecast warmer surface waters, which will contribute to an increase in salinity due to evaporation in shallow water systems (Pearl & Huisman 2008). These ecosystems, including Mediterranean lakes and ponds, will be more impacted by stochastic episodes of gradual and repetitive increased salinity (due to higher periods of drought) rather than drastic salinity shocks (Nielsen *et al.* 2008). It is believed that oscillations in precipitation, including periods of intensive rainfall (i.e. floods) and increase in land runoff, might affect salinity levels of sensitive ecosystems such as estuarine areas, wetlands, lagoons and brackish areas (Cloern & Jassby 2012; Teikaki *et al.* 2019). Such events could affect the distribution of bloom-forming species from freshwater to coastal areas (Lehman *et al.* 2005; Reguero *et al.* 2015) – especially if they are able to tolerate moderate to high salt levels (Paerl & Otten 2013). Thus, rapid runoff, including toxic cyanobacterial transport, may contaminate and thus impair aquaculture, fisheries, and vegetation in downstream waters (Preece *et al.* 2017; Robson & Hamilton 2003).

Planktothrix agardhii (Gomont) Anagnostidis & Komàrek is one of the most common freshwater MC-producers in temperate areas (Chomerat *et al.* 2007) and has been reported to produce blooms in brackish waters (from 3 to 11 g l^{-1} NaCl; Rojo & Alvarez Cobelas 1994; Villena & Romo 2003). However, there are few data regarding the influence of salinity on MC production because these widespread MC-producing species are encountered mainly in freshwater. While *P. agardhii* can persist in brackish areas, it is important to investigate whether a rise in salinity affects MC production in *P. agardhii*, as meteorological models tend to predict increased salinisation in shallow Mediterranean areas due to climatic changes (Nielsen *et al.* 2008); however, experiments on salt stressors *in vivo* are lacking. Therefore, we investigated the response of a dominant cyanobacterial strain of *P. agardhii* originating from an oligohaline pond to a salinity gradient, aiming to determine (1) the influence of salinity of various concentrations on *P. agardhii* bloom development (growth and morphological changes) in batch cultures over a period of 18–20 days; and, (2) the influence of salinity on effective MC production by *P. agardhii*.

MATERIAL AND METHODS

Strain isolation and culture conditions

The strain of *P. agardhii* used in this study was collected from Olivier Pond, near Istres, just outside the city of Marseille (in the south of France; 43°30.46N and 4°59.17E). Olivier Pond is a eutrophic, oligohaline waterbody (average salinity 3 g l^{-1}), area of *c.* 225 ha and maximum depth of 10 m. *P. agardhii* is the dominant cyanobacterium throughout the year (Vergalli 2013). Water samples were collected at the pond surface during a *P. agardhii* bloom to isolate filaments. After isolating a single filament (Rippka 1988), five clones were purified and only one was microcystin positive. The MC-producing strain was maintained for several years under non-axenic conditions in Z8 liquid medium (Kotai 1972), at 22 °C, using a light:dark cycle of 14:10 h with constantly bubbling air to ensure homogeneity and to provide sufficient inorganic carbon. The 'Brack' strain was assigned to *P. agardhii*, according to morphological criteria as per Komárek & Anagnostidis (2005) and maintained in the Paris Museum Collection (PMC-MNHN) under reference PMC1014.18.

Experimental setup

NaCl was added to Z8 medium to obtain salinities of 3, 5, 7.5, 10, 12, and 15 g l^{-1} and transferred into 250-ml Erlenmeyer flasks. Five replicates of each salinity treatment were established and checked with a conductivity metre (WTW LF330, Weilheim, Germany). The control ($n = 3$) corresponded to a culture maintained in Z8 medium only (NaCl = 0 g l^{-1}). Each flask was then inoculated with *P. agardhii* PMC1014.18 pre-culture in exponential growth phase and adjusted to obtain an initial $OD_{750} = 0.1$ measured using a Shimadzu UV-1700 (Kyoto, Japan) spectrophotometer. Batch cultures were maintained in growth chambers under the same experimental conditions described above. Flasks were regularly replaced to homogenize the light exposure in the growth chambers.

Growth measurements

The growth kinetics of *P. agardhii* PMC1014.18 strain cultures were monitored by measuring optical density at 750 nm. Growth rate (μ) was calculated as follows:

$$\mu = \ln x2 - \ln x1 / t2 - t1$$

where $t1$ and $t2$ are time [t: start of the exponential phase; t2 (day): end of the experiment]; and $x2$ and $x1$ correspond to biomass (expressed in OD values) at time t (with t2 > t1).

Biovolumes (BV) were assessed in μm^3 based on the cylindrical shape of filaments, according to Sun & Liu (2003):

$$BV = 0.5\Pi \, lw$$

where l is filament length (μm) and w is filament width (μm). A mean of 20 filaments was randomly measured in a counting chamber, using a micro-scale with a Nikon Labphoto2 microscope (Tokyo, Japan).

Fluorescence microscopy was performed with a Zeiss Primo Star microscope equipped with an AxioCam IcC1

camera. Epifluorescence images were recorded with specific filter (CY3) for chlorophyll *a* and acquired with the same time exposure setting (AxioVision LE software).

Characterisation of MC variants

Three ml of culture (OD = 0.6) of the PMC1014.18 strain was centrifuged at $4000 \times g$ for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 ml of methanol:water (90:10, v:v) followed by four pulses of sonication on ice for 30 sec. The mixture was centrifuged at $8000 \times g$ for 15 minutes at 4 °C. Supernatant was collected, filtered (GF/C 1.2 μm pore size x 2.1-mm diameter), and evaporated using a speed-vac concentrator at 40 °C. The extract was then resuspended with 100 μl water with 0.1% formic acid and centrifuged at $4000 \times g$ for 5 min at 4 °C. The supernatant was saved and transferred into a 200-μl conical insert and directly injected into a LC/ESI–MS (Liquid Chromatography/ Electrospray Ionisation–Mass Spectrometry) system.

LC/ESI–MS and LC/ESI–MS/MS experiments were performed on a liquid chromatograph (LC; UltiMate 3000®, Dionex, San Diego, California, USA) coupled to a quadrupole-time of flight (Q-TOF) hybrid mass spectrometer (Pulsar, Applied Biosystems-Foster City, USA) equipped with an electrospray ionization source (ESI). Chromatographic separation was conducted on an ACE3-C18 reverse-phase column (100 mm × 1 mm × 3 μm). Mobile phases contained MilliQ water 0.1% (v/v) + formic acid (A), and acetonitrile 0.07% + formic acid (v/v) (B). LC separation was achieved at a flow rate of 40 μl min^{-1} using a gradient elution from 10% to 30% of solvent B in 5 min; then, from 30% to 70% B in 17 min; held at 70% B for 5 min; returned from 70% to 10% B in 3 min; and held at 10% B for 15 min. The MS was operated with an ESI source in positive ion mode. For mass spectra, capillary voltage was set to 2500 V with a declustering potential of 20 V. Full scan mass spectra were performed from 100 to 1500 m/z for 1 sec per scan in continuum mode. Fragmentation spectra were obtained in automatic mode using nitrogen as a collision gas, with collision energy automatically determined by the software according to mass-to-charge ratio (m/z) values.

MS/MS spectra were analysed manually for highlighted spectra which contained the fragment ion (m/z = 135.1) characteristic of MC fragmentation. All other ion fragments present on the fragmentation spectra were used to elucidate the structure of the MCs. LC/ESI-MS data were processed using BioAnalyst 1.1. Molecular weight distribution of species (ranging from 100 Da to 1500 Da) observed in each sample was generated using the LC-MS reconstruct option. As the signals observed for both MC standards were close, the proportion of each variant was calculated by comparing the peak area corresponding to a given MC variant to the total peak area of all MC variants in a sample.

MC concentration

Microcystin concentration (MC) was determined by Enzyme Linked Immuno-Sorbent Assay (ELISA) using an MC-ADDA ELISA kit (Abraxis LLC-Warminster, Pennsylvania, USA). ELISA tests were applied to supernatants from the cultures previously disrupted by sonication on ice (2 pulses of 1 min, maximum speed) according to a previous protocol (Combes *et al.* 2013). Mixture was then centrifuged at $8000 \times g$ for 15 min at 4 °C. Supernatant was collected and cleaned, according to instructions specific to seawater samples ['Technical bulletin for microcystins in brackish and seawaters samples' (Abraxis)] to avoid certain matrix effects and potential salt interference. Measurements were performed in duplicate on different samples exposed to each salinity treatment on days 0, 2, 6, 8, 10 and 18. The limit of detection was *c.* 0.26 ppb (μg l^{-1}). MC content was expressed in μg l^{-1} equivalent to MC-LR. Due to the positive correlation between biovolume (i.e. quantitative unit) and biomass (i.e. OD$_{750}$ values; r2 = 0.80; *n* = 40, Fig. S2), MC content was converted and normalised per biomass (OD$_{750}$) as a proxy of MC quota to compare different MC patterns over time by minimising the growth factor.

Statistical analyses

Growth curves were fitted with the best trend approximation from absorbance measurements over time, following the equation of Kahm *et al.* (2010):

$$y(t) = A/1 + \exp(4\mu/A(\lambda - t) + 2)$$

where A is the asymptote of the curve, an estimation of maximal population density reached during the life cycle; μ is maximum slope of the growth curve and characterises exponential growth phase (days 6 to 18); λ is lag phase period of growth (i.e. day 2).

Two parameters (growth rate and maximal density), obtained from the logistic curves generated with the 'grofit' package (Kahm *et al.* 2010), were used to compare growth under various salinities. Normality and homoscedasticity were systematically checked using the Shapiro-Wilk and the Fligner-Killeen tests, respectively. Consequently, significant differences in growth (growth rate, biomass and filament length) between salinities were identified by one-way analysis of variance (ANOVA; *n* = 5) and Tukey's *post-hoc* test. Differences in MC concentrations were evaluated by Kruskal-Wallis test (among salinity treatments) and by Mann-Whitney U statistic when MC patterns were compared to the control. Pearson correlation coefficients were calculated between growth variables and MC concentrations. All statistical tests were carried out in the R-2.14.0 environment and Statview (Roth *et al.* 1995).

RESULTS

Cell growth and morphological changes induced by various NaCl concentrations

Optical density (OD$_{750}$) was recorded daily (from t0 to t18) to monitor culture growth and calculate growth rates (μmax). While the highest salinity (15 g l^{-1}) had a drastic effect on growth (Fig. 1), the PMC1014.18 strain persisted and grew in low to high salinity (from 3 to 12 g l^{-1}) over the 18-day experiment.
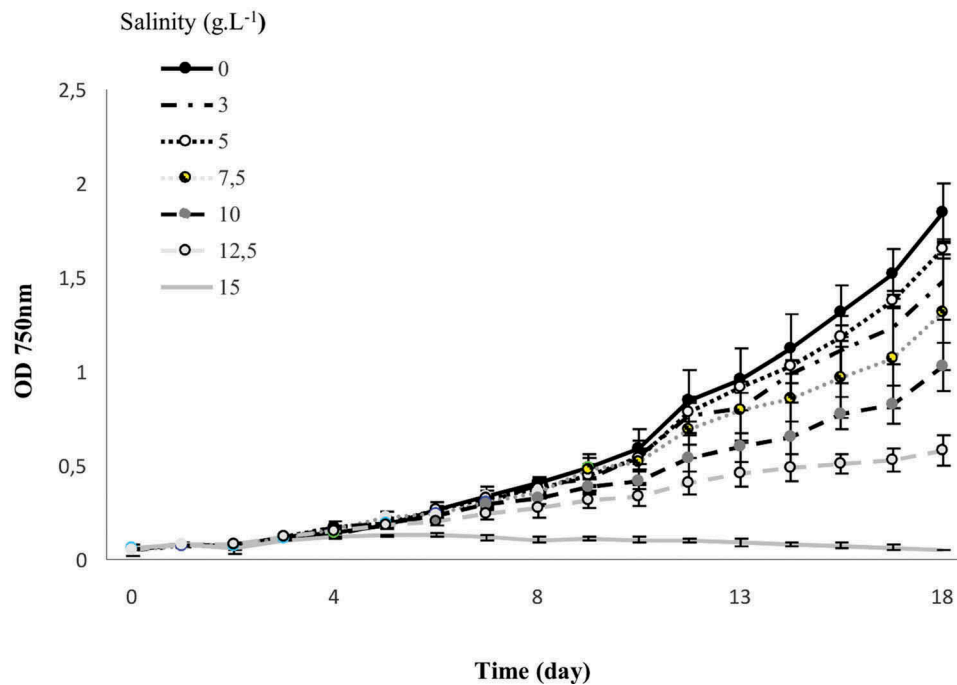
Fig 1. Growth dynamics of *Planktothrix agardhii* PMC1014.18 at different NaCl concentrations, obtained using OD750 values (n = 5, ± SD) from day 0 to day 18 (A) and fitted with the 'grofit' package.

Two growth profiles were distinguished: one included the control and the low salt concentrations and corresponded to a progressive increase in growth and a similar growth rate (ANOVA, $P > 0.05$); and another that corresponded to the high salt concentrations (7.5 to 12.5 g l^{-1}) that revealed a significant decrease in growth rate, and cell density (Table 1), especially at day 8.

Additionally, microscopic observations, performed every two days to estimate filament size, detected morphological changes in whole cells and filament lengths (Fig. 2). The physiological state of filaments was assessed by light and epifluorescent microscopy, taking intact morphology and chlorophyll autofluorescence intensity as indicators of survival (Fig. 3–10, S3–10). The decrease in chlorophyll content with increasing salinity was correlated with total biovolumes, i.e. following morphological changes, and to a reduction in filament size (Fig. 2). While filaments were *c.* 240 μm long at the beginning of the experiment (t0), morphological variation was noted as early as days 2 to 8 for moderate treatments (5 to

7.5 g l^{-1}), with a significant increase in filament length up to a maximum (521 μm vs the control; ANOVA, $P < 0.05$). During these morphological changes, all filaments had bright chlorophyll *a* fluorescence (Figs S5, S6) corresponding to the integrity of the cells and to photosynthetic pigments which led to the maintenance of primary metabolic functions after several days under moderate salinities.

Filament elongation was observed for cultures at 5 to 7.5 g l^{-1} NaCl between days 2 and 8 (ANOVA, $P < 0.01$), followed by reductions in length beyond day 10 (not significantly different from the control; Figs 2, S3–10). For high salinities (12.5 to 15 g l^{-1} NaCl) a significant reduction in filament length was detected after 14 days until the end of the experiment (day 20), with a mean length not exceeding 60 μm (day 20) vs the usual 350 μm for the control (Figs 2, 4, 5), 78). A remarkably high number of short fragments with only 5–10 cells were observed at 15 g l^{-1} NaCl by the end of the experiment (Figs 9, 10). Typical morphologies are shown in Figs 5, 8–10. Nevertheless, high intensity chlorophyll autofluorescence was still detected in short filaments even after 20 days of incubation (Figs 8, 10), suggesting the continued viability of most cells within the broken filaments.

Characterisation of MC profile in *P. agardhii* PMC1014.18

Characterization of the MC variant composition (Figs S1–S3) was performed by LC coupled to ESI mass spectrometry (LC/ESI–MS/MS) to identify the chemo-type profile of the strain grown under optimal conditions (i.e. the control), as MC diversity may exist within the same species (Shimizu *et al.* 2014; Tonk *et al.* 2005). Five MC variants (two major and
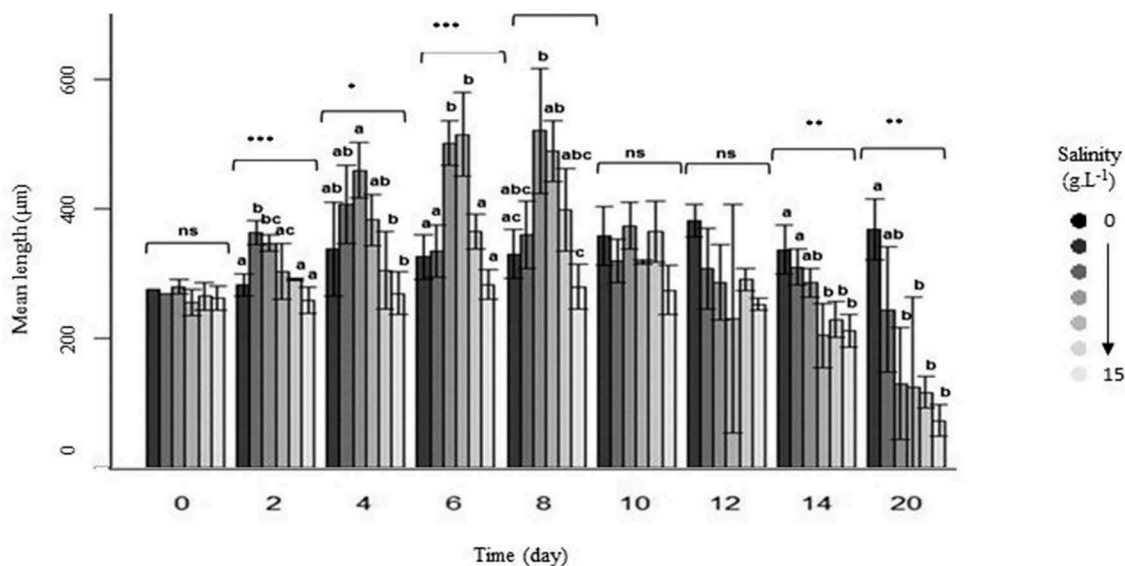
Table 1. Descriptive parameters, including the growth rate and cell density, were determined from growth curves fitted by the "grofit" model. The different letters mentioned above (a, b, c) indicate significant differences between the salinity treatments (Tukey's test, $P < 0.01$).

Salinity (g.L^{-1})	Growth rate μ(day^{-1})			Max. Density (10^3 cell/ml)		
0	0,25	±0,05	a	2,4	±0,1	a
3	0,19	±0,02	a	2,3	±0,4	b
5	0,20	±0,01	a	2,6	±0,2	a
7.5	0,15	±0,02	ab	2,3	±0,5	b
10	0,12	±0,02	ab	2,2	±0,6	bc
12.5	0,05	±0,01	b	0,9	±0,6	c
15	0	-		0,01	-	

Fig 2. Variations in filament length of *Planktothrix agardhii* PMC1014.18 for cultures grown at different NaCl concentrations. Means ± s (n = 60) with different letters (a, b, c) show significant differences between NaCl concentrations ($P < 0.05$, ANOVA, Tukey's post-hoc test). Asterisks (*) indicate differences from control (day = 0) sizes (NS = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).
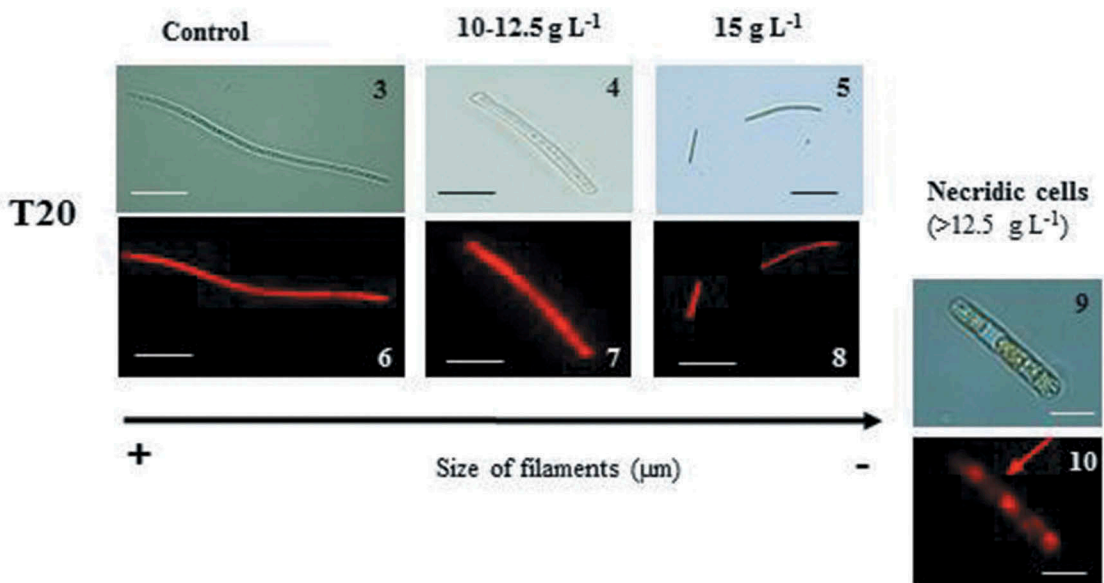
Figs 3–10 Bright field and epifluorescence microscopy of *Planktothrix agardhii* PMC1014.18 showing different sized filaments at day 20 (T20) based on chlorophyll autofluorescence (CY3 filter).

Figs 3–5. Bright field microscopy after 20 days.
Fig. 3. Filament size in control. Scale bar = 20 μm.
Fig. 4. Filament size at 10–12.5 g l^{-1}. Scale bar = 10 μm.
Fig. 5. Filament size at 15 g l^{-1} NaCl. Scale bar = 30 μm.
Figs 6–8. The same filaments as in Figs 3-5 with epifluorescence microscopy.
Fig. 6. Filament size in control. Scale bar = 20 μm;
Fig. 7: Filament size at 10–12.5 g l^{-1} Scale bar = 10 μm;
Fig. 8: Filament size at 15 g l^{-1} NaCl. Scale bar = 30 μm.
Fig. 9. Short fragment with necridic cells observed in bright field at >12.5 g l^{-1} . Scale bar = 10 μm.
Fig. 10. Short fragment with necridic cells observed with epifluorescence microscopy; red arrow indicates necridia (non-fluorescent cells). Scale bar = 10 μm.

three minor) were determined from the cultured PMC1014.18 strain (Table 2).

Based on Adda fragment signals (m/z 135.1), the MS/MS spectra, and the retention times of other ions (identical to MS standards and their MS/MS spectra), two major doubly-charged ions [M + 2H]$^{2+}$ (Table 2) were identified as [Asp3] MC-RR and [Asp3]MC-LR, respectively Together, these represent 93% of MCs present in this strain. Among the three minor ions, only one was clearly characterised as demethylated [Asp3]MC-HtyR (Table 2), while the doubly-

Table 2. LC-ESI-MS determination of individual MC-variants detected in the 'Brack' strain. The identification of the MC-variant corresponds to ions detected on the mass spectra (m/z) and retention time (RT) compared to the standards. The proportions for each MC-variant are also included in the table. The m/z = 512.8; 491.3 and 523.3 with the RT time of 16.8; 19.2 and 18.7 min were identified and confirmed using the corresponding MC standard. *Variants obtained under optimal conditions. Microcystins were quantified using [Asp3] MC-RR and [Asp3] MC-LR standards.

Peak	m/z	MH$^+$	rTmin – rTmax (in min)	Variants of MCs	Proportion (%) of MC variants*
1	**512.8**	1024.6	16.7–17.1	[Asp3]MC-RR	**83**
2	523.3	1045.6	18.6–18.9	[Asp3]MC-HtyR	2
3	**491.3**	981.6	18.9–19.4	[Asp3]MC-LR	**10**
3	502.2	1003.4	19 – 19.4	Undetermined MC	1
4	516.2	1031.4	20.8–21.2	DeMC-YR ou [Asp3, Dha7] MC-HtyR	4

charged [M + 2H] $^{2+}$ (m/z = 516.2 with a retention time (RT) of 21 min) was assigned to be one of either [Asp3]MC-YR or [Asp3, dha7]MC-HtyR. The ion m/z = 502.2 was undetermined and could not be assigned to [Asp3]MC-LR, as referred to in Yuan *et al.* (1999).

Impact of salinity treatments on MC concentration

To simplify analysis, and to obtain a better idea of the MCs present in the cell cultures, quantification of total MCs, including both extra- and intracellular MC fractions, was performed using a microtiter plate MC-Adda ELISA for all salinity treatments. Total MC concentrations (μg l^{-1}) were positively correlated with biomass (i.e. OD values) as suggested by the high r^2 values for all salinity treatments except 15 g l^{-1} (r^2 = 0.037; Fig. 11). All salt conditions led to similar MC concentration profiles, including a progressive increase in MC content from days 0 to 12, followed by a maximal concentration at day 18 for 0 to 12.5 g l^{-1} NaCl (Kruskal Wallis, $P > 0.05$). At 15 g l^{-1}, a maximal peak was present at day 18, contrasting with a concomitant arrest in cell growth (μ = 0).

Since a positive correlation was obtained between biovolumes (μm^3) and biomass (OD values, Fig. S4) for all samples (r^2 = 0.85, n = 70), we were able to normalise MC content (μg equivalent) per biomass as a proxy of MC quota to discriminate MC profiles at various salinities and over time so as to minimise the biomass factor. Here, four distinct MC concentration profiles were obtained, depending on the time frame and salinity concentrations involved (two-way ANOVA, $P < 0.05$). A first group was observed between 3 and 7.5 g l^{-1} and the control (Kruskal-Wallis, $P > 0.05$), where the MC quota peaked at day 2 followed by a progressive decline from day 8 to 18. Some slight differences were detected for the 5 g l^{-1} treatment (group 2) at days 6 and 8 ($P < 0.05$; Fig. 12) for which a high MC value was still noted at day 2, but without the constant decline of MC levels previously observed in group 1 and the control. For these groups, the MC quota was negatively correlated with the logarithm of biomass (r = −0.82, $P = 5.8^{e-11}$, n = 42) and biovolumes (r = −0.76, $P = 5.2^{e-09}$, n = 42) throughout the experiment (days 2 to 18). A third MC profile was characterised for the 10 and 12.5 g l^{-1}

treatments, including a rather stable MC concentration throughout the experiment with a moderate increase at days 6 and 8 (Fig. 12) that differed significantly from the control ($P < 0.01$). The last group involved the highest salinity level (15 g l^{-1}) and exhibited an increase in MC concentration from day 2 to day 10 with a maximal value seven times higher than control ($P < 0.001$) at the end of the experiment (Fig. 12). As ELISA was applied to the whole culture (i.e. extracellular and intracellular fractions), it was not possible to confirm whether the MC concentrations arose from viable cells or from the media, suggesting a release of MCs after cell death.

DISCUSSION

Effects of salinity on growth and morphology of *P. agardhii* PMC1014.18

Our results highlight differences in growth phases of *P. agardhii* related to salt concentration, including a general decrease in growth rate as salinity increased (from 7.5 g l^{-1} to 12 g l^{-1}, with growth heavily impaired at 15 g l^{-1} salinity. However, the *Planktothrix* strain can maintain its growth under brackish water conditions (from 0 to 9 g l^{-1}; Chomerat *et al.* 2007), revealing a higher tolerance to salinity than previous studies (Orr *et al.* 2004; Chomerat *et al.* 2007; Komárek & Anagnostidis 2005). Nevertheless, the low (observed at 10 to 12 g l^{-1}) to very low growth of the strain (at 15 g l^{-1}) coupled with morphological changes observed throughout the experiment, suggest that salt stress was still insufficient to produce drastic effects on population survival. While no significant differences were detected for the different salinities during the lag phase ($P > 0.05$ from 0 to 2 days), a rapid increase in filament length occurred from days 4 to 8 for cultures exposed to 10 and 12.5 g l^{-1} salinity (Figs 2, S5–6). Elongation could be the signal for cellular growth dysfunction or disruption in development (Singh & Montgomery 2013a, 2013b), which prevent proper cell division in the presence of this stressor. This temporary step was followed by a contrasting significant reduction in filament length after 12 days for 10 to 15 g l^{-1} (Fig. 2), and the appearance of an increasing number of short filaments (one-sixth of controls) at the end of experiment (day 20) for higher salinities (up to 10 g l^{-1}, Fig. 2). Some studies demonstrated that salt stress inhibits cell division after several days at 0.5 M NaCl (Ferjani *et al.* 2003), which could explain the increasing frequency of small filaments observed here. Moreover, the presence of broken filaments suggests cell damage (Montgomery 2015), and has already been reported as a cyanobacterial stress response to environmental pressures (Poulickova *et al.* 2004; Singh & Montgomery 2013a). Under these conditions, the shortened filaments could be a defence mechanism used by cells to preserve energy (Romo & Miracle 1993) and maintain integrity of the few cells remaining. Indeed *in vivo* chlorophyll autofluorescence revealed a high intensity fluorescence signal in the short fragments at 15 g l^{-1} (Figs 8–10), which, combined with light microscopy, indicated that cell integrity, as well as the active photosynthetic pigments within cells, were maintained (Figs 5–9). Although the best way to assess cell viability is the use of fluorescent dye-staining methods (Pouneva 1997), assessing pigment autofluorescence may be
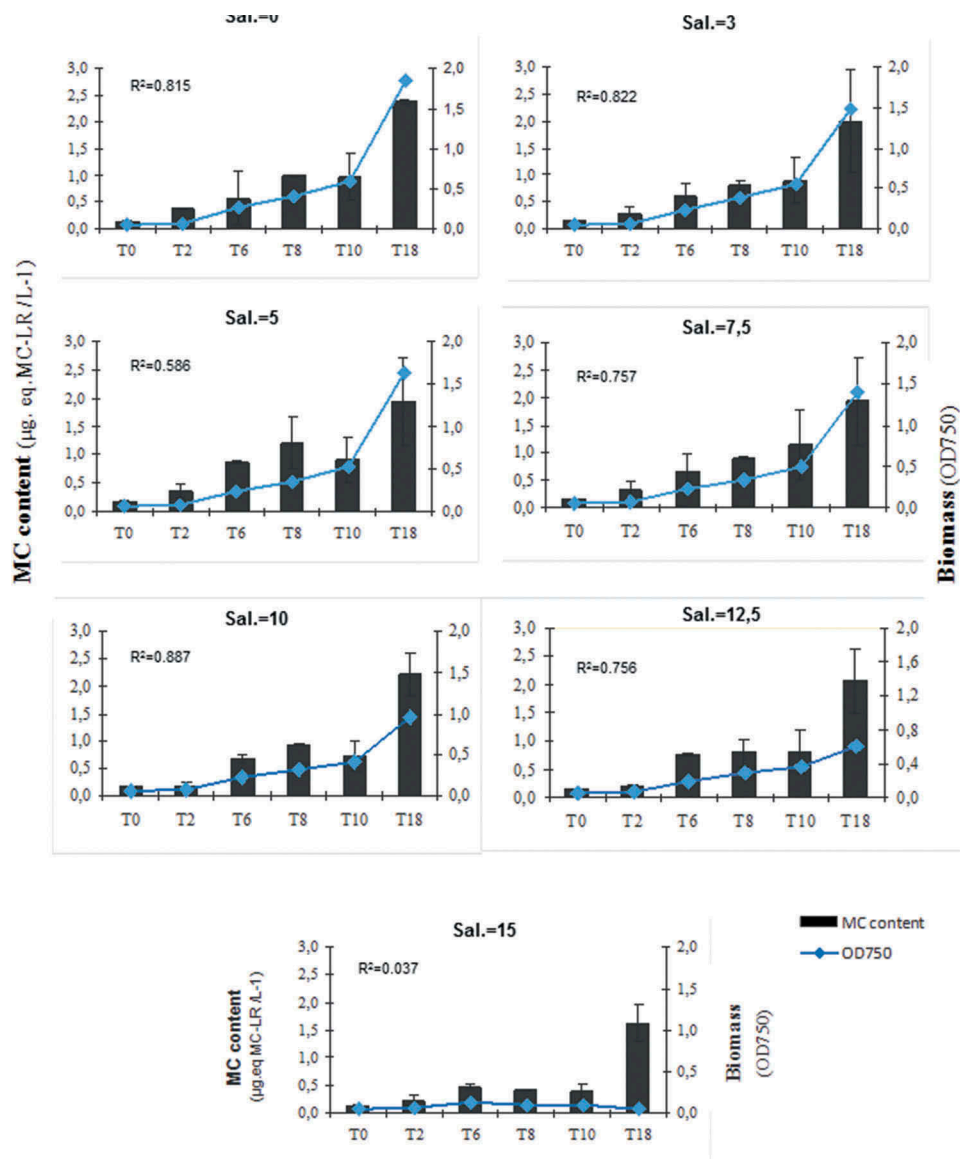
Fig. 11. Variation in MC content (µg eq. MC-LR/l^{-1}) of *Planktothrix agardhii* PMC1014.18 (means ± *s*, *n* = 3) compared to biomass (OD$_{750}$ values) over time (18 days).

a convenient method to evaluate the physiological state of cells (Corrobé *et al.* 2017).

At 12.5 to 15 g l^{-1} salinity, there was intense fluorescence in most short fragments, whereas a small number of cells exhibited non-photosynthetic fluorescent signals (dark cells; Figs 9, 10), which corresponded to necridia or 'suicidal-cells' (Figs 9, 10; Yuan *et al.* 1999), which split filaments in two (Castenholtz & Waterbury 1989). Interestingly, these suicidal cells can be considered a dispersal strategy, increasing the chance of finding a more suitable habitat to restart a population (Komárek & Anagnostidis 2005).

Effects of salinity on total MC concentration

Identification of MC profiles of the PMC1014.18 strain (performed by ESI-LC MS/MS) revealed that MC-LR is one of two major variants which is, with the dominance of Asp3 MC-RR,

characteristic of *P. agardhii* (Fastner *et al.* 1999; Kurmayer *et al.* 2005). In our study, total MC concentration was positively correlated with biomass of *P. agardhii* in all but one salinity treatment (15 g l^{-1}), and corroborates previous studies (Dolman *et al.* 2012; Lyck 2004; Mazur-Marzec *et al.* 2008; Paerl & Otten 2013). The relatively constant proportion of MC content from days 2 to 10 for the 15 g l^{-1} treatment revealed no minute-lethal effects of the PMC1014.18 strain, suggesting a rather high tolerance of this strain to the salt stressor. Indeed, no drastic effects were recorded immediately, or within 24 h, as reported for other cyanobacteria when exposed to a pulsed salt treatment (Tolar 2012) that released massive intracellular MCs linked to osmotic shock and cell death (Allakhverdiev & Murata 2008).

In our study, MC quotas were not significantly different from 10 to 15 g l^{-1}, suggesting that cells coped with this stressor for a long period (from 2 to 18 days; Fig. 12). Some studies have shown that salinities up to 10 g l^{-1} do not affect MC quota for *Microcystis*,
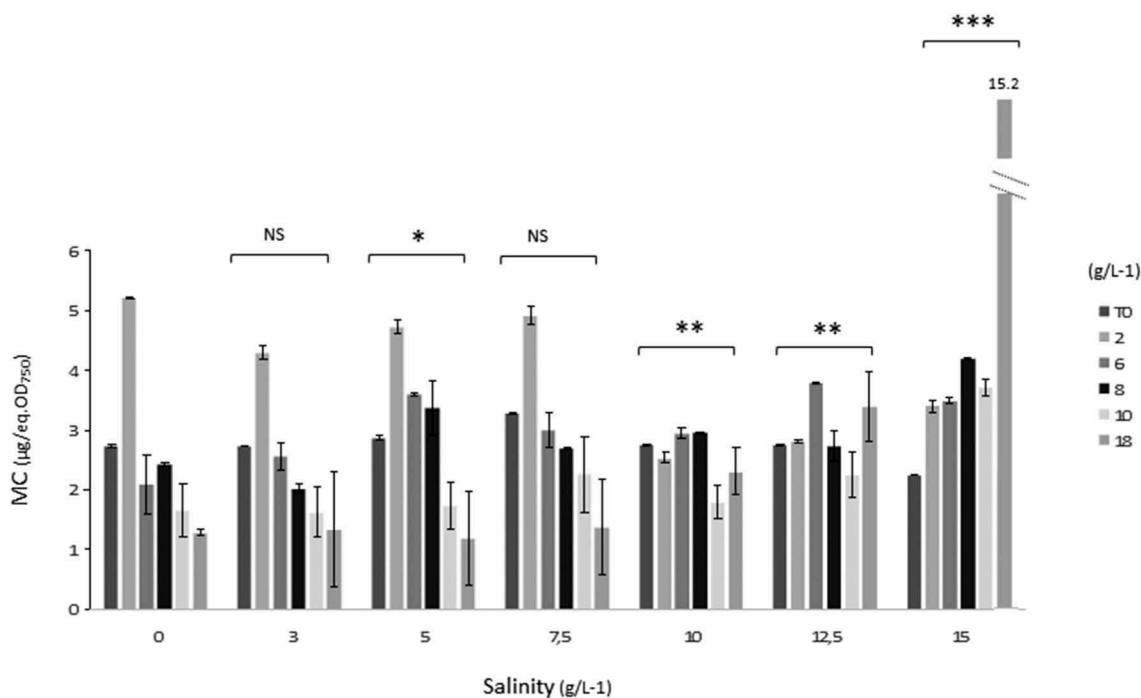
Fig. 12. Evolution of MC content (expressed as eq MC-LR µg/biomass) in the *Planktothrix agardhi* PMC1014.18, for each salinity treatment, from day 0 to day 18; asterisks indicate significant differences compared to control (Mann-Whitney U test). NS, Not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Anabaena or *Anabaenopsis* (Black *et al.* 2011; Martin-Luna *et al.* 2015). Surprisingly, at the end of the experiment for the 15 g l⁻¹ treatment, a maximal MC peak was detected, which was seven times higher than control, and five times higher than the previous MC amount at day 10. Considering the decline in cell density and the increased number of short fragments, this suggests that massive cell disruption had occurred, releasing a large quantity of free MC (only free-MC fraction is detected by ELISA). Thus, MC may accumulate over several days with a stable MC fraction in the medium. Indeed, some MC variants can be detected and remain intact for several months (Miller *et al.* 2010; Zastepa *et al.* 2014), and may directly affect all organisms in the environment (Preece *et al.* 2017). Because ELISA was performed on both the extra- and intra-cellular MC fractions throughout the experiment, it is possible that the high level of MC production by living cells contributed to total MC concentration.

Ecological implications and perspectives

Salinity is an important abiotic factor that affects physiological and ecological performance of phytoplankton species, including cyanobacteria (De Pace *et al.* 2014; Teikaki *et al.* 2018; Tonk *et al.* 2007). We focused on the morphological and physiological changes of *P. agardhii*, a dominant species from a brackish pond that might therefore be pre-adapted to varying salinities (Vergalli 2013). Over the last decade, several episodes of increasing salinities (3 to 8 g l⁻¹) were recorded in this pond, which may have selected ecotypes adapted to the changing environment, as suggested by Kirkwood *et al.* (2008). Therefore, the highest salt concentration that we tested, i.e. 15 g l⁻¹, did not seem to be a threshold limit, at least after 20 days. Accordingly, the MC-producing strain PMC1014.18 can be proposed as a good candidate to further

transcriptomic analyses to better understand its ability to adapt to increased salinity. Identification of salt-tolerant genotypes are still few in literature (Tanabe *et al.* 2018), and there are no reports for *P. agardhii*. *Microcystis* (another potential MC-producing cyanobacterium) strains that proliferate in brackish water have genes for synthesis of the osmolyte sucrose (Tanabe *et al.* 2018). Other reports suggest that low halotolerant strains can produce various compatible solutes such as trehalose and sucrose (Klähn & Hagemann 2011) while estuarine cyanobacterial species, i.e. those acclimated to high salt concentrations near full seawater, have genes for sucrose synthesis with a suite of general and salt-stress-specific genes and regulatory systems enabling them to cope with changing environments (Tanabe *et al.* 2018; Teikaki *et al.* 2018, 2019). Earlier studies reported controversial results on cyanobacterial responses and capacity to cope with salt-stress (De Pace *et al.* 2014; Tolar 2012; Tonk *et al.* 2007), even at the intraspecific level (Otsuka *et al.* 1999). While some *Microcystis* strains resisted 10 g l⁻¹ of NaCl (Orr *et al.* 2004; Tonk *et al.* 2007), others reached their limit at 2 g l⁻¹, leading the authors to consider this as a strain-specific halotolerance rather than as a species-specific trait (Orr *et al.* 2004).

Genomic plasticity associated with ability to tolerate salt variations raises the question of persistence in downstream waters. These include coastal areas after meteorological-drifting events caused by strong rainfall or floods, and/or frequent water intrusion into estuarine ecosystems (De Pace *et al.* 2014; Nielsen *et al.* 2008). Indeed, the salinity gradients of sensitive ecosystems such as lagoons, wetlands, bays and estuaries are highly variable with significant changes in NaCl levels associated with tidal and seasonal cycles (Cloern & Jassby 2012; Schumann *et al.* 2006). These salt fluctuations in the freshwater-estuarine-marine continuum are a driving force that could make aquatic species respond and

adapt to salinity pressures. This could be a serious issue, as MC-producing cyanobacterial species such as *P. agardhii* (in this study), *Microcystis aeruginosa* (Teikaki *et al.* 2018) and *Dolichospermum spp.* (Teikaki *et al.* 2019) can survive and still maintain (and even increase) their toxin-producing ability under varying salinities. This will contribute to serious aquaculture and fishery farm contamination along the freshwater-marine continuum (Bergmann *et al.* 2008). Finally, as climate models predict warmer surface waters and higher evaporation in shallow water systems (Pearl & Huisman 2008), it will be crucial to evaluate the impact of salinity oscillations on cyanobacterial expansion and MC production in a broader context, i.e. in other systems around the world.

Increasing salinity seems to prevent cyanobacterial bloom formation as elevated concentrations of NaCl (up to 12.5 g l^{-1}) affect growth and morphology of *P. agardhii* PMC1014.18, including lower growth rates and an increase in the number of short broken filaments. However, examination of chlorophyll fluorescence within these shortened filaments demonstrate that the cells inside the fragments are still viable. This could be associated with a defence mechanism used by cells to preserve energy and survive unfavourable conditions. Indeed, *P. agardhii* can tolerate brackish water (from 1 to 9 g l^{-1}) and even higher levels of salinity, such as 15 g l^{-1}, without lethal salt-shock, at least for 18 days. This highlights a possible survival mechanism of cyanobacteria in brackish to coastal environments under climate change conditions, and raises the issue of their effect on human health, as we found a constant amount of free MC products over time in this investigation. Finally, there is a need to further investigate the effects of gradual and repetitive drought, rather than drastic salinity shocks, as an indirect effect of global warming changes on the acclimation and/or adaptive response of these filamentous toxic cyanobacteria.

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