Use of macrophytes allelopathy in the biocontrol of harmful *Microcystis aeruginosa* blooms

Zakaria Tazart, Mountasser Douma, Lamiaa Tebaa and Mohammed Loudiki

ABSTRACT

In recent years macrophytes have been considered promising tools in the biocontrol of harmful cyanobacteria blooms (cyanoHABs). In this study, the inhibitory effect of aqueous extracts of *Ranunculus aquatilis* and *Nasturtium officinale* on *Microcystis aeruginosa* growth was assessed via six treatments (0%, 0.1%, 0.25%, 0.50%, 0.75%, and 1% extracts). Chlorophyll a and carotenoid content were analyzed and changes in cell and colonial morphology of *M. aeruginosa* cultures were observed. Also, to reveal potential allelochemical compounds, total phenols (TPs), total flavonoids (TFs), and tannins (TTs) were analyzed in both extracts. The obtained results showed that *M. aeruginosa* growth was significantly inhibited by *R. aquatilis* and *N. officinale* aqueous extracts in a concentration-dependent way. After 8 days of treatment, the highest inhibition rates reached 100% and 75.74% respectively. The Chlorophyll a and carotenoid concentrations were decreased compared to the control group. Colonial and cell and colonial morphology changes were observed under the treatment group with 1% of aqueous extract accompanied by sedimentation of the cyanobacterial cells. This study shows that *M. aeruginosa* growth inhibition was induced by the total polyphenol, flavonoids and tannins. It was concluded that theses macrophytes may control *M. aerugi*nosa and may be useful to control harmful blooms in lake-reservoirs.

Key words | allelopathy, aqueous extract, biocontrol, growth inhibition, macrophytes, *Microcystis aeruginosa*

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INTRODUCTION

Harmful cyanobacterial blooms (cyanoHABs) have become a common occurrence in water bodies worldwide (Harke *et al.* 2016). CyanoHABs have significant socioeconomic and ecological costs that impact drinking water, irrigation, fisheries, recreational activities, food web resilience, and ecosystem integrity, and contribute to fish deaths and aquatic invertebrate mortality (Carmichael & Boyer 2016). In Moroccan freshwaters, fish and waterfowl observed mortalities often coincide with regular and persistent toxic *Microcystis* blooms (Loudiki *et al.* 2002; Douma *et al.* 2010). Therefore, it has become urgent to seek methods to mitigate and eliminate these toxic blooms (Ni *et al.* 2012; Pei *et al.* 2014).

The most commonly adopted methods for the elimination of cyanobacteria in aquatic ecosystems are primarily chemical methods (Wu *et al.* 2010; Yan *et al.* 2011); however, the persistence of chemicals in the environment induces a potentially toxic secondary pollution that affects both aquatic organisms and humans (Zhang *et al.* 2013). This explains the growing interest in recent years in biological control methods (e.g., fish introduction or algaecide bacteria) as alternatives to chemicals.

Recent studies have shown that the allelopathic activities of macrophytes against phytoplankton and cyanobacteria were reported for at least 40 macrophyte species. However, it seems that the most frequent submerged

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macrophytes in shallow lakes, such as *Myriophyllum, Ceratophyllum, Elodea, Najas,* and *Stratiotes,* or certain charophytes, are the most allelopathically active species, as shown by their high polyphenol and flavonoid content and the presence of other allelochemicals besides tannins (Nakai *et al.* 2000; Zhu *et al.* 2010). This work aims to assess the allelopathic effect of the aqueous extracts of two macrophytes (*Ranunculus aquatilis*, *Nasturtium officinale*) on *Microcystis aeruginosa* growth. In addition, it evaluates the potential allelochemical substances that may be responsible for the inhibition mechanism. These two species were selected because *R. aquatilis* is one of Moroccan endemic macrophytes and *N. officinale* dominates in a small mountain lake (high Atlas) where the *Microcystis* bloom has not been observed. The obtained results may be used as solutions for the biocontrol of cyanoHABs.

MATERIALS AND METHODS

Materials and culture conditions

Ranunculus aquatilis and *Nasturtium officinale* were collected in Oued Oukaimeden, Marrakesh area (31° 12′ 19″ N, 7° 51′ 44″ W). Sampling was carried out during the flowering period (April 2016). The samples were rinsed with their original water and then with distilled water to remove debris. The leaves were used for the extraction procedure.

The *M. aeruginosa* strain was sampled, during bloom occurrence, in the Lalla Takerkoust eutrophic reservoir (31°36′ N, 8°2′ W), one of the oldest Moroccan reservoirs without aquatic macrophytes; situated at 600 m, it is around 35 km SSW of Marrakesh. Its maximum depth is 25 m, with a total volume of 69×10^6 m^3 and an area of 584 ha. The main uses of the reservoir are for irrigation, provision of drinking water and leisure activities (Samoudi *et al.* 2016). The sampling of *Microcystis* was carried out on October 2015 (bloom period) and then the strain was isolated and maintained in culture in Z8 medium under controlled conditions. The isolation of *M. aeruginosa* was conducted through a series of subcultures on solid and liquid media. The cultures were placed in a culture room under an illumination intensity of 63 μmoles·m^{-2}·s^{-1} and a light/dark regime of 15:9 h at 25 ± 2°C.

Preparation of aqueous extract

Leaf aqueous extracts of *R. aquatilis* and *N. officinale* were prepared according to the methods described by Ball *et al.* (2001), as slightly modified by Li *et al.* (2016). 10 g of leaves (fresh biomass) were cut into small pieces in 100 ml of distilled water under agitation (37°C; 40 min). The solution was filtered through a glass fiber paper (Whatman GF/C, 0.22 μm). Afterward, the filtrate was adjusted with sterilized distilled water to a total volume of 100 ml and maintained at 4°C as an aqueous extract.

Experimental design

The antialgal activity of the plant extracts was examined by the addition of the extracts (0.1%, 0.25%, 0.50%, 0.75%, or1%) in Z8 medium (biological test 400 ml) into flasks of 500 ml. The medium was inoculated with a culture volume of *M. aeruginosa* (2×10^6 cells/mL in the exponential growth phase) to each vial. All concentrations of the extract were tested in three replicates and the treatment cultures were incubated in a controlled room under the same conditions described in the section titled Materials and culture conditions.

Effects of aqueous extracts on *Microcystis aeruginosa* growth

M. aeruginosa growth was estimated by cell counting using a Malassez counting cell (Sbiyyaa *et al.* 1998). The calculation of the cell density was derived as follows: algal density (cells/mL) = Moy(Nb) × 10^5

With Moy (Nb): the average of cells counted in 10 rectangles (volume of 0.1 μl).

The inhibition rate (IR inhibition rate %) was calculated by the following equation:

IR (%) = ((N0-Ns))/N0 × 100; N0 and Ns (cells/mL) are the cell densities in the control and treatment samples, respectively.

Pigment quantification

The concentrations of Chlorophyll a and carotenoids were measured by spectrophotometry (Wang *et al.* 2010) and calculated using the method described by Lichtenthaler & Wellburn (1983) as follows:

$$\text{Chl a} = 13.95\text{x}OD_{665} - 6.88\text{x}OD_{649};$$

$$\text{Carot} = [(1000 \times OD_{470}) - (2.05\ \text{Ch} - \text{a})]/229$$

where Chl a and Carot represents the concentration of Chlorophyll a and carotenoids (μg/ml) respectively; OD665, OD649 and OD470 are wavelengths absorbance (nm).

Determination of total phenols (TPs), flavonoids (TFs), and tannin contents (TTs) in aqueous extracts

The concentration of TPs was determined using the Folin-Ciocalteu method (Singleton *et al.* 1965). 0.5 ml of the aqueous extract was added to 0.5 ml of Folin-Ciocalteu reagent (Sigma-Aldrich) in water, and then 0.5 ml sodium carbonate solution (20% w/v) was added. The mixture was left for 1 h at room temperature and absorbance was measured at 760 nm.

The concentration of TFs was determined using the method described by Bahorun *et al.* (1996). 500 μl of aqueous extract was mixed with 500 μl distilled water. Then 150 μl sodium nitrite solution (5%) was added, followed by 150 μl aluminum chloride solution (10%) after 5 min. Test tubes were incubated for 11 min at ambient temperature, and then 500 μl sodium hydroxide (1 M) was added. The mixture was vortexed and absorbance was determined at 510 nm.

The concentration of TTs was determined using the Folin-Denis test described by Joslyn (1970). 1 ml of the aqueous extract was added to 75 ml distilled water and then 5 ml Folin-Denis reagent (Sigma-Aldrich) solution and 10 ml sodium carbonate solution was added. The mixture was vortexed and absorbance was determined after 30 min at 760 nm.

Statistical study

Statistical analysis was performed by SPSS V23 software and Microsoft Excel 2016. All experiments were done in three replicates. The results are expressed as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) with the Tukey test was used to analyze the growth and physiological parameters. The significance of the results is compared at $\alpha < 0.05$.

RESULTS

Inhibitory effect on growth of *M. aeruginosa*

Figure 1(a) shows that the aqueous extract of *N. officinale* inhibits the growth of *M. aeruginosa*. Unlike the control group, the cell densities of *Microcystis* were significantly reduced ($p < 0.05$) during the 8 days of exposure at the different tested concentrations. The inhibition rate (IR) appeared to be dose-dependent (Figure 1(b)). The results in Figure 1(c) show that the inhibitory effect of the *R. aquatilis* extract is well marked and statistically significant for the 0.25%, 0.5%, 0.75%, and 1% concentrations, compared to the control group ($P < 0.05$). However, the 0.1% treatment group was not significantly different from the control group. All treatment groups (except 0.1%) showed significant inhibition of *Microcystis aeruginosa* growth (Figure 1(d)).

Morphological changes of *M. aeruginosa* cells

Visual observation of *Microcystis* cultures under *R. aquatilis* aqueous extract treatment shows that after 3 days of exposure, a yellowing and a clear depigmentation appeared in the treated group but not the control group (Figure 2). These morphological changes are accompanied by the coagulation and sedimentation of cyanobacterial cells, especially after 4 days of exposure, with yellowing colors (Figure 2).

Effects of aqueous extract on pigment concentrations of *M. aeruginosa*

The Chlorophyll a content of *M. aeruginosa* was significantly decreased ($p < 0.01$ and $p < 0.05$) by aqueous extract of *R. aquatilis* and *N. officinale* within the initial 5 days (Figure 3(a) and 3(c)). Compared to the control, all the treatment groups showed significant decrease of Chlorophyll a content during the 8 days' experimental period. For

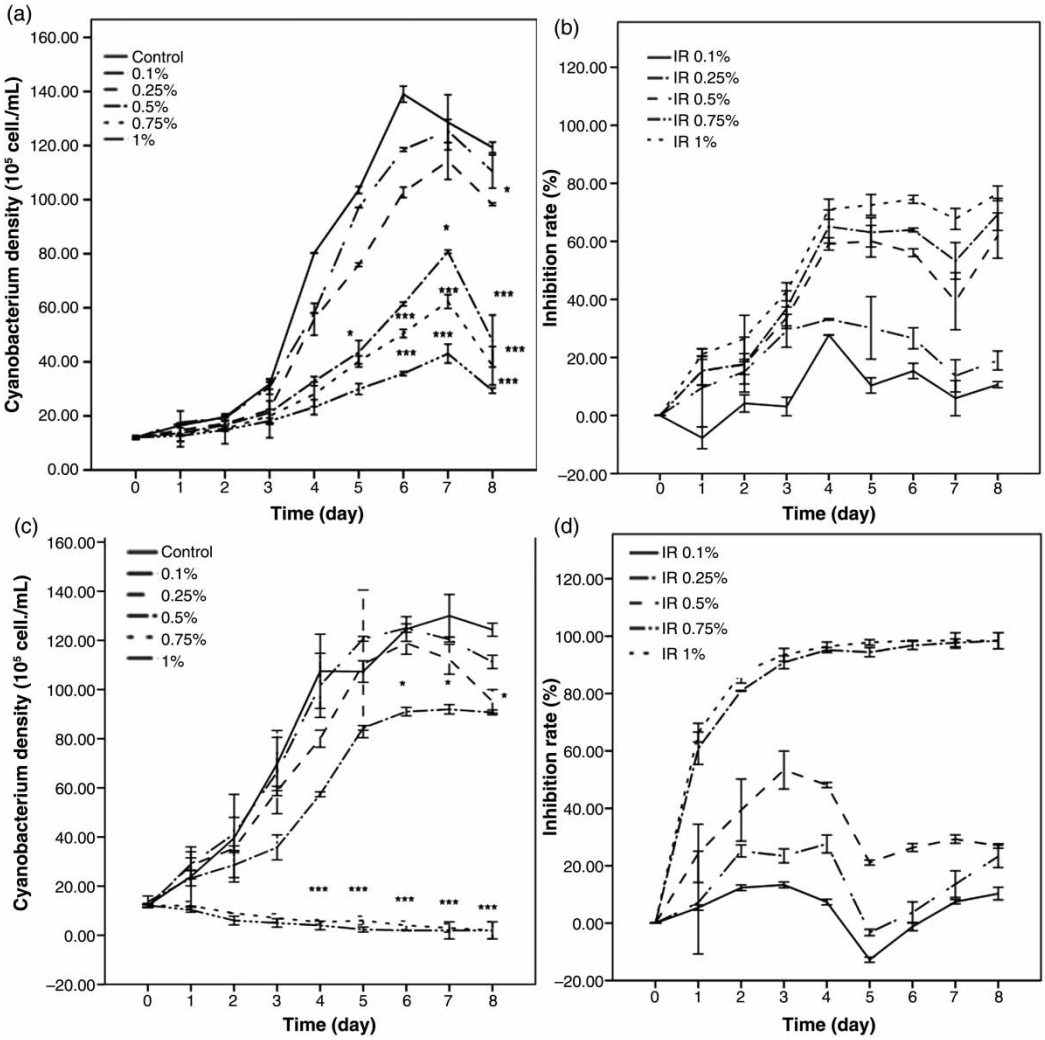

Figure 1 | Effects of *N. officinale* (a and b) and *R. aquatilis* (c and d) aqueous extracts on the growth of *Microcystis aeruginosa*. (a and c) Growth curves and (b and d) inhibition curves. Data are means ± SD ($n = 3$). *$p < 0.05$ and ***$p < 0.001$ indicate significant differences compared with the corresponding controls.

example, the Chlorophyll a content decreased by 80% and 100% respectively for both plant extracts at 1%.

Figure 3(b) and 3(d) show that aqueous extract of both macrophytes also induce a significant reduction ($p < 0.01$ and $p < 0.05$) in carotenoid concentration after at least 6 days of exposure.

The cells quota of the chlorophyll (Figure 3(e)) was decreased in the treatment groups by 1% of aqueous extract of *R. aquatilis* and *N. officinale* compared to the control group. Figure 3(f) shows that the treatments with 1% of aqueous extracts of the two macrophytes also induce a reduction of cell-specific carotenoid, which is more significant for *R. aquatilis* extracts.

Inhibitory effect of potential allelochemical substances of aquatic macrophytes on *M. aeruginosa* growth

To investigate the potential allelochemical substances that may be responsible for the inhibitory mechanism of *M. aeruginosa* growth, the TPs, TFs, and TTs in the two aquatic macrophytes' extracts were determined. Correlation analysis was carried out between phenolic compound concentration and the IR (%) of *M. aeruginosa* after 8 days' exposure to 0.1, 0.25, 0.5, 0.75 and 1% aqueous extracts. Although the correlation coefficients for different compound classes are identical for each IR (%), Table 1 shows that the IR (%) of *M. aeruginosa* after 8 days of exposure to aqueous plant

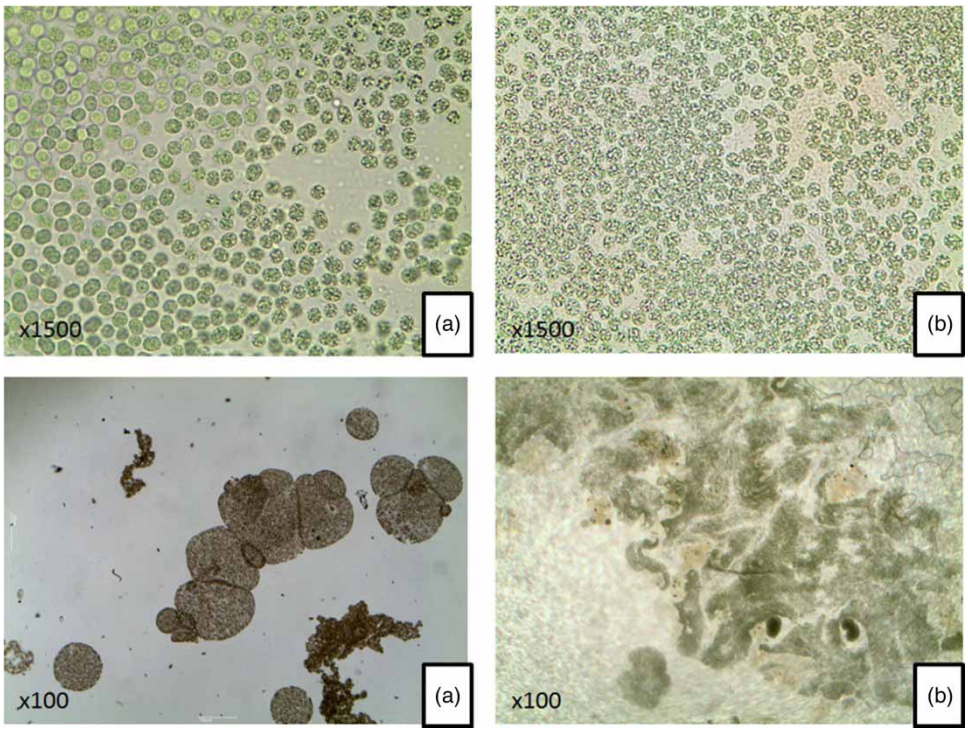

Figure 2 | Microscopic images of *M. aeruginosa* (a) control; (b) under treatment groups with 1% of *R. aquatilis* aqueous extract after 3 days.

extracts was positively correlated especially with the highest concentrations 0.75 and 1%, which means that probably flavonoids, polyphenols and tannins are involved in this allelopathic activity. These compounds, plus other non-identifying allelochemical molecules, probably induce the inhibitory mechanism of cyanobacterium growth. Also, a significantly negative correlation for the 0.5% treatment was observed, which suggests that the inhibition in the latter group of treatment is probably due to other unknown factors.

DISCUSSION

Growth inhibition and the physiological responses of cyanobacteria to macrophytes vary under stress conditions (Li *et al.* 2016). This variation was determined by the different sensitivities of the species to the stressors, as well as by the treatment processes and extract preparation. Some studies used co-cultures between *M. aeruginosa* and macrophytes by the addition of aqueous or organic solvent extracts of active fractions of plants to the culture medium of the cyanobacteria or by the direct addition of autoclaved plant tissues (Chen *et al.* 2012). In our study, the treatment was carried out with aqueous extracts of the leaves of *R. aquatilis* and *N. officinale*, which induced similar growth inhibition and physiological changes in *Microcystis* as those reported in other studies (Xiao *et al.* 2010; Zak *et al.*, 2012; Li *et al.* 2016). However, the inhibition rate at the 1% concentration seems to be relatively higher, especially for *R. aquatilis* (100%), compared to those reported for other plants (Nakai *et al.* 1999, 2000). Furthermore, the aqueous extract inhibited *M. aeruginosa* growth in a dose-dependent manner. However, the 0.10% concentration of the two aqueous extracts appeared to slightly stimulate the growth of *M. aeruginosa* during the first three days; this stimulation at low doses was already reported in other studies (Chen *et al.* 2012; Li *et al.* 2016).

On the other hand, the results of our experiments show the potential algaecide effect of macrophytes and confirm that the leaves are probably the part of plant that excrete compounds responsible for cyanobacterial growth inhibition. Indeed, the antialgal activity of plant leaves has

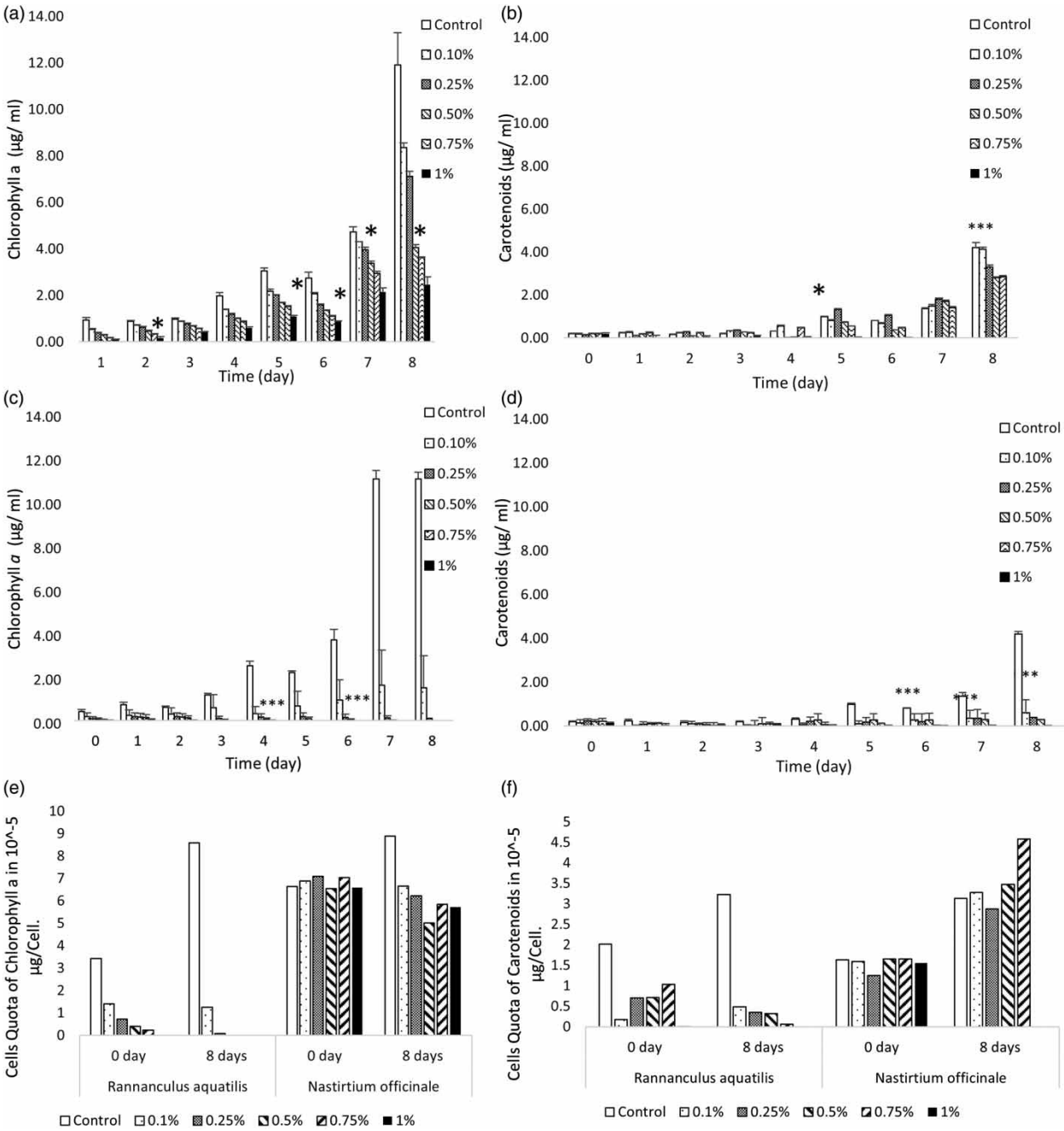

Figure 3 | Chlorophyll a (a and c) and carotenoids (b and d) content of *M. aeruginosa* cells after treatment with *N. officinale* (a and b) and *R. aquatilis* (c and d) aqueous extract for 8 days. Cells quota of chlorophyll and carotenoids in the control and the treatment groups between 0 and 8 days, respectively (e and f). Results are presented as mean ± SD of three independent assays (*indicates $p < 0.05$ and ***indicates $p < 0.01$ relative to the control by ANOVA).

already been demonstrated by other studies (Chen *et al.* 2012; Li *et al.* 2016).

The high inhibition rate, especially observed in *R. aquatilis* extract, could be explained by the plant's richness of secondary metabolites that inhibit the growth of *M. aeruginosa* as has already been demonstrated by other studies (Nakai *et al.* 2000; Chen *et al.* 2012; Meng *et al.* 2015; Li *et al.* 2016).

The morphological changes observed in *Microcystis* colonies (coagulation, sedimentation, and yellowing) could

Table 1 | Correlation coefficient and probability between total phenolic compound, flavonoid, and tannin levels of aquatic macrophytes and inhibition rate after 8 days of exposure to 0.1, 0.25, 0.5, 0.75 and 1% aqueous extracts

	Polyphenols	Flavonoids	Tannins
Inhibition rate 1%			
r	0.993**	0.993**	0.993**
P	0.000	0.000	0.000
Inhibition rate 0.75%			
r	0.990**	0.990**	0.990**
P	0.000	0.000	0.000
Inhibition rate 0.5%			
r	−0.989**	−0.989**	−0.989**
P	0.000	0.000	0.000
Inhibition rate 0.25%			
r	0.786	0.786	0.786
P	0.064	0.064	0.064
Inhibition rate 0.1%			
r	−0.214	−0.214	−0.214
P	0.684	0.684	0.684

**The correlation is significant at the 0.01 level.

be explained by the action of the aqueous extract on the polysaccharide compounds of colonial mucilage, inhibition at the level of the mechanism of biosynthesis of this mucilage, or by an action on the phospholipids of the cell walls (Chen *et al.* 2017; Naceradska *et al.* 2017). This latter hypothesis was confirmed by Meng *et al.* (2015) by observations of cellular damage under the scanning electron microscope. They showed that after 2 days' exposure to *Aliantus altissima* leaf extracts, *Microcystis* cells were deformed with cell surface narrowing due to the destruction of membrane phospholipids and wall constituents by reactive oxygen species derived from the oxidative stress of the cells.

However, recent studies (Li *et al.* 2013) have shown that positive correlations were found between the specific growth rate, extracellular polysaccharide content, and morphology of *M. aeruginosa.* Under conditions of stress, this cyanobacterium forms an aggregate of small colonies (maximum size of colonies is about 100 μm) and has low specific growth rates. In contrast, standard culture conditions yielded single or paired colonies with high specific growth rates.

Chlorophyll a and carotenoids are major pigments in microalgae photosynthetic systems (Yang *et al.* 2012). Their concentrations can be used indirectly to evaluate the biomass of cyanobacteria (Lawton *et al.* 1999) and could be used as biomarkers of the physiological state and possible cell damage of *M. aeruginosa* (Chen *et al.* 2012; Li *et al.* 2016). The decrease of the cells quota of chlorophyll in all the treatment groups compared to the control might be explained by the inhibition of photosynthetic pigments and protein synthesis by the allelochemicals of the extracts leading to malfunctions of normal physiological metabolism in cyanobacterial cells. This assumption was in accordance with previous study, and might be one of the algal inhibiting mechanisms (Ni *et al.* 2012). Allelochemicals from many macrophytes significantly reduce Chlorophyll a and carotenoids contents in algae, disturb PSI and destroy PSII of cyanobacteria (Leu *et al.* 2002). The effect of reducing the cell-specific carotenoid after 8 days of exposure, especially by the aqueous extract of *R. aquatilis*, could be explained by the action of the allelochemical substances contained in the aqueous extract. Indeed, the inhibition of the biosynthesis of carotenoids could be explained either by enzymatic inhibition or by damage to the precursors of biosynthesis of these pigments (Chen *et al.* 2012). This hypothesis deserves to be confirmed, especially since no previous study has analyzed the effect of aqueous extracts on the carotenoid content in *Microcystis* cells.

The released allelochemicals excreted by macrophytes are the main cause of algal inhibition (Nakai *et al.* 1999, 2000; Zhu *et al.* 2014; Li *et al.* 2016). Following the correlation analysis carried out between the concentration of phenolic compounds of the two tested plants and the IR of *M. aeruginosa*, it appears that TPs, TFs, and TTs are probably involved in this allelopathic activity in addition to other non-identifying allelochemical molecules. Whittaker & Feeny (1971) have shown that allochemical compounds mainly include terpenes, steroids, alkaloids, and organic cyanide in addition to phenolic compounds.

CONCLUSION

The results show that the aqueous extracts of the leaves of the two macrophytes *R. aquatilis and N. officinale* exhibited an allelopathic effect and dose-dependently inhibited the growth of *M. aeruginosa. R. aquatilis* had the highest IR of the two macrophytes tested. The quantified photosynthetic

pigments were significantly reduced in the highest extract concentrations. This physiological change may be explained by the allelopathic effect of the allelochemical compounds of the aqueous extracts of the macrophytes. These results suggest that the tested extract probably contains active allelochemicals that are responsible for inhibitory effect on *Microcystis* growth. The phytochemical characterization of *R. aquatilis and N. officinale* aqueous extracts revealed relatively high levels of total phenols (167.7 and 142.9 μg equivalent of gallic acid/ml), TFs (201.8 and 106.5 μg equivalent of catechin/ml) and tannins (0.11 and 0.07 μg equivalent of tannic acid/ml) respectively. These compounds, and probably other specific secondary metabolites not yet identified, have algaecidal activity leading to a de-structuration of the photosynthetic system and the membrane complex of the cyanobacterium cells.

In order to better understand the potential inhibitory effect of these two aqueous extracts, further research will need to be conducted against other macrophytes and/or phytoplankton in macrocosms and natural field conditions, with a focus on the study of the nature and stability of the specific compounds and their potentially synergistic interactions in the aquatic ecosystem.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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