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Effect of algal surface area and species interactions in toxicity testing bioassays

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ABSTRACT

Single and multispecies algal bioassays were assessed using copper toxicity with three green algae (*Scenedesmus subspicatus, Scenedesmus quadricauda and Ankistrodesmus angustus*) and one blue-green algae species (*Oscillatoria prolifera*). Single and multispecies toxicity tests were conducted based on cell density as per standard toxicity testing, and on equivalent surface area. A higher copper sulfate toxicity was registered for *O. prolifera*, followed by *S. subspicatus*, *S. quadricauda*, and *A. angustus* in single-species toxicity tests based on cell density. Single species toxicity tests based on surface area showed increased copper toxicity with increasing algal surface area except for *A. angustus*. In multispecies control bioassays, the growth *of A. angustus* was inhibited in the presence of other species in surface area-based tests. As compared to single species bioassays, *O. prolifera*, and *S. quadricauda* showed a decreased sensitivity to copper sulfate in both cell density and surface area based multispecies tests. However, for the algae species with the smallest surface area, *S. subspicatus*, 96h-EC_{50} value decreased in multispecies bioassays based on surface area as compared to the single species test, while it increased in multispecies bioassays based on cell density. The difference in *S. subspicatus* sensitivity to copper between tests based on cell density and surface area supports the need to adopt multispecies toxicity testing based on surface area to avoid the confounding effect on copper toxicity of increased biomass for metal binding. 96h-EC_{50} values for all species combined in the multispecies test based on cell density and on surface area were significantly different from 96h-EC_{50} values obtained in single species bioassays. These results demonstrate that single-species bioassays may over- or underestimate metal toxicity in natural waters.

1. Introduction

To assess the risk of pollutants in aquatic ecosystems, numerous laboratory scale toxicity tests have been developed. Algae inhibition toxicity tests, characterized by four-day static tests, are used to determine the effects of contaminants on the growth of a unicellular green-algal species after exposing algae population to a series of algaecidal concentrations (OECD, 1984; Beelen and Fleuren-Kemila, 1999; EPA, 2002). In different studies, most metal toxicity testing included single-species laboratory bioassays based on cell density to evaluate the concentration at which the chemical is deemed environmentally unsafe (Petersen, 1982; Swartzman et al., 1990; Wagner and Lokke, 1991; Beelen and Fleuren-Kemila, 1999; Sanderson et al., 2004). However, toxicity tests on the basis of algae surface area have seen little investigation.

The surface area of organisms proved to be highly significant as it provides a new approach in the analysis of microalgae. The initial toxicant loading can be determined by assessing the adsorption of metal ions to algal surfaces. It is both dependent on the cell surface area and on the nature of metal binding sites (Dahl, 1973; Franklin et al., 2004). As algal cell surface is composed of complex and heterogeneous binding sites for metals and protons, it is capable of absorbing nutrients and bioaccumulating heavy metals, such as copper (Xia et al., 2016). Further, agitation and mixing rate of nutrients is affected by the size and shape of microalgae. In this context, cell size is linked to several processes, including “nutrient uptake, light affinity, photosynthesis, respiration, settling rates, physical transport and plant-herbivore interactions” (Vadrucci et al., 2013).

In algae inhibition toxicity tests, individual species are evaluated for their sensitivity to a specific algaecide under controlled laboratory setup (temperature, light, nutrients, and water quality). However, these tests do not represent the interactions among organisms as part of complex communities in real scenarios. The resulting effect of these single species toxicity tests reflects the sensitivity of the monitored species to direct algaecidal effects only and excludes potential effects of other coexisting algal organisms (Franklin et al., 2004; De Laender

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et al., 2009). Some microalgae can produce secondary metabolites when under chemical stress and influence the growth and toxicants induced sensitivity of other algae species present in the aquatic environment. These effects are hard to simulate in isolated tests (Metaxas and Lewis, 1991; Gross, 2003; Legrand et al., 2003; Granéli et al., 2008). Hence, multispecies toxicity tests provide a means to identify the combined effect of algal-algal interactions and the influence of toxicants, which are difficult to determine in isolated algae toxicity bioassays (Swartzman et al., 1990; Franklin et al., 2004; Yu et al., 2007; Picone et al., 2016). Aside from affecting the activity of the test chemical, algal-algal interactions provide more realistic judgment on algaecide treatment dosages in natural waters. Thus, conducting multispecies toxicity tests could present an efficient way to assess precise metal toxicity mechanisms in complex systems (Suter, 1983; Franklin et al., 2004; Yu et al., 2007; De Laender et al., 2009; Bautista-Chamizo et al., 2019).

For many years, copper was used as a reference toxicant to control algae in ponds (Bartley, 1976) and to reduce the abundance of algae that causes off-flavor in catfish (Haughey et al., 2000). Copper is an essential micronutrient for algae metabolism at very low levels. It functions as an enzyme co-factor and plays a role in electron transport in the photosynthetic process (Bossuyt and Janssen, 2004; Garlich et al., 2016; Wang et al., 2017). Lack of copper interferes with photosynthesis, respiration, protein synthesis, and decreases algal resistance and reproduction (Bossuyt and Janssen, 2004). However, at high concentrations, copper becomes toxic to algae and may be potentially detrimental to recipient aquatic ecosystems, with the cupric ion (Cu^{2+}) being the primary toxic form. Cupric ion affects the efficiency of photosynthesis, chloroplast structure and lipid biosynthesis (Lobban et al., 1985; Cooke et al., 1993; Sunda and Huntsman, 1983; Andrade et al., 2004; Garcia-Villada et al., 2004; Dewez et al., 2005; Jancula and Maršálek, 2011). It also causes disarrangement of the plasma membrane (Ferreira et al., 2018), the destruction of the chloroplast membrane of green plants (Tiecher et al., 2017) and interferes with cell permeability on the binding of essential metals (Gardea-Torresdey et al., 2004). Hence, it is of outmost importance to determine the effective lethal copper concentration to algal communities in eutrophicated waters (DeFilipis, 1979; Stauber and Florence, 1987; Bossuyt and Janssen, 2004; Garlich et al., 2016; Wang et al., 2017). On the other hand, copper toxicity is species specific. Studies demonstrated that blue-green algae are highly sensitive to copper, whereas green algae have wide range of resistance to the metal toxicant (Illinois state water survey, 1989; Zhang et al., 2016). It is therefore critical to determine the minimal dose of copper algaecide necessary to effectively treat algae while minimizing the drawbacks of excessive unneeded algaecide levels.

This study aims at determining growth interactions in single and multi-species bioassays of *Scenedesmus subspicatus, Scenedesmus quadricauda, Ankistrodesmus angustus,* and *Oscillatoria prolifera* under control and copper toxicity conditions. The test species were selected for their previous appearance in irrigation channels in Lebanon and common occurrence in lakes and ponds (BAMAS, 2005; Fawaz et al., 2018). Their different representation of shape and size render them ideal and well-aligned with the study objectives. The effect of copper on individual species and algal mixtures was determined to identify whether the interaction between different species affects contaminant toxicity. Copper sulfate was used as an inhibitory agent on the four algal species. The difference between cell density and surface area considerations in both single and multi-species bioassays was assessed.

2. Materials and methods

2.1. Algae culturing medium

Scenedesmus quadricauda (UTEX B 76 strain*), Scenedesmus subspicatus (UTEX 2532* strain*), Ankistrodesmus angustus (UTEX 189* strain), and *Oscillatoria prolifera (UTEX B 1270* strain) were selected for this study (Fig. S1 in SI). Algae species were provided on solid culture media and were transferred aseptically to BG-11 and EPA autoclaved aqueous culture media. Algal growth was then monitored in both media to determine the most suitable culture medium for each algal species. BG-11 was selected as the culture medium in which a starter culture of at least 10^6 cells/mL of healthy, reproducible growing algal cultures was achieved for all species over a period of six weeks (EPA, 1994). The cultures were incubated in a controlled environmental chamber at 25 ± 1 °C under continuous illumination provided by cool white-type fluorescent lamps (Orsam daylight L36W/765, 120 cm, 76 Hz) allowing an energy level output varying between 380 and 440 cd. Transfers of stock algal cultures to fresh nutritive medium was performed once per week under sterile conditions, to maintain a supply of cells in the logarithmic growth phase.

2.2. Determination of algae cell surface area

A ZEISS inverted fluorescence microscope (Axiovert 200) was used to determine the mean surface area (SA) of each algae species by image analysis. Cell dimensions (diameter, width, and length) were measured for 65 cells of each species and the surface area was determined as the corresponding mean value. Approximated geometrical shapes were assumed for each species such as an oval for *S. subspicatus* ($SA = 4 * \pi * R_1 * R_2$; where R_1 and R_2 are the corresponding radiuses), a rectangle for *S. quadricauda* ($SA = 2 * L * W$; where L is the length and W is the width), a crescent for *A. angustus* (SA = area of a crescent*2), and a filamentous shape for *O. prolifera* (SA = area of a filament*2). For the latter two species, more accurate average areas were modeled (Fig. S2 in SI). Cells thickness was considered negligible. The calculated mean surface area per cell were 47, 108, 742 and 2348 μm^2 for *S. subspicatus*, *S. quadricauda*, *A. angustus* and *O. prolifera* respectively.

2.3. Experimental setup

Algae growth inhibition bioassays were carried out in 250 mL Erlenmeyer glass flasks which were thoroughly rinsed with acetone and a 10% solution of reagent grade hydrochloric acid, followed by two times rinsing with MILLI-Q water according to the EPA 96-h static chronic algal toxicity test (EPA, 1994). Two sets of experiments were carried out. In the first set, individual algae species were tested in single species toxicity bioassays and in the second set of experiments, algae were combined and tested in multispecies cultures. In each case, two treatments were performed based on cell density and on equivalent surface area.

In toxicity bioassays based on cell density, single species bioassays were composed of 10,000 cells/mL for each species separately (EPA, 1994). In the case of multispecies toxicity tests, the initial density was 10,000 cells/mL for all combined species, whereby 2500 cells/mL of each species were cultured in the test flask.

In toxicity bioassays based on surface area, the equivalent surface area of 10,000 cells/mL of the species with the largest measured cell surface area was determined and used for single species testing. In this case, the initial cell density of the smaller species were correspondingly increased to match the surface area of the largest species in the single species tests. As such, and knowing that *O. prolifera* has the largest average area/cell of 2348 μm^2, the total algal surface area adopted in the toxicity testing was computed to 23,480,000 μm^2. Thus, mono-algal toxicity tests conducted with *S. quadricauda, S. subspicatus, A. angustus* and *O. prolifera,* were performed with an initial cell density equivalent to 23,480,000 μm^2 in each case (Table 1). The required culture starter for each algae species (cells/mL) was calculated using Eq. (1).

$$\text{Initial Starter} \quad (cells/mL) = \frac{23{,}480{,}000}{SA_{species}}. \tag{1}$$

In the case of multispecies toxicity test based on algal surface area, the initial density used for each of the four algal species was equivalent

Table 1
Initial inoculum of each algae species in single-species (SS) and multi-species (MS) toxicity bioassays based on surface area (SA) and cell density (CD).

Bioassays	Initial cell inoculum (cells/mL)				Surface area (μm^2) of a species in 1 mL	Total surface area (μm^2)
	S. subspicatus	*S. quadricauda*	*A. angustus*	*O. prolifera*[2]		
SS/SA[1]	499.60×10^3	217.40×10^3	31.60×10^3	10.00×10^3	23.48×10^6	23.48×10^6
MS/SA	122.30×10^3	53.20×10^3	7.70×10^3	2.50×10^3	5.87×10^6	23.48×10^6
SS/CD	10.00×10^3	10.00×10^3	10.00×10^3	10.00×10^3	–	–
MS/CD	2.50×10^3	2.50×10^3	2.50×10^3	2.50×10^3	–	–

[1] Initial inoculum for each species in tests based on surface area was computed based on a total surface area of 23,480,000 μm^2.
[2] *O. prolifera* bioassays were conducted once as the initial inoculum for tests based on CD and SA was the same.

to 25% of the total defined surface area of the starting inoculum ($23{,}480{,}000 \times 25\% = 5.87 \times 10^6$ μm^2). Table 1 indicates the initial cell count in the test flasks for each species in mono and multi-species toxicity testing based on surface area and cell density.

Control flasks with no added copper were prepared in triplicates for each set of toxicity bioassays. In the case of multispecies tests, two types of controls were prepared. The first type consisted of the combined species, and the second type consisted of separate species with an initial cell density equal to the corresponding species' density in the algal mixtures. The two types of controls were necessary to allow a direct comparison of control growth rates in the presence and absence of other species, accounting thus for the effect of algal-algal interactions on individual species growth.

2.3.1. Algae inhibition tests

For each treatment, eight copper concentrations (50, 100, 200, 350, 500, 1000, 1500, and 2000 µg/L as $CuSO_4$) were tested in triplicate cultures for 96 h. The test concentrations were in accordance with EPA (2002) algal growth inhibition test and selected in the recommended range by Cook et al. (1993) and based on previous toxicity results on similar algal groups (Fawaz et al., 2018). The test cultures were aseptically prepared by adding algal inoculum to 40 mL copper test solutions to provide the corresponding cell densities in the flasks depending on the type of test. Test flasks were incubated under continuous illumination (55.48–64.24 µmol.photons.m^2/s) at 25 ± 1 °C, and were shaken twice daily to keep the algae in suspension and facilitate the transfer of CO_2. Flasks positions in the incubator were randomly rotated to minimize potential spatial differences in illumination and temperature on algal growth rate. Algae growth was measured at the end of 24, 48, 72, and 96 h of incubation, by performing algae cell counts using direct microscopic observation and Improved Neubauer counting chamber (0.1 mm depth, 1.8 µl volume). An ultrasonic bath was used to detach algal cells from the culture flasks' walls and to disperse cell clumps in the solutions. pH was measured at the beginning and at the end of the single and multispecies bioassays (Tables S1 and S2 in SI).

Copper toxicity on the different tested species was measured as % growth reduction of each species at the end of each exposure day. The average specific growth rates of the exponentially growing cultures in the different test media were calculated at 96 h as follows:

$$Growth\ Rate = \frac{\ln N_4 - \ln N_0}{d_4 - d_0}$$

Where by, N_0 is the nominal number of cells/mL at day 0, N_4 is the measured number of cells/mL at day 4, d_0 is the time of measurement before beginning of test, and d_4 is the time of measurement at 96 h.

Residual free copper in test solutions was measured for each species alone in tests based on cell density and surface area, as well as in multispecies tests using atomic absorption spectrophotometry.

2.4. Statistical analysis

Copper toxicity was expressed as 96h-EC_{50} value, which represents the concentration of copper that is required to inhibit algal growth by 50% after 96 h compared to the controls. A total of nine independent experiments were conducted to determine copper toxicity to algae in single and multispecies tests using cell density and surface area. Algae count data from triplicate bioassays were pooled and a combined concentration-response curve was plotted using weighted linear regression analysis on Probit transformed data. 96h-EC10 (the concentration of copper that is required to inhibit algal growth by 10% after 96-h compared to the controls) was obtained using the same method. The effect of copper dosage and the potential changes in growth rates at 96 h were assessed for each of the treatments. In each case, growth rates were found to follow a normal distribution using the Shapiro-Wilks' test. Student's *t*-test was then used to evaluate statistically significant differences. The R software (R Core team, 2015) was used to conduct the statistical analysis. A significance level of 5% was used in all tests. Significant difference between 96h-EC_{50} values in the different treatments was determined depending on confidence interval (CI) overlap. EC_{50}s were considered significantly different when their respective CIs were not overlapping. However, EC_{50}s with overlapping confidence intervals were considered not significantly different only when the following condition was satisfied: $(EC50_1 - EC50_2) - (1.96\sqrt{SE_1^2 + SE_2^2}) < 0$, where 1.96 is the critical t-value (Knezevic, 2008).

3. Results

3.1. Single species tests

3.1.1. Tests based on cell density

In tests based on cell density, controls of algae species achieved at least 10^6 cells/mL for each species (Fig. S3a in SI) which confirms healthy growth of algae.

96h-EC_{50} values obtained for *O. prolifera, S. subspicatus, S. quadricauda and A. angustus* were respectively 126 µg/L, 198 µg/L, 359 µg/L and 1809 µg/L as copper sulfate, and were significantly different (*p-value* < 0.05) (Table 2).

O. prolifera was the most sensitive to copper (96 h-EC_{50} = 126 µg/L) among all species in single toxicity tests. At copper sulfate concentrations of only 100 µg/L, more than 60% growth inhibition occurred at 96 h relatively to the control. Almost complete inhibition was observed at the highest copper sulfate dosages of 1500 and 2000 µg/L and exceeded 97% (Fig. 1). *S. subspicatus* reached a 96h-EC_{50} value of 198 µg/L. While showing no significant effect (p-values > 0.05) at dosages of 50 and 100 µg/L, as compared to the control, copper sulfate proved to be effective in inhibiting *S. subspicatus* at higher dosages (*p-value* < 0.05). Growth inhibition of *S. subspicatus* reached around 88% at 350 µg/L and exceeded 96% at higher dosages. Compared to *S. subspicatus*, copper sulfate proved to be a less toxic algaecide to *S. quadricauda* (96h-EC_{50} = 359 µg/L). The maximum attained growth inhibition of *S. quadricauda* was 82% after 96 h at the highest copper sulfate concentration. Among all single species, *A. angustus* was the most resistant to copper sulfate in tests based on cell density (96h-EC_{50} = 1809 µg/L). At the lowest copper sulfate dosage (50 µg/L), cell density of *A. angustus* was statistically not different from the control (*p-*

Table 2
EC_{50} and EC_{10} values of algal species after 96 h of copper sulfate exposure, across treatments.

	96h-EC_{50} (μg/L)			
	Tests based on cell density		**Tests based on surface area**	
Algal species	**Single species**	**Multispecies**	**Single species**	**Multispecies**
S. subspicatus	198 (152–259) ag*	321 (224–461) hs	1123 (708–1781) bm	462 (370–563) ns
S. quadricauda	359 (214–604) ci	1695 (729–3939) jt	692 (389–1230) do	1011 (667–1531) pu
A. angustus	1809 (979–3342) ek	235 (160–345) lv	190 (188–391) fq	60 (15–232) rw
O. prolifera	126 (79–202) x	454 (205–1002) y	126 (79–202) x	471 (209–1064) y
Mixed species		389 (263–577) z		462 (288–741) Ω
	96h-EC_{10} (μg/L)			
	Tests based on cell density		**Tests based on surface area**	
Algal species	**Single species**	**Multispecies**	**Single species**	**Multispecies**
S. subspicatus	93 (72–122) ag	80 (55–114) hr	178 (112–283) bl	28 (16–49) ms
S. quadricauda	38 (23–64) ci	44 (19–101) it	57 (32–101) dn	200 (132–302) ou
A. angustus	156 (84–288) ej	53 (36–77) kv	41 (28–61) fp	0.1 (0.04–0.5) qw
O. prolifera	20 (13–33) x	13 (6–28) y	20 (13–33) x	12 (5–27) y
Mixed species		80 (54–119) z		63 (39–100) Ω

* a to Ω denote whether EC_{50}s or EC_{10}s of the different algae species are significantly different ($p < 0.05$) across treatments. Same letter means no significant difference ($p > 0.05$).

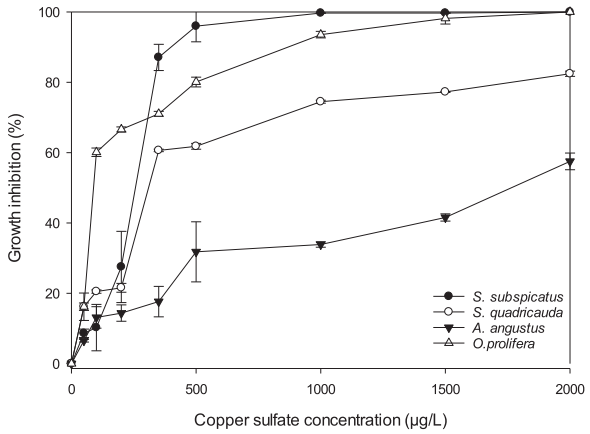

Fig. 1. Induced percent growth inhibition of *S. subspicatus*, *S. quadricauda*, *A. angustus* and *O. prolifera* exposed to copper sulfate after 96 h in single-species bioassays based on cell density.

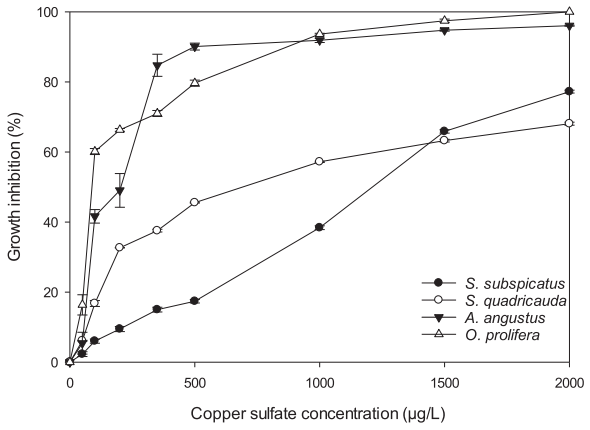

Fig. 2. Induced percent growth inhibition of S. subspicatus, S. quadricauda, A. angustus and O. prolifera exposed to copper sulfate after 96 h in single-species bioassays based on surface area.

value = 0.69). At the highest copper sulfate dosage of 2000 μg/L, density of *A. angustus* cells was statistically lower than the control after 96 h of treatment, but only reached 57.6% inhibition.

3.1.2. Tests based on surface area

the respective 96h-EC_{50} of *S. subspicatus, S. quadricauda, A. angustus* and *O. prolifera* were 1123, 692, 190 and 126 μg/L as copper sulfate (Table 2). A trend between cell surface area of each species and its corresponding 96h-EC_{50} could be deduced in this case. For the same total surface area, as algae species single cell area increases, 96h-EC_{50} decreases. Thus, the higher the algae cell surface area, the more the species is sensitive to copper.

In tests based on cell density, controls of algae species achieved cell count ranging between 10^6–$1.4 * 10^6$ cells/ mL (Fig. S3a in SI), while a much higher growth was attained in control bioassays based on surface area where cell counts of *S. subspicatus*, *S. quadricauda* and *A. angustus* reached respectively $4855 * 10^3$ cells/mL,$1811 * 10^3$ cells/mL and $6716 * 10^3$ cells/mL after 96 h (Fig. S3b in SI). This is due to the increased initial cell density in the case of bioassays based on surface area and highlights the effect of cells starting density in toxicity results.

In toxicity test flasks, *S. subspicatus* was the most tolerant species to copper sulfate in single species-tests based on surface area with a 96h-EC_{50} value of 1123 μg/L as copper sulfate. Growth inhibition of only 38% was reached at copper sulfate concentration of 1000 μg/L (Fig. 2).

S. quadricauda was more sensitive to copper sulfate than *S. subspicatus* and reached a 96h-EC_{50} value of 692 μg/L. However, *S. quadricauda* reduction of only 68% was observed at 96 h at the highest applied copper sulfate dose (2000 μg/L). *A. angustus* registered a 96h-EC_{50} value of 190 μg/L. Copper sulfate showed to be efficient through all dosages at 96 h in tests of *A. angustus* which reached an inhibition percentage of around 96% at 2000 μg/L. As mentioned previously, one test was conducted for *O. prolifera*, being the reference species with the largest cell surface area. A measured EC_{50} value of 126 μg/L was reached after 96 h. Thus, *O. prolifera* with the largest cell surface area, showed the highest sensitivity to copper sulfate.

3.2. Multispecies tests

3.2.1. Controls of multispecies tests

In multispecies tests, the initial inoculum of all species together was equal to 10,000 cells/mL in tests based on cell density (2500 cells/mL/ algae species), whereas in tests based on surface area, the total surface area was equivalent to 23,480,000 μm^2 (5,870,000 μm^2/algae species). To identify possible interactions between *O. prolifera, S. subspicatus, S. quadricauda, and A. angustus*, control growth (in the absence of copper sulfate) were compared for each species alone and in the presence of the other species and are presented in Table 3.

In multispecies controls based on cell density, S. *subspicatus, S.*

Table 3
Average control growth rate of each species alone and when mixed with other species at 96 h.

Treatments	Initial inoculum (cells/mL)	Average growth rate of species in single species control (cells/mL/day)	Average growth rate of species in mixed species control (cells/mL/day)
S. subspicatus CD[1]	2.50×10^3	1.27 ± 0.01 a[3]	1.27 ± 0.01 a
S. subspicatus SA[2]	122.34×10^3	0.54 ± 0.01 b	0.51 ± 0.02 b
S. quadricauda CD	2.50×10^3	0.96 ± 0.01c	0.95 ± 0.01c
S. quadricauda SA	53.24×10^3	0.59 ± 0.01 d	0.61 ± 0.01 d
A. angustus CD	2.50×10^3	1.03 ± 0.01 e	1.01 ± 0.01 e
A. angustus SA	7.75×10^3	1.28 ± 0.01 f	1.11 ± 0.01 g
O. prolifera CD	2.50×10^3	1.09 ± 0.01 h	1.09 ± 0.02 h
O. prolifera SA	2.50×10^3	1.10 ± 0.01 i	1.09 ± 0.01 i

[1] CD = Cell density.
[2] SA = Surface area.
[3] a to i denote whether growth rates single species controls are significantly ($p < 0.05$) different from rates in mixed species controls (same letter means not significantly [$p > 0.05$] different).

quadricauda, A. angustus and *O. prolifera* did not show significantly different growth compared to single species control tests. However, in multispecies controls based on surface area, only *A. angustus* control growth was reduced significantly at 96 h in the presence of S. *subspicatus, S. quadricauda,* and *O. prolifera* compared to single species control tests. Growth rate at 96 h dropped from 1.28 ± 0.01cells/mL/d in single species control tests to 1.11 ± 0.009 cells/mL/d in the presence of other species. For *S. subspicatus, S. quadricauda,* and *O. prolifera*, similar growth rates were obtained in the presence and absence of other algal species in tests based on surface area (*p-values* > 0.05).

3.2.2. Multispecies test samples

In both multispecies copper sulfate toxicity tests based on cell density and surface area, *A. angustus* was the most sensitive to copper sulfate in the presence of other species in comparison to single species tests (Table 2). In tests based on cell density, 96h-EC_{50} value dropped from 1809 µg/L in single species tests to 235 µg/L in multispecies tests and from 190 µg/L to 60 µg/L in tests based on surface area. Copper was significantly less toxic to *S. quadricauda* in the presence of other species compared to single species bioassays in both cell density and surface area based-tests (*p-value* < 0.05). The EC_{50} value after 96 h increased from 359 µg/L and 692 µg/L in single species bioassays based on cell density and surface area respectively, to 1694 µg/L and 1010 µg/L in the corresponding multispecies bioassays. For *O. prolifera*, 96h-EC_{50} significantly increased from 126 µg/L in single species bioassays, to 454 and 472 µg/L in multispecies bioassays based on cell density and surface area, respectively. Copper was significantly less toxic to *S. subspicatus* in the presence of other species compared to single species bioassays based on cell density. The 96h-EC_{50} value significantly increased from 198 µg/L in single species bioassays based on cell density to 321 µg/L in the corresponding multispecies bioassays. However, in toxicity tests based on surface area, 96h-EC_{50} values of *S. subspicatus* decreased from 1123 µg/L in single species bioassays to 461.6 µg/L in the multispecies bioassays. Fig. S4 in SI shows multispecies algae growth curves of the two conducted bioassays in the presence of copper.

For all species combined in the multispecies test based on cell density and surface area, respective 96h-EC_{50} were 389 µg/L and 462 µg/L.

4. Discussion

4.1. Single species tests

O. prolifera was the most sensitive to copper (EC_{50} = 126 µg/L) among all species in toxicity tests. It is a common knowledge that blue-green algae are more sensitive to the effects of copper than other algae groups, and consequently this type of algae is the most easily controlled by copper sulfate applications (McGuire et al., 1984; Illinois state water survey, 1989; Saikia et al., 2011).

In tests based on cell density, *S. subspicatus, S. quadricauda* and *A. angustus* were less sensitive to copper sulfate than *O. prolifera. Scenedesmus* sp. have the ability to avoid metal toxicity by excreting biotic factors (Levy et al., 2007; Magdaleno et al., 2014). In particular, *S. subspicatus* produces glutathione, a peptide of high metal binding capacity which might have contributed to the complexation of copper ions, reducing its toxicity to the test species (Hullebusch et al., 2002; Kalinowska and Pawlik-Skowronska, 2010; Le Faucheur et al., 2006). Similarly, *S. quadricauda* produces and accumulates intracellular and extracellular metal-chelating exudates. These include carbohydrates and proteins among which proline and are produced by *S. quadricauda* as a self-protective mechanism against copper toxicity stress (Maršálek and Rojíčková, 1996). *Ankistrodesmus* sp. excrete high molecular weight nitrogenated and oxygenated compounds which complex copper as suggested by Henriques Vieira and Nascimento (1988). This might have led to the reduced metal toxicity to Ankistrodesmus in this study.

In tests based on surface area, *S. subspicatus, S. quadricauda* and *A. angustus* were more resistant to copper sulfate toxicity than *O. prolifera* owing to their metal binding ability. Compared to single species tests based on cell density, significantly ($p < 0.05$) higher 96h-EC_{50} values were measured for *S. subspicatus* and *S. quadricauda* in tests based on surface area (EC_{50} values of 1123 and 692 µg/L for S. subspicatus and *S. quadricauda*, respectively, as compared to 198 and 359 µg/L). *S. subspicatus* and *S. quadricauda* initial density varied from 10,000 cells/mL in tests based on cell density to $499.6 * 10^3$ and $217.4 * 10^3$ cells/mL respectively in tests based on surface area. The significantly higher initial inoculum density of the two tested *Scenedesmus* species in surface area-based toxicity bioassays in comparison with tests based on cell density may have contributed to the reduction of copper toxicity. For the same concentrations of copper sulfate present in both tests, higher initial inoculum densities of *S. subpicatus* and *S. quadricauda* led to an increased production of metal chelating exudates which contributed to an increased copper complexation (Hullebusch et al., 2002; Xue et al., 1988). In addition, faster surface adsorption onto the cells binding sites by higher biomass density, produced lower metal ion levels in solution and positively affected algal growth (Crist et al., 1990). This was demonstrated by the lower measured labile cupric ion concentrations (Table S3 in SI) in bioassays conducted with of *S. subspicatus* and *S. quadricauda* with higher initial biomass density (tests based on surface area).

In contrary to *S. subspicatus* and *S. quadricauda*, *A. angustus* showed a significant decrease in 96h-EC_{50} value from 1809 µg/L in tests based on cell density to 190 µg/L in tests based on surface area with higher initial cells density. This unexpected increase in copper toxicity at higher initial cell density was associated with a pH increase of nearly 2.2 units in control samples (Table S1b in SI), rendering the test invalid (acceptable pH increase in the control of the tests should not exceed 1.5; OECD, 2011). Therefore, the corresponding test was no further discussed.

4.2. Multispecies tests

Multispecies bioassays were conducted to determine the effect of algal-algal interactions on copper toxicity to the different tested species.

4.2.1. Controls of multispecies tests

In multispecies controls based on cell density, S. *subspicatus, S. quadricauda, A. angustus,* and *O. prolifera* showed similar control growth in the presence and absence of other algal species. In multispecies controls based on surface area, only *A. angustus* showed a reduced growth after 96 h in the presence of other species compared to the single species control. This growth reduction could be due to competition for nutrients which became significant in surface area based-multispecies controls with higher initial biomass density. *S. subspicatus, S. quadricauda,* and *O. prolifera* which showed similar control growth in the presence and absence of other algal species, had the capability to outcompete *A. angustus* for nutrients. Kayser (1979) demonstrated that the main element controlling the growth of several species in multi-species cultures is nutrient competition. Sakshaug and Olsen (1986) found that *Staurastrum luetkemuellerii* outcompetes *Microcystis aeruginosa* when the nutrient supply is continuous and concluded that inter-specific nutrient competition depends on the nutrient requirement of individual species and the mode of nutrient uptake. Franklin et al. (2004) found that in multispecies controls, *P. tricornutum* and *H. niei* competed successfully with *M. pusilla* for the nutrient uptake inducing its growth reduction. Yu et al. (2007) found that growth inhibition may be more influenced by algal–algal interactions than by the contaminant effect itself. Further, Friebele et al. (1978) observed that for similar surface areas, smaller cells (like *S. Subspicatus* and *S. quadricauda*) have faster nutrient uptake rates which grant them a competitive advantage over larger cells (like *A. Angustus*). Another potential reason which could have significantly reduced *A. angustus* growth in multispecies control flasks based on surface area is the allelopathic effect that *O. prolifera* might have induced on *A. Angustus*. In fact, Araoz et al. (2010) demonstrated that *Oscillatoria* sp. produces anatoxin-a under stress and several studies described the effect of anatoxin-a from *Oscillatoria* sp. on other algal species. Leao et al. (2009) showed that *Oscillatoria* sp. has allelopathic potential on green algae such as *C. Vulgaris*. Also, Kearns and Hunter (2001) reported that the green alga *Chlamydomonas reinhardtii* was paralyzed by exposure to anatoxin-a. *O. Prolifera* could thus have secreted anatoxin-a which might have significantly affected the growth of *A. Angustus*. Unlike *A. Angustus* both *Scnenadesmus* species were not affected by the allelopathic signaling of *O. Prolifera*. Sedmak and Kosi (1998) studied how toxins secreted by cyanobacteria (like filamentous *Oscillatoria* sp.) can selectively negatively affect algal species growth. They found that algal species like *C. erosa* showed cell loss and therefore growth inhibition in the presence of cyanobacterial toxins, while other species like *M. contortwn* and *S. quadricauda* showed enhanced proliferation. Similarly, in this study, *Scenedesmus* sp. showed to be unaffected by the toxin exudates of the cyanobacteria.

4.2.2. Effect of copper sulfate toxicity

The effect of copper on the growth of *S. subspicatus, S. quadricauda, A. angustus*, and *O. prolifera* in multispecies bioassays based on both cell density and surface area was measured as 96h-EC_{50} values reported in Table 3. The difference in copper toxicity in single and multispecies bioassays will be discussed to determine the effect of algal interactions on the metal toxicity.

96h-EC_{50} of *A. angustus* in multispecies bioassays based on cell density and surface area decreased in comparison with their corresponding single species bioassays. In addition to copper stress, the significant ($p\text{-value} < 0.05$) reduction of *A. angustus* growth is probably due to the inhibitory effect which other micro-algal species could have induced through competition for nutrients or secretion of toxins with inhibitory effect.

Decreased toxicity of copper to *S. quadricauda* in the presence of other species may be due to the reduction of free copper ions due to self-production of exudates. As discussed in the single species toxicity tests, *S. quadricauda* may be producing and accumulating intracellular metabolic products that play an important role in the protection of the algae against copper toxicity (Maršálek and Rojíčková, 1996; Magdaleno et al., 2014). Carbohydrates and proteins that *S. quadricauda* produced, might have contributed to the complexation of copper and to the reduction of copper toxicity. Nonetheless, given the higher toxicity of copper to *S. quadricauda* in single species bioassays, a more eventual justification for the reduced copper toxicity on *S. quadricauda* in the presence of the other species would be the production and release of exudates by *S. subspicatus*.

Toxicity of copper was also reduced to *O. prolifera* in the presence of *S. subspicatus* and *S. quadricauda*. This might be correlated to the production and extracellular algal exudates by these latter species in response to copper stress (Le Faucheur et al., 2006; Kalinowska and Pawlik-Skowronska, 2010). These exudates may complex copper ions, making them less bioavailable to *O. prolifera* in multispecies bioassays.

Copper was significantly less toxic to *S. subspicatus* in the presence of other species compared to single species bioassays based on cell density. Decreased toxicity of copper to S. *subspicatus* may be due to the complexation of copper by the exudates produced by *S. quadricauda*. It is worth mentioning that a mutually profitable interaction between *S. quadricauda* and *S. subspicatus* may be concluded whereby, each species releases exudates which protect itself and the other species from copper toxicity. However, in toxicity tests based on surface area, S. *subspicatus* showed to be less tolerant to copper in the presence of other species. Quigg et al. (2006) reported that in surface area tests, small cells that have large surface area to volume ratios are more sensitive to copper than larger species. The difference in *S. subspicatus* sensitivity to copper between tests based on cell density and tests based on surface area could be explained by the positive effect that bigger algal cells have on smaller ones in the form of increased protection against copper toxicity (Franklin et al., 2002). In multispecies toxicity tests based on cell density, the same initial inoculum was used for the different algal species (2500 cells/mL each). The different species present in the medium have different total surface areas each (*O. prolifera, A. angustus, S. quadricauda, and S. subspicatus* present respective single cell surface areas of 5,870,000 μm^2, 1,855,000 μm^2, and 270,000 μm^2 and 117,500 μm^2). Having the same initial cell inoculum but different surface areas per species, the algae with the largest cell surface area, *O. prolifera,* will occupy the biggest surface area, followed by *A. angustus, and S. quadricauda.* The 2500 cells/mL of *S. subspicatus* will occupy the smallest surface area of the medium in the presence of the other algal species (Fig. S5 in SI). This suggests that an increased copper uptake by active sites present on the cell membrane of bigger cells that occupy most of the medium area might have decreased copper uptake by the smallest algal cells and subsequently reduced copper toxicity to *S. subspicatus* in multispecies bioassays based on cell density (Dahl, 1973; Vadrucci et al., 2013). This shielding effect of bigger cells on smaller ones, suggests the necessity of conducting toxicity tests based on equivalent surface area rather than on cell density to avoid the confounding effect on copper toxicity of increased biomass for metal binding.

For all species combined in multispecies tests based on cell density and on surface area, 96h-EC_{50} was reported to be 389 μg/L and 462 μg/L, respectively. These results are significantly higher or lower than 96h-EC_{50} values recorded for each species separately in monoalgal bioassays based on cell density and on surface area. This demonstrates that single-species bioassays may over or underestimate metal toxicity in natural waters.

5. Conclusion

Results from this study showed that, the growth *of A. angustus* was inhibited in the presence of other species in surface area-based tests due

to nutrients competition. Moreover, copper sulfate toxicity to algae could increase or decrease in the presence of other species as compared to when the species is isolated. Hence, single-species bioassays may over or underestimate metal toxicity in natural waters. Improvements to the environmental realism of laboratory toxicity testing is thus gained from the development of bioassays incorporating multispecies rather than standard individual species bioassays. Further, large algal cells could induce shielding effects on smaller ones decreasing copper toxicity in multispecies bioassays. To avoid the confounding effect on copper toxicity of increased biomass for metal binding, it is thus recommended to conduct multispecies toxicity bioassays based on equivalent surface area rather than based on cell density.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2019.03.032.

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