Isobolographic analysis of the interaction between cadmium (II) and sodium sulphate: toxicological consequences

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ABSTRACT

Sulphate is an essential nutrient for autotrophic organisms and has been shown to have important implications in certain processes of tolerance to cadmium toxicity. Sodium sulphate is the main salt of sulphate in the natural environments. The concentration of this salt is increasing in the aquatic environments due to environmental pollution. The aim of this study was to investigate, using an analysis of isobolograms, the type and the degree of the interaction between Cd(II) and sodium sulphate in the freshwater microalga *Chlamydomonas moewusii*. Two blocks of experiments were performed, one at sub-optimal sodium sulphate concentrations (<14.2 mg/L) and the other at supra-optimal concentrations (>14.2 mg/L). Three fixed ratios (2:1, 1:1, and

1:2) of the individual EC_{50} for cadmium and sodium sulphate were used within each block. The isobolographic analysis of interaction at sub-optimal concentrations showed a stronger antagonistic effect with values of interaction index (γ) between 1.46 and 3.4. However, the isobologram with sodium sulphate at supra-optimal concentrations revealed a slight but significant synergistic effect between both chemicals with an interaction index between 0.54 and 0.64. This synergic effect resulted in the potentiation of the toxic effects of cadmium, synergy that was related to the increase of the ionic strength and of two species of cadmium, $CdSO_4$ (aq), and $Cd(SO_4)_2^{2-}$, in the medium. Results of the current study suggest that sodium sulphate is able to perform a dual antagonist/synergist effect on cadmium toxicity. This role was concentration dependent.

KEYWORDS Cadmium toxicity; Sodium sulphate; Interaction; *Chlamydomonas moewusii*; Isobolographic analysis; Cadmium speciation

INTRODUCTION

Cadmium is one of the most toxic metals for the environment. It has been listed in the so-called “Black-list” of the European Community (Mason 2002; Mislin and Ravera 1986). This metal has been distributed into aquatic environments mainly from anthropogenic sources. In fact, the major cadmium sources in water come from the use of fertilizers and mining industries (phosphate fertilizers, 41 %; use of fossil fuels, 22 %; and iron and steel production, 17 %) (Van Assche 1998). This metal is usually considered as a non-essential element, which is a real risk because it shows a high toxicity and capacity of bioaccumulation in the food chain (Raskin et al.1997; Sanità di Toppi and Gabbrielli 1999). As result, cadmium is a serious hazard to human health, plants, and living species in general (Järup and Åkesson 2009). For these reasons, the study of the factors influencing the toxicity of this element is important from an environmental point of view. As in other metals, external factors such as pH, salinity, and organic matter, or internal factors such as species-specific tolerance mechanisms influence the degree of toxicity of this metal. Recently, sulphate is emerging as an environmental factor to be considered in the toxicity of cadmium for autotrophic organisms (Mera et al. 2014; Nocito et al. 2002).

Plants, algae, yeast, and most prokaryotes cover their demand for reduced sulphur by reduction of sulphate. Therefore, sulphate is an important nutrient for autotrophic organisms and is the most stable sulphur form in oxidizing environments of the Earth. In addition to this well-known function, sulphate has been shown to have important implications in certain processes of tolerance to environmental stress in different organisms, mainly related to cadmium toxicity. A higher bioavailability of sulphate has been associated with a lower toxicity of this metal (Anjum et al. 2008; Gill and Tuteja 2011; Mendoza-Cózatl et al. 2005; Mera et al. 2014). With this in mind, it becomes evident that there is some type of interaction between them. Both chemicals can be found simultaneously in the environment, especially in those polluted with cadmium. In most cases, sulphate is a common compound in aquatic natural environments and is well accessible. The concentration of sulphate is usually fairly constant in marine environments. On the contrary, in freshwater environments, a greater variation occurs (about 0.96–48 mg/L) (Holmer and Storkholm 2001).

The most common salt of sulphate is sodium sulphate. The concentration of this salt in aquatic ecosystems is increasing due to anthropogenic discharges (fertilizers in agriculture, mining industry, atmospheric deposition, etc.) since this salt is one of the most important minerals in the chemicals industry. For this reason, sodium sulphate can be the dominant contributor to salinity (Bowell 2000). Because of this and of the relationship between cadmium and sulphate, it would be interesting to study what would happen when an environment polluted with this metal undergoes an increase in the concentration of sodium sulphate. However, there is little available information about the type of interaction between cadmium and sulphate and its intensity. The study of the combined toxicological effects (interaction) that may occur from exposures to different chemicals in mixtures is a field that is currently having a great relevance because most toxicological studies focus only on individual compounds. Thus, the results obtained can be essential for evaluating the combined effects of cadmium and sodium sulphate. In addition, this information would be important to improve the tolerance of these organisms to cadmium stress and the algae-based biotechnologies for bioremediation of this metal (Hamdy2000; Radway et al. 2001; Torres et al. 1998; Travieso et al. 1999).

The analysis of isobolograms is a method proposed for the assessment of combined effects of chemicals (Loewe 1927). This method provides a rigorous

evaluation of the interaction between two active substances. The isobolographic analysis provides a basis for evaluating whether biological responses induced by mixtures of chemicals are additive, synergistic, or antagonist reactions (Gessner and Cabana 1970). The isobologram (derived from *iso*, equal + *bol*, effect) of binary mixtures constitutes a graphical representation of the interaction in two dimensions (Gessner 1995). In general, if the chemical pair has improved its potency relative to each chemical alone, the combination is synergistic; if potency remains unchanged, the effect is additive, and if potency is reduced, the effect is antagonistic.

In the present study, the type and the degree of interaction between cadmium (II) and sodium sulphate were investigated by an isobolographic analysis, using the freshwater microalga*Chlamydomonas moewusii* as test microorganism. The degree of interaction was evaluated from an interaction index.

MATERIAL AND METHODS

Microorganism and culture conditions

The microalgal species chosen for this study was *C. moewusii* Gerloff (strain CCAP 11/5B). This strain was obtained from the Culture Collection of Algae and Protozoa (Cumbria, UK). Cells of this freshwater microalga were cultured and maintained in modified Bristol medium sterilized at 121 °C for 20 min. The composition of the culture medium is shown in Table 1. Different amounts of Na_2SO_4 were added depending on the experiments. Cultures were maintained at 18 ± 1 °C under a light intensity of 68 μmol/(m^2s) using cool fluorescent light with a light/dark cycle of 12:12 h. Natural sterile air was constantly bubbled at a flow rate of 10 L/min.

Chemicals

All chemicals used for the culture medium (Table 1), cadmium chloride 2 ½-hydrate ($CdCl_2 \cdot 2½\ H_2O$), and Lugol's solution (I_2-KI) were of the highest purity available, and they were purchased from Sigma-Aldrich® (St. Louis, MO, USA). The different reagents, dilutions, and culture media were prepared with Milli-Q® water obtained from a Milli-Q Plus system (Millipore Ibérica, Spain).

Growth measurement

Growth of the microalgal cultures was measured as cell concentration by counting aliquots in an improved Neubauer hemocytometer chamber (Marienfeld-Superior, Germany) after fixation with Lugol's solution and using a phase-contrast light microscope, Nikon Labophot (Nikon, Japan).

Table 1 Composition of the culture medium for the experiments

Compound	g/L
$NaNO_3$	0.250
KH_2PO_4	0.175
K_2HPO_4	0.075
Na_2SO_4	Variable
$MgCl_2$	0.029
$CaCl_2 \cdot 2H_2O$	0.029
NaCl	0.025
$CoCl_2 \cdot 6H_2O$	$4.0x10^{-3}$
$MnCl_2 \cdot 4H_2O$	1.8×10^{-3}
$FeCl_3 \cdot 6H_2O$	5.1×10^{-4}
$MoO_4Na_2 \cdot 2H_2O$	3.9×10^{-4}
H_3BO_3	2.0×10^{-4}
$ZnCl_2$	1.1×10^{-4}
$CuCl_2$	4.3×10^{-5}
$pH = 6.3 \pm 0.1$	

Experimental design and EC_{50} determination

Since previous results with this microalga showed that the optimal concentration of sodium sulphate for this microalga was 14.2 mg/L (0.1 mM) (Mera et al. 2014), the interaction experiments of cadmium with this salt were divided in two blocks of experiments, one at sub-optimal concentrations of sodium sulphate and the other at supra-optimal.

Both blocks of experiments were conducted in the same way. *C. moewusii* was cultured with the conditions listed above in sterilized 250-mL Pyrex glass bottles, previously washed with hydrochloric acid and distilled water. Different nominal concentrations of sodium sulphate or cadmium (or both) were added to the culture medium. Finally, the inoculum, taken from a culture that was in a 21-day free-sulphate culture medium to decrease the cellular pool of organic sulphur, was added. All

procedures were performed under aseptic conditions of handling and transfer. The initial cell density in the assays was 4×10^5 cells/mL.

After 96 h of culture, the growth was evaluated as previously described and used as an endpoint. The inhibitory effect on the growth of this microalga was expressed as percentage of inhibition. The effect selected was the median effective concentration value (EC_{50}, concentration of the chemical that reduces the population growth to 50 % of the control). The EC_{50} of cadmium, sodium sulphate, and combinations of both for this microalga was calculated by non-linear regression of the sigmoidal dose–response function (four-parameter logistic regression) whose equation is

$$y = A + \frac{B-A}{1 + \left(\frac{x}{C}\right)^D}$$

where A is the value for the minimum asymptote, B is the value for the maximum asymptote, $C$is the point of inflection (estimated value of EC_{50}), and D is the Hill's slope of the curve.

Analysis of the interaction

An isobolographic analysis was used to characterize the interaction between cadmium and sodium sulphate (Gessner 1995; Tallarida et al. 1989). Isobolographic analysis of interaction is a mathematical method that allows the precise characterization of interactions between drugs or chemicals. Five groups of experiments were assigned to each of the two blocks (sub-optimal and supra-optimal) consisting of different proportions (fixed ratio) of sodium sulphate and cadmium. Table 2 shows the nominal concentrations used in each group of experiments for the block of sub-optimal concentrations of sodium sulphate. Groups A and E were used to determine the EC_{50} of the individual compounds. The group A was established with a minimum concentration of sodium sulphate (0.04 mg/L) in which the growth allowed the significant calculation of the cadmium EC_{50}. In the same way, Table 3 shows the nominal concentrations used for each group of experiments in the block of supra-optimal concentrations of sodium sulphate. The groups A and E were also used to determine the EC_{50} of the individual compounds and, in this case, the group A was established with the optimal concentration of sodium sulphate (14.2 mg/L). The concentrations of the groups B, C, and D in both blocks were obtained using different

ratios (2:1, 1:1, 1:2) of the EC_{50} values obtained from the individual compounds in their respective blocks, as explained below. Each group of experiments was carried out in triplicate. In total, $68 \times 3 = 204$ pairs of combinations were used in the experiments.

The isobologram was constructed using a standard method (Tallarida 1992). The individual EC_{50} values for cadmium and sodium sulphate were plotted on the *x*- and *y*-axes, respectively. A straight line joining these values is the theoretical additive line. All points on this line represent the pair of theoretical additive concentrations for the compounds administered together that produce the same level of effect (EC_{50}) that the compounds administered individually. These theoretical additive concentrations (Z_{add}) for each combination in the same component ratio (2:1, 1:1, 1:2) were computed from the concentrations of EC_{50} of the single compounds according to the equation described by Tallarida (1992) but with a modification:

$$Z_{add} = f * [Cd]_{50} + (1 - f) * ([Na_2SO_4]_{50} + [Na_2SO_4]_0) \quad (1)$$

where $[Cd]_{50}$ is the cadmium concentration corresponding with the EC_{50}, $[Na_2SO_4]_{50}$ is the EC_{50} of sodium sulphate, $[Na_2SO_4]_0$ is the minimum concentration of sodium sulphate used in each block of experiments, and f is the fraction of cadmium EC_{50} in the combination. f is equal to 1/3 in the combination 1:2, 1/2 in the combination 1:1, and 2/3 in the combination 2:1. For example, the combination 1:2 was composed of 1/3 of the cadmium EC_{50} and 2/3 of the sodium sulphate EC_{50}. The introduction of the term $[Na_2SO_4]_0$ is because a minimum concentration of sodium sulphate was necessary to perform the experiments and thus be able to calculate the cadmium EC_{50} in the sub-optimal range. This minimum value was established in 0.04 mg sodium sulphate/L. Similarly, the experiments in the supra-optimal range were established on the basis of the optimal concentration of sodium sulphate, 14.2 mg/L.

Five concentrations (twofold serially diluted) around each fixed-ratio combination (2:1, 1:1, and 1:2, groups B, C, and D, respectively) were prepared (Tables 2 and 3), maintaining the component ratio constant, in order to generate a range of six concentrations, which were then analyzed under standard growth conditions of the microalga to provide dose–response curves and a EC_{50} for each component in the combination. The experimental concentration (Z_{exp}) is the total

concentration of both components in the combination that produces this EC_{50} effect. These experimental values obtained from the three fixed-ratio combinations assayed, which cause the EC_{50} effect, were plotted against the theoretical additive line and compared with the theoretical values of additivity (Z_{add}) obtained with the Eq. 1 for each of the three combinations assayed. It is considered that when the point falls below the additive line, the mixture is synergistic, and when the point falls above the line, the combination is antagonistic.

The interaction index, denoted by γ, was calculated by the isobolar relation:

$$\gamma = [a] / [Cd]_{50} + [b] / [Na_2SO_4]_{50} \quad (2)$$

where $[Cd]_{50}$ and $[Na_2SO_4]_{50}$ are the individual concentrations of cadmium and sodium sulphate that produce the specific effect (EC_{50}), and $[a]$ and $[b]$ are relative concentrations of cadmium and sodium sulphate in their combinations that produce this same effect. If $\gamma = 1$ the interaction is additive, if $\gamma < 1$ it is super-additive (synergistic) and if $\gamma > 1$ it is sub-additive (antagonistic).

Effect of different concentrations of sulphate ion at the same ionic strength

To determine the effect of the sulphate ion, an experiment was conducted with different concentrations of sulphate at the same initial ionic strength. Two sets of experiments, one with 0 mg Cd(II)/L and another with 2 mg Cd(II)/L, were performed. Ionic strength of the media was calculated by means of Visual MINTEQ software, assuming ideal behavior and molarity concentrations. The theoretical ionic strength used as reference for these experiments was that calculated from the culture medium containing a concentration of 1000 mg/L of sulphate; this value was 37 mM. The sulphate concentrations tested were 0, 0.01, 0.02, 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5, 10, 25, 50, 100, 200, 300, 500, and 1000 mg/L. The ionic strength of each of these media was previously calculated with Visual MINTEQ. The difference in the theoretical ionic strength with respect to the reference culture was adjusted by adding sodium chloride to the simulation with Visual MINTEQ to achieve the same value of ionic strength than the reference. These amounts of sodium chloride were finally added to the respective culture media. The pH was experimentally measured to verify that all the cultures had the same pH. The pH was measured with an Orion 720A+ pH

meter (Thermo Electron Corporation, UK); the average value obtained for all the cultures was 6.3 ± 0.1.

Table 2 Nominal concentrations of cadmium (II) and sodium sulphate used to determine the interaction at sub-optimal sodium sulphate concentrations

Sub-optimal Group	[Cd(II)] (mg/L)	[Na_2SO_4] (mg/L)	Fixed ratio (cadmium: sodium sulphate)
A	0.01	0.04	–
	0.02	0.04	–
	0.05	0.04	–
	0.08	0.04	–
	0.10	0.04	–
	0.25	0.04	–
	0.50	0.04	–
B	0.29	1.64	2:01
	0.15	0.82	2:01
	0.07	0.41	2:01
	0.04	0.20	2:01
	0.02	0.10	2:01
	0.01	0.05	2:01
C	0.88	9.46	1:01
	0.44	4.73	1:01
	0.22	2.36	1:01
	0.11	1.18	1:01
	0.06	0.59	1:01
	0.03	0.30	1:01
D	0.15	3.09	1:02
	0.07	1.55	1:02
	0.04	0.77	1:02
	0.02	0.39	1:02
	0.01	0.19	1:02
	0.01	0.10	1:02
E	0.00	0.04	–
	0.00	0.10	–
	0.00	0.15	–
	0.00	0.30	–
	0.00	0.70	–
	0.00	1.50	–
	0.00	3.50	–
	0.00	7.00	–
	0.00	14.20	–

The fixed ratio is around the combination of the EC_{50} values obtained individually for both chemicals

Table 3 Nominal concentrations of cadmium (II) and sodium sulphate used to determine the interaction at supra-optimal sodium sulphate concentrations

Supra-optimal Group	[Cd(II)] (mg/L)	[Na_2SO_4] (mg/L)	Fixed ratio (cadmium: sodium sulphate)
A	0.00	14.20	–
	0.50	14.20	–
	1.00	14.20	–
	2.00	14.20	–
	4.00	14.20	–
	6.00	14.20	–
	8.00	14.20	–
B	8.26	1733.69	2:01
	4.13	866.85	2:01
	2.07	433.42	2:01
	1.03	216.71	2:01
	0.52	108.36	2:01
	0.26	54.18	2:01
C	6.20	2572.13	1:01
	3.10	1286.06	1:01
	1.55	643.03	1:01
	0.78	321.52	1:01
	0.39	160.76	1:01
	0.19	80.38	1:01
D	4.13	3410.57	1:02
	2.07	1705.28	1:02
	1.03	852.64	1:02
	0.52	426.32	1:02
	0.26	213.16	1:02
	0.13	106.58	1:02
E	0.00	14.20	–
	0.00	35	–
	0.00	70	–
	0.00	300	–
	0.00	700	–
	0.00	1400	–
	0.00	3500	–
	0.00	7000	–
	0.00	20000	–

The fixed ratio is around the combination of the EC_{50} values obtained individually for both chemicals

Statistical analysis and software used

Microsoft's Excel spreadsheet was used to perform calculations. Data are presented as mean ± standard error. The dose–response curves and the EC_{50} values were obtained using SigmaPlot for Windows 12.5 (Systat Software, Inc., Chicago). In the isobolographic analysis, the statistical analysis of the data was performed using the unpaired Student's *t* test to compare the significance between the theoretical additive EC_{50} values and the experimentally derived EC_{50}values. Differences were considered significant with $p < 0.05$. The absence of a significant difference between experimental and theoretical values was interpreted as no interaction and, therefore, an additive relationship in the combination. Statistical analyses were performed using the SPSS statistical package (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

Visual MINTEQ software (Version 3.1) for Windows (Gustafsson 2013) was used to calculate the theoretical ionic strength and the distribution of cadmium species.

RESULTS AND DISCUSSION

The present work was carried out to study the type and the degree of interaction between cadmium (II) and sodium sulphate using the freshwater microalga *C. moewusii* as test microorganism. Combinations with different ratios of both chemicals were bioassayed to evaluate the combined effect of both by means of an isobolographic analysis. Since it is a proven fact that environmental factors affect the cadmium uptake and, therefore, its toxic effect on the aquatic organisms, it is interesting to understand the interaction between cadmium and sodium sulphate from an environmental point of view, especially because of the current increase of the concentrations of cadmium and sodium sulphate in the environment due to pollution. The experiments were performed with S-deprived *C. moewusii* cells for 21 days. In these conditions, the cells are more sensitive to the sulphate variations due to changes in regulatory elements (González-Ballester et al. 2010). This allows to assess more adequately the effect of different bioavailabilities of sulphate (from its absence until high concentrations) and how this variation affects the toxicity of cadmium.

Sodium sulphate at sub-optimal concentrations

Individual effects of sodium sulphate and cadmium

Sulphate is an essential nutrient for autotrophic organisms and therefore is required for their growth (Giordano et al. 2005). It is the primary source of sulphur for these organisms, and for this reason, the growth of the microalga *C. moewusii* improved when the concentration of this compound increased, reaching a maximum value in the optimal concentration (14.2 mg/L of sodium sulphate). With this in mind, it was possible to calculate the effective concentration that allowed to achieve the 50 % growth compared to the growth obtained at the optimal concentration. Thus, sodium sulphate administered alone (without cadmium, group E in Table2) in the culture medium of this microalga at concentrations ranging between 0.04 and 14.2 mg/L produced a clear increase in the final cell density of the cultures, and therefore a decrease in the percentage of inhibition with respect to the optimal concentration. The EC_{50}(96 h) obtained from the dose–response curve (Fig. 1) was 1.14 ± 0.05 mg/L of sodium sulphate. That is, 1.14 mg/L of sodium sulphate allowed to reach half of the growth achieved in the cultures with the optimal concentration of this compound.

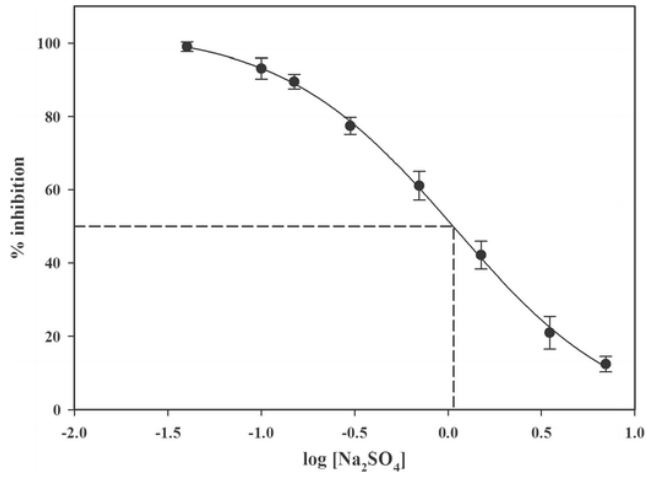

Fig. 1 Dose–response curve for sodium sulphate at sub-optimal concentrations (<14.2 mg/L) in*C. moewusii* cells after 96 h of growth. The *dashed line* indicates the value of the EC_{50}. Each data point represents mean ± standard error ($n = 3$)

Conversely, cadmium is considered one of the most toxic elements to living organisms (Hasanuzzaman and Fujita 2013). When cadmium was administered at concentrations ranging between 0 and 6 mg/L and with a sodium sulphate

concentration of 0.04 mg/L (group A in Table 2), and EC_{50} (96 h) of 0.11 ± 0.02 mg Cd(II)/L in the growth of this microalga (Fig. 2) was produced. In these experiments, it was necessary to use a minimum concentration of sodium sulphate in order for the microalga to grow, and thus calculate the EC_{50} for cadmium. As indicated above, sulphate is an essential nutrient and its absence would avoid the growth of the microalga, making impossible the study of the interaction. For this reason, it was decided to use the concentration of 0.04 mg sodium sulphate/L because the growth achieved was enough to obtain a suitable dose–response curve for cadmium, calculate the EC_{50}, and use this value in the construction of the isobologram.

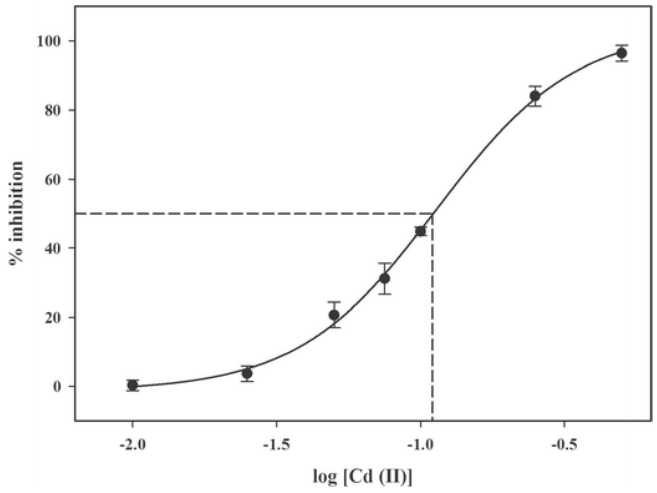

Fig. 2 Dose–response curve for the inhibition of growth in *C. moewusii* after 96 h of exposure to different cadmium (II) concentrations and with a sodium sulphate concentration of 0.04 mg/L. The *dashed line* indicates the value of the EC_{50}. Each data point represents mean ± standard error ($n = 3$)

Combined effect of cadmium and sodium sulphate

The EC_{50} values obtained individually for both chemicals were used in the construction of the isobologram. The isobolographic analysis of interaction, based in three different combinations of both EC_{50}, revealed that the mixtures of sodium sulphate at sub-optimal concentrations and cadmium (II) exerted a strong antagonistic interaction. The experimentally derived values of EC_{50} of the combinations (B_{exp}, C_{exp}, D_{exp}) for the three fixed ratios were distinctively ($p < 0.01$ and $p < 0.001$) above the Loewe additivity line on the isobologram (Fig. 3). Such antagonistic effects

were further confirmed with the interaction index γ, with values significantly >1, between 1.46 and 3.4 (Table 4). These values show that the antagonistic interaction was high in the range of sub-optimal sodium sulphate concentrations with a stronger antagonistic effect in the combination 2:1. From this result, it can be deduced that the presence of increasing concentrations of sodium sulphate mitigated the toxic effect of cadmium.

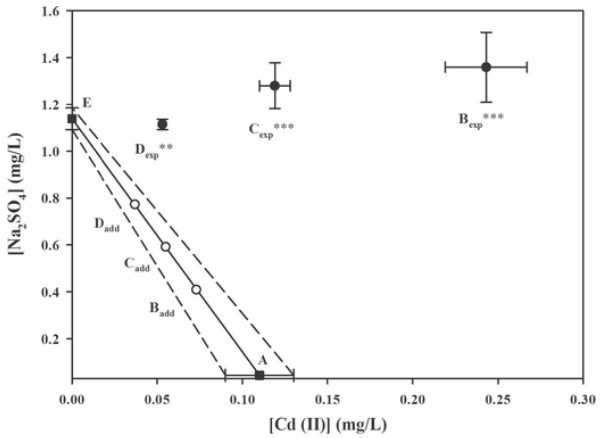

Fig. 3 Isobolographic analysis of the interaction between cadmium (II) and sodium sulphate at sub-optimal concentrations. The individual EC_{50} (*square*), the theoretical calculated EC_{50}value (Z_{add}) for each fixed ratio (2:1, 1:1, and 1:2; B_{add}, C_{add}, and D_{add}, respectively) (*open circle*), and its corresponding experimental EC_{50} values (B_{exp}, C_{exp}, and D_{exp}) (*filled circle*) are represented in the figure. *Horizontal* and *vertical bars* indicate S.E.M. The values of Z_{exp} were significantly different from Z_{add} (**$p < 0.01$, ***$p < 0.001$), indicating a strong antagonistic interaction for the three studied combinations

Table 4 Summary of the isobolographic analysis of interaction between cadmium (II) and sodium sulphate at the three fixed ratios tested and for the two ranges of sodium sulphate concentrations

Range of sodium sulphate concentrations	Fixed ratio	Theoretical EC_{50}	Experimental EC_{50}	Interaction index
Sub-optimal (<14.2 mg/L)	B (2:1)	0.48 ± 0.15	1.60 ± 0.17***	3.40 ± 0.35
	C (1:1)	0.65 ± 0.13	1.40 ± 0.11***	2.21 ± 0.17
	D (1:2)	0.81 ± 0.10	1.17 ± 0.02**	1.46 ± 0.03
Supra-optimal	B (2:1)	435.49 ± 83.05	231.36 ± 28.72*	0.54 ± 0.07
(>14.2 mg/L)	C (1:1)	644.58 ± 82.25	386.18 ± 51.22*	0.60 ± 0.08
	D (1:2)	853.67 ± 81.45	542.92 ± 5.74*	0.64 ± 0.01

Data are presented as EC_{50} values (mg/L) ± S.E.M. Statistical analysis was performed with Student's *t* test

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. the respective theoretical additive groups

It is a known fact that numerous internal and external factors influence the toxicity of metals (Rai et al. 1981). The bioavailability and toxicity of these elements is strongly dependent of two main factors, chemical speciation (Langston 1990; Niyogi and Wood 2004; Veltman et al. 2010) and mechanisms of tolerance that the organisms may have to counteract this toxicity (Hossain et al. 2012; Torres et al. 2013). With this in mind, sulphate is an important nutrient that must be taken into account as a factor influencing cadmium toxicity (Alves de Oliveira et al. 2009; Anjum et al. 2008; García-García et al. 2012; Mendoza-Cózatl et al. 2005; Nocito et al. 2006). The results of the present work indicate that this compound (at sub-optimal concentrations) acts by counteracting the toxicity of this metal through a very significant antagonistic effect (Table 4). Under conditions of very low concentrations of sodium sulphate, this microalga was more susceptible to cadmium toxicity. When the sodium sulphate concentration was only 0.04 mg/L, the EC_{50} for cadmium was 0.01 ± 0.02 mg Cd(II)/L (Fig. 2), while when the concentration of sodium sulphate was the optimal (14.2 mg/L), this EC_{50} value increased up to 3.1 ± 0.21 mg Cd(II)/L (Fig. 4). This antagonistic effect of the combinations of cadmium with sodium sulphate can also be observed on the isobologram (Fig. 3), where the experimental EC_{50}values of the combinations (B_{exp}, C_{exp}, D_{exp}) were above the theoretical additive EC_{50} values (B_{add}, C_{add}, D_{add}) and were significantly different ($p < 0.01$ or $p < 0.001$, Table 4).

This antagonistic effect can be explained because when cadmium is present in the medium, sulphate is the source of sulphur necessary for the biosynthesis of phytochelatins. Phytochelatins are polypeptides, synthesized from glutathione (GSH) that acts as an intracellular mechanism of defense against cadmium toxicity, chelating this metal and making it unavailable to the cellular targets (Cobbett 2000; Grill et al. 1987; Mishra et al. 2006; Pérez-Rama et al. 2006; Vatamaniuk et al. 2000). Since these compounds and their precursor (GSH) are rich in cysteine (a sulphur amino acid), their biosynthesis is directly influenced by the bioavailability of sulphate (Carfagna et al. 2011; García-García et al. 2012). In fact, the expression of genes involved in reductive sulphate assimilation pathway and its enzyme activities are stimulated by cadmium in plants (Ernst et al. 2008). Under conditions of sulphur deficiency, the amount of sulphur amino acids (methionine and cysteine) limits the

biosynthesis of proteins and GSH (Ahmad and Abdin 2000; Bochenek et al. 2013). Higher bioavailability of sulphate involves a higher cysteine biosynthesis and therefore higher possibilities for the biosynthesis of GSH and phytochelatins. In fact, it is known that cadmium interacts with the sulphur assimilatory pathway and upregulates the genes involved in the biosynthesis of GSH and phytochelatins (Mendoza-Cózatl et al. 2005). There is evidence suggesting that freshwater organisms are affected by cadmium at lower concentrations than marine organisms (Baścik-Remisiewicz et al. 2011; Folgar et al. 2009; Källqvist 2009; Pérez-Rama et al. 2006; Suárez et al.2010; Torres et al. 2000). One of the reasons for this observation might have its basis precisely in this antagonistic interaction cadmium-sulphate; freshwater media generally have a lower bioavailability of sulphate than seawater.

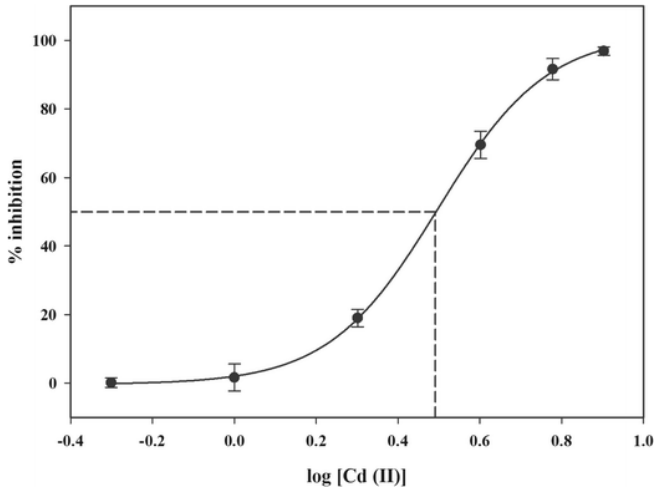

Fig. 4 Dose–response curve for the inhibition of growth in *C. moewusii* after 96 h of exposure to different cadmium (II) concentrations and with a sodium sulphate concentration of 14.2 mg/L. The *dashed line* indicates the value of the EC_{50}. Each data point represents mean ± standard error ($n = 3$)

This antagonistic effect, as a mechanism that reduces the toxicity of cadmium, was also observed with other inorganic nutrients in several autotrophic organisms. This is the case of calcium (Rivetta et al. 1997; Suzuki 2005; Wang and Song 2009) and micronutrients as zinc (Cataldo et al. 1983; Lavoie et al. 2012) and selenium (Filek et al. 2008; Zembala et al. 2010) because their addition to the nutrient solution leads to decrease the uptake of cadmium and its accumulation into cells, or in some cases, these nutrients can also exert a protective effect through their antioxidative activity. In this regard, the cadmium removal capacity of *Pichia kudriavzevii* also

decreased significantly after a preincubation with sodium chloride (Ma et al.2015). In this case and in the present study with *C. moewusii*, sodium appears to be another important cation in reducing cadmium toxicity; the increase of this cation with the increase of the concentration of sodium sulphate could additionally contribute to the reduction of this toxicity. In fact, studies with *Amaranthus mangostanus* showed that the competition between sodium and cadmium for the passage through calcium ion channels might explain a low uptake of cadmium (Mei et al. 2014). In addition, it has been demonstrated in different plants that the increase of sodium chloride reduces the toxicity of cadmium through several possible mechanisms such as the increase of sodium concentration, the reduction of cadmium uptake, and with this, the reduction of oxidative stress generated by this metal (Mariem et al. 2014; Zhang et al. 2013). In the case of sodium sulphate, these mechanisms could act in a similar way, but there would be an additional mechanism, as indicated above; sulphate improves the biosynthesis of phytochelatins (Mera et al. 2014). Therefore, it would be logical to think that sodium sulphate at appropriate concentrations has a stronger effect than sodium chloride in decreasing the effects of cadmium toxicity. In the present work, a strong antagonistic effect was obtained. Considering this and in a general sense, different studies with plants have showed that the severity of the cadmium toxicity can be reduced through the optimization of macronutrients and micronutrients, which induce physiological responses for the mitigation of this stress (Nazar et al. 2012). All this suggests that sulphate at appropriate concentrations should be added to the list of compounds that act by reducing the toxic effects of cadmium in aquatic autotrophic organisms.

Sodium sulphate at supra-optimal concentrations

Surprisingly, the opposite phenomenon was observed at the zone of higher concentrations of sodium sulphate, above the optimal concentration. In this zone, both cadmium and sodium sulphate were toxic for the microalga and interacted not as a simple additive interaction but synergistically.

Individual effects of sodium sulphate and cadmium

When sodium sulphate was used in concentrations above the optimal, the obtained EC_{50} in absence of cadmium (experiment E, Table 3) was

1271.86 ± 79.27 mg/L of sodium sulphate (Fig. 5). In this case, sodium sulphate acted as a toxic compound. It is a known fact that excessive levels of nutrients may be toxic to the organisms. Thus, elevated sodium sulphate concentrations can cause detrimental effects on aquatic ecosystems due to the creation of an unsustainable osmotic imbalance between autotrophic organisms and their surrounding environment (Davies 2007).

In the case of cadmium, administered alone, the reference to study its toxicity was set at the optimal concentration of sodium sulphate because this was also the minimum concentration of this compound in this range. Thus, the experiment to obtain the EC_{50} for cadmium (experiment A, Table 3) was performed with a sodium sulphate concentration of 14.2 mg/L. The EC_{50} value obtained under these conditions was 3.1 ± 0.21 mg Cd(II)/L (Fig. 4). This result agrees with the values obtained in other studies with the same microalga exposed to this metal (Mera et al.2014; Suárez et al. 2010).

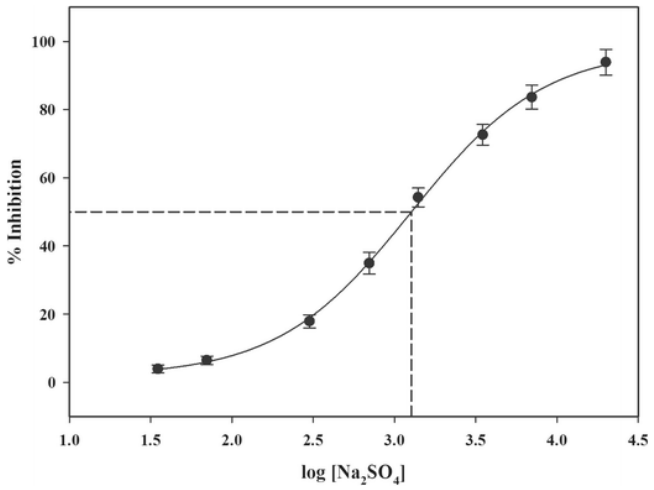

Fig. 5 Dose–response curve for sodium sulphate at supra-optimal concentrations (>14.2 mg/L) in *C. moewusii* cells after 96 h of growth. The *dashed line* indicates the value of the EC_{50}. Each data point represents mean ± standard error ($n = 3$)

Combined effect of cadmium and sodium sulphate

As in the previous isobologram, the new obtained EC_{50} values were used in the construction of the isobologram, and to determine the type of interaction and the interaction factors, again three different fixed ratios were assayed. The isobolographic

analysis of the combination (Fig. 6) shows a synergistic effect between both chemicals. All the points of the combinations appear to the left of the theoretical straight line of additivity on the isobologram, which is the characteristic patter of synergy. The values of the EC_{50} obtained in the combinations (B_{exp}, C_{exp}, D_{exp}) were significantly ($p < 0.05$) lower than those expected in a purely theoretical additive interaction (B_{add}, C_{add}, D_{add}). In addition, the interaction indices (γ) were also significantly <1 (Table 4). In this case, the significance was lower than in the case of the antagonism observed at sub-optimal concentrations of sodium sulphate and with values closer to 1. Therefore, the antagonistic effect between cadmium and sodium sulphate can be considered stronger than the synergistic effect.

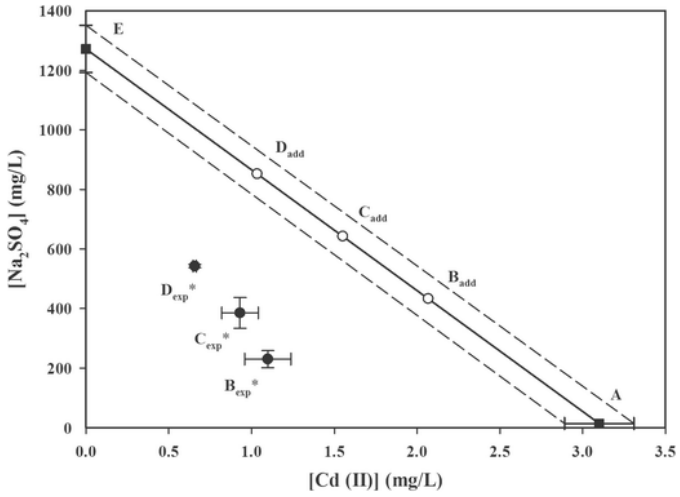

Fig. 6 Isobolographic analysis of the interaction between cadmium (II) and sodium sulphate at supra-optimal concentrations. The individual EC_{50} (*square*), the theoretical calculated EC_{50} value (Z_{add}) for each fixed ratio (2:1, 1:1, and 1:2; B_{add}, C_{add}, and D_{add}, respectively) (*open circle*), and its corresponding experimental EC_{50} values (B_{exp}, C_{exp}, and D_{exp}) (*filled circle*) are represented in the figure. *Horizontal* and *vertical bars*indicate S.E.M. The values of Z_{exp} were significantly different from Z_{add} (*$p < 0.05$), indicating a synergistic interaction for the three studied combinations

These data suggest that the excess of sodium sulphate enhances the toxic effect of cadmium through a synergistic effect between them. It is known that one of the mechanisms of cadmium toxicity is the deficiency generated in certain essential metals (Hasanuzzaman and Fujita 2013). If we consider that there are evidences indicating that sulphate at high levels inhibits the uptake of molybdenum, which

competes for the same specific transporter in the plasma membrane (Marino et al. 2003), the result of this would be an alteration in the cellular levels of several essential metal ions that could explain this synergism.

However, it is necessary to note that in the isobolographic experiments, at the same time that the sodium sulphate concentration varied, also varied the ionic strength of the medium from a value of 7.79 mM in the sodium sulphate concentration of 14.2 mg/L up to a value of 51.4 mM in the concentration of 3410.57 mg/L. Therefore, this synergistic effect may be due not only to the variation of sulphate but also to the variation of the ionic strength of the medium.

Effect of different concentrations of sulphate ion at the same ionic strength

Therefore, since in these experiments there was a variation in the ionic strength of the medium as a result of adding different sodium sulphate concentrations, it was decided to carry out experiments varying the sulphate concentration but maintaining the ionic strength constant in order to determine whether the sulphate or the ionic strength is responsible of the increased toxicity.

Figure 7 shows the response of the microalga to different concentrations of sulphate at the same ionic strength with 0 and 2 mg Cd(II)/L. It can be seen that, after 96 h of culture, at sub-optimal concentrations of sulphate, this compound, as expected, acted as a nutrient. The final cell density of the cultures increased with the increase of sulphate concentration in the medium. However, the cell density at supra-optimal concentrations remained constant despite the increase in the concentration of this anion (Fig. 7a), where with these concentrations it was expected a toxic effect (Fig. 5). Thus, the range of tested concentrations of sulphate ion (up to 1000 mg/L) at ionic strength of 37 mM showed no toxic effect on the final density of *C. moewusii*. Therefore, the toxicity observed with the increase of sodium sulphate concentration above the optimum was not due to the increase in the sulphate ion concentration, but it was due to the increase in the ionic strength. However, this result was different when the culture medium had 2 mg Cd(II)/L (Fig. 7b). In the supra-optimal range of concentrations of sulphate, the final cell density decreased as the concentration of sulphate ion increased.

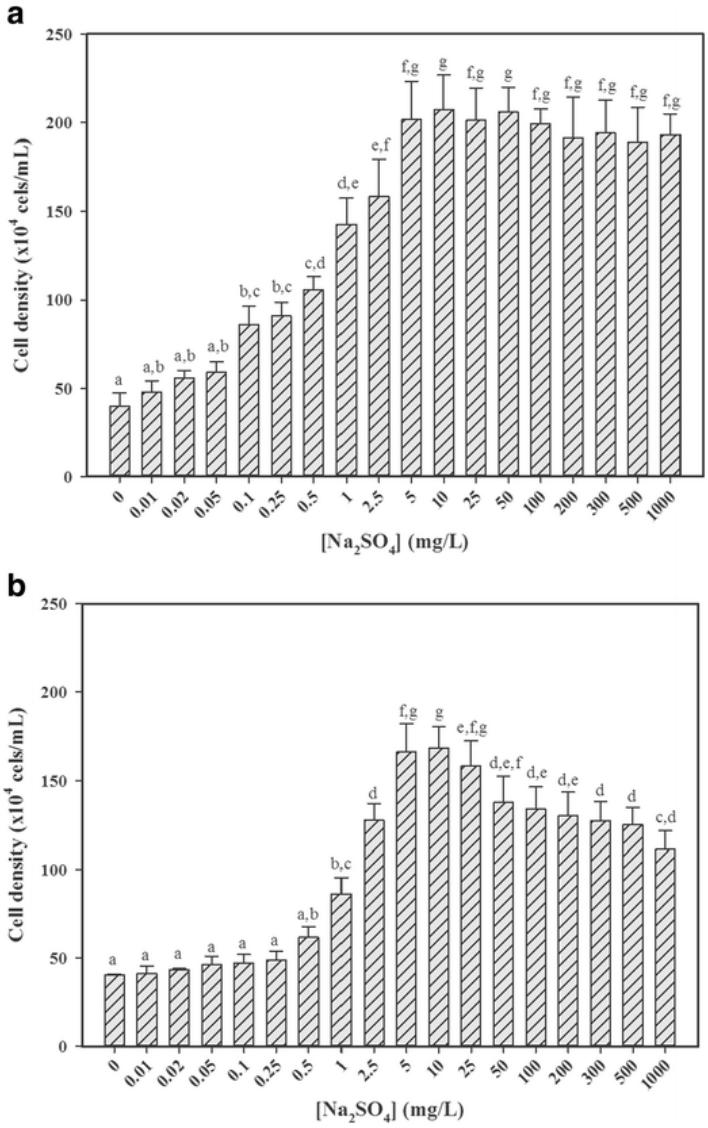

Fig 7 Effect of different sulphate concentrations at the same initial ionic strength with 0 mg Cd(II)/L (**a**) and 2 mg Cd(II)/L (**b**) on the final cell density of *C. moewusii* after 96 h of culture. The *x*-axis is not to scale. Different *letters* mark values significantly different at $p < 0.05$

It is important to highlight that the increase in the observed cadmium toxicity due to the increase in ionic straight was not a simple additive effect. The increase of ionic strength due to the addition of sodium sulphate above the optimum showed a cadmium toxicity higher than expected in an additive effect. This fact is easily deductible from the isobolographic analysis (Fig. 6 and Table 4). A similar result was obtained with sodium chloride in plants of *Triticum aestivum*; the combined effect of sodium chloride and cadmium was larger than that of both sodium chloride and

cadmium alone (Shafi et al. 2009). One of the important factors influencing the toxicity of a metal in the aquatic environment is speciation. The biotic ligand models (BLMs) predict metal toxicity by accounting for metal speciation in the abiotic environment and competitive binding of protective cations at the site of toxic action (Niyogi and Wood 2004; Veltman et al. 2010). For this reason, the initial distribution of cadmium species in the two blocks of experiments and for the three tested fixed ratios was predicted using the Visual MINTEQ software. Figure 8 shows the proportion, expressed as a percentage, of the main species of cadmium found under the conditions of culture used (Table 1). To make this graph, the concentrations of both chemicals, corresponding to the values of EC_{50} of the combinations obtained experimentally (B_{exp}, C_{exp}, D_{exp}), were chosen as representative for each fixed ratio. The cadmium speciation showed that Cd^{2+}, $CdHPO_4$, $CdCl^{+}$, and Cd-SO_4 complexes were the dominant forms in the nutrient solution (>95 %). It is interesting to note that in the supra-optimal region of sodium sulphate appear two species of cadmium, $CdSO_4$ (aq) and $Cd(SO_4)_2^{2-}$, which do not appear in the sub-optimal region. The activity of Cd-SO_4 complexes increased significantly with the increase of sulphate concentration in the solution. Taking into account that the synergistic toxic effect is localized in this range, these species could be related to that effect. These species of cadmium could be more easily assimilated by the microalga than others, as $CdHPO_4$, which is also in a high percentage. In fact, the percentage of $CdHPO_4$ and of Cd^{2+}decreased with the increase of the sulphate concentration in the culture medium due to the formation of Cd-SO_4 complexes; however, despite this reduction, the toxic effect of cadmium did not decrease. As shown in Figure 9, the significant appearance of these complexes occurred at concentrations of sodium sulphate above the optimal. This increase in the Cd-SO_4 complexes would lead to a further increase in the intracellular content of cadmium and in its toxicity. In this case, the concomitant increase of the sodium concentration would have less effect than that obtained in the sub-optimal region of sodium sulphate. That is, as discussed above, the high levels of sodium would prevent the entry of cadmium ions into the cell through channels of calcium; however, the decline of free-cadmium species in favor of the Cd-SO_4 complexes would prevent that this mechanism of tolerance was effective. These complexes could enter the cell through another mechanism with which the intracellular concentration of cadmium would increase. Although, in these conditions, sulphate also would be entering the cells together with cadmium, the biosynthesis of

GSH and phytochelatins would not be fast enough to counteract the amount of cadmium that would be entering. In addition, it should be taken into account that the cells would be more damaged by their own toxic effect of the excess of sodium sulphate in the medium. In fact, in plants, there are data that suggest that the $CdSO_4^0$ complexes are taken with equal efficiency than the free Cd^{2+} ion (McLaughlin et al. 1998). Results obtained by Lopez-Chuken and Young (2010) suggest that, in addition to the free ion, $CdSO_4^0$ complexes are important factors in determining cadmium uptake in nutrient solution by maize plants. Higher nominal concentrations of sulphate in solution generally resulted in a higher cadmium accumulation by these plants. At sub-optimal level of sodium sulphate, the species of cadmium more assimilable and therefore considered more toxic would be only Cd^{2+} (Fig. 8); the other main species present in the solution ($CdHPO_4$) seems not to be so easily taken up by the cells. This would allow that the mechanisms of cadmium tolerance can work efficiently, even better with the increase of the sulphate concentration (antagonism) but reaching a limit with the significant appearance of the Cd-SO_4 species (synergism).

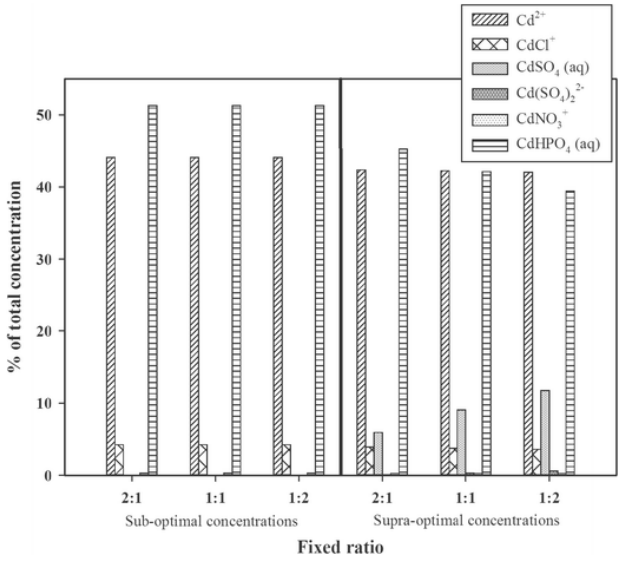

Fig. 8 Major fractions of cadmium (%) for each fixed ratio (cadmium/sodium sulphate) tested at sub-optimal and supra-optimal concentrations of sodium sulphate

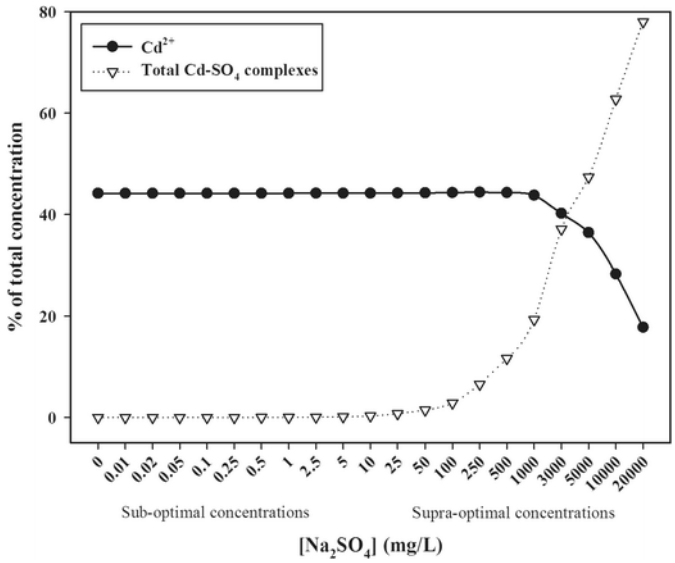

Fig 9 Visual MINTEQ simulation to investigate the initial speciation of cadmium as function of sodium sulphate concentration in the culture medium. The *x*-axis is not to scale

CONCLUSION

Results of the current study by an isobolographic analysis suggest that sodium sulphate was able to perform a dual antagonist/synergist role on cadmium toxicity in cells of *C. moewusii*. This dual role was concentration dependent. Sodium sulphate alleviated/aggravated cadmium toxicity in relationship to its concentration in the culture medium. The combinations of sodium sulphate at sub-optimal concentrations with Cd(II) exerted a strong antagonistic interaction, counteracting the toxicity of this metal. As the concentration of sodium sulphate increased, this compound not only promoted the growth of the microalga but, when cadmium was present in the medium, also promoted the tolerance mechanisms to this metal. However, the addition of concentrations of sodium sulphate above the optimal concentration showed a synergistic effect between both chemicals. This synergic effect resulted in the potentiation of the toxic effects of cadmium, synergy that seemed to be related to the increase of the ionic strength and of two species of cadmium, $CdSO_4$ (aq) and $Cd(SO_4)_2^{2-}$, in the medium. Therefore, the excessive increase in the concentration of sodium sulphate in the natural environments with presence of cadmium may potentiate the toxic effect of this metal.

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