Ecotoxicology
DOI 10.1007/s10646-015-1561-2

Interference of heavy metals on the photosynthetic response from a Cr(VI)-resistant *Dictyosphaerium chlorelloides* strain

A. D'ors[1] · A. A. Cortés[2] · A. Sánchez-Fortún[1] · M. C. Bartolomé[2] · S. Sánchez-Fortún[1]

Accepted: 28 September 2015

Abstract The successful selection of a particular type of bioelement and its association to the appropriate transducer determines the specificity of a biosensor. Therefore, from a strain of chloroficea *Dictyosphaerium chlorelloides*, modified in laboratory to tolerate high Cr(VI) concentrations, the possible interferences of other heavy metals on photosynthetic activity were studied. After exposing wild type and Cr(VI)-resistant cells to increasing Ag^{+1}, Co^{+2}, Hg^{+2}, Cr^{+3}, Cu^{+2}, Zn^{+2}, Fe^{+3} and Cd^{+2} concentrations, both photosynthetic quantum yields was compared. Photosynthetic electron transport rates were measured with a TOXY-PAM chlorophyll fluorometer, non-linear regression analysis of each of the toxicity tests was done, and means of both groups were compared using unpaired *t* test. The results show no significant differences between both cell types when they were exposed to Ag^{+1}, Co^{+2}, Hg^{+2}, Cr^{+3}, Cu^{+2}, Fe^{+3} and Cd^{+2} metal ions, and extremely significant differences ($p < 0.0001$) to Zn^{+2} exposures. These results demonstrate the suitability of this Cr(VI)-resistant type *D. chlorelloides* strain as a suitable bioelement to be coupled to a biosensor based on dual-head microalgae strategy to detect and quantify Cr(VI) in water courses and waste water treatment plants. However, some disturbance may be expected, especially when certain analyte species such as zinc are present in water samples tested. The analysis of binary mixtures between Zn^{+2} and other heavy metals showed a slight antagonistic phenomenon in all cases, which should not alter the potential Zn^{+2} interference in the Cr^{+6} detection process.

Keywords *Dyctiosphaerium chlorelloides* · Photosynthetic quantum yield · Selective bioelement · Cr(VI)-resistant strain · Heavy metals interference

✉ S. Sánchez-Fortún
fortun@ucm.es

[1] Department of Toxicology and Pharmacology, Faculty of Veterinary Sciences, Complutense University, s/n Puerta de Hierro Ave., 28040 Madrid, Spain

[2] School of Chemistry-Pharmacology, Michoacana de San Nicolás de Hidalgo University, 43 Santiago Tapia St., 58000 Morelia, Michoacán, Mexico

Introduction

Metals are introduced in aquatic systems as a result of the weathering of soils and rocks, from volcanic eruptions, and from a variety of human activities involving the mining, processing, or use of metals and/or substances that contain metal pollutants. During evolution, aquatic and terrestrial organisms have developed diverse strategies to maintain an equilibrated relation with heavy metal ions present and available in the surrounding medium. Cells face two tasks, the first is to select those heavy metals essential for growth and exclude those that are not, and the second to keep essential ions at optimal intracellular concentrations (Cobbett and Goldsbrough 2002).

Deterioration of the aquatic environment by pollutants is an important problem. Among all of them, heavy metals constitute a priority preoccupation: they are frequently found in surface and ground waters and are harmful to aquatic organisms (Davis et al. 2001; Stankovic and Jovic 2012). Environmental monitoring of heavy metals with automatic systems applied online and allowing rapid response is one of the best ways to control the quality of the environment. Biosensors mean a step forward in the quest for early-warning analytical systems (Kissinger 2005). They are able to carry out in situ on-line measurements,

Published online: 12 October 2015

replacing more sophisticated, expensive and slower laboratory analytical methods (Marks et al. 2007a, b).

Among the wide range of microorganisms which have been employed to develop biosensor technology, microalgae possess a number of features that suite perfectly the requirements of an early-warning environmental biosensor (Giardi and Piletska 2010). Microalgae are especially sensitive to all those pollutants that work as inhibitors at the photosystem II (PSII) level, such as heavy metals (Brayner et al. 2011).

Regardless the transducer employed, the lack of specificity is a common drawback of almost every microalgae biosensor described so far, owing to the large amount of pollutants that affect the microalgae metabolism. Some of the researchers developing microalgae biosensors have solved this problem by using a battery of different microalgae cells, on the basis that dissimilar species render distinct sensitivities to a particular toxicant (Podola et al. 2004; Peña-Vazquez et al. 2009; Tatsuma et al. 2009).

Numerous papers have reported that heavy metals have a rapid effect on microalgae, causing massive destruction of sensitive cells (Rai et al. 1981; García-Villada et al. 2004), and however some heavy metal-resistant algal variants may have succeeded by means of physiological or genetic mechanisms. The biological consequences of heavy metal contamination would be very different depending on the ability of microalgae to adapt to chromium. It seems that microalgal populations can be tolerant to the presence of heavy metals if they have had previous exposures (Corradi et al. 1995, 1998).

Previous studies demonstrated that wild-type *Dictyosphaerium chlorelloides* was very sensitive to the effect of hexavalent chromium [Cr(VI)], but it was verified that after destruction of most algae following to these chromium exposures, some rare, resistant algal variants are able to grow in an environment with high levels of chromium (Sánchez-Fortún et al. 2009). However, these clonal cultures continued being very sensitive to trivalent chromium exposures, with similar inhibition ratios between wild-type and Cr(VI)-resistant *D. chlorelloides* strains (D'ors et al. 2010).

The aims of this work were to estimate the toxic effect of selected heavy metals on Cr(VI)-sensitive and Cr(VI)-resistant strains of the green algae *D. chlorelloides*, and to evaluate the specific tolerant level obtained by the Cr(VI)-resistant clone respect to other heavy metals.

Materials and methods

To determine the toxicity of metals (Ag^{1+}, Co^{2+}, Hg^{2+}, Cu^{+2}, Cr^{+3}, Zn^{2+}, Fe^{+3} and Cd^{2}), on the photosynthetic quantum yield rate of both strains, silver nitrate ($AgNO_3$), cobalt nitrate ($Co(NO_3)_2{\cdot}6H_2O$), mercury chloride ($HgCl_2$), chromium chloride ($CrCl_3{\cdot}6H_2O$), copper sulphate ($CuSO_4$), zinc sulphate ($ZnSO_4{\cdot}7H_2O$), cadmium nitrate ($Cd(NO_3)_2{\cdot}4H_2O$), and iron chloride ($FeCl_3{\cdot}6H_2O$) were used as ion donors. A range of nominal concentration of test medium was prepared (from 0.03 to 0.2 mg L^{-1} for Ag^{1+}, from 0.24 to 0.44 mg L^{-1} for Co^{2+}, from 0.20 to 0.90 mg L^{-1} for Hg^{2+}, from 1 to 10 mg L^{-1} for Cu^{2+}, from 1 to 5 mg L^{-1} for Cr^{+3}, from 2.30 to 14.80 mg L^{-1} for Zn^{2+}, from 4.50 to 13.50 mg L^{-1} for Fe^{3+} and from 3.70 to 29.00 mg L^{-1} for Cd^{2+}). All heavy metals analyzed in this work were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All drugs were dissolved in distilled water. In all assays, the working solutions were freshly prepared each time.

Hexavalent chromium sensitive (Dc^{wt}) and resistant ($Dc^{Cr(VI)R25}$) strains of *D. chlorelloides*, from the algal culture collection of the Environmental Toxicology Laboratory (Toxicology and Pharmacology Department, UCM, Madrid, Spain), were used in these assays. Dc^{wt} strain (wild type Dc1 M) grew axenically in culture flasks (Greiner, Bio-One, Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany), while $Dc^{Cr(VI)R25}$ *D. chlorelloides* strain was cultured in BG-11 medium supplied with 25 mg L^{-1} CrO_3. The BG-11 culture medium was obtained from commercial concentrate (×50), which was reconstituted with deionized water, adjusting the pH to 7.5 using 1 N HCl. After autoclaving and cooling the pH was about 7.1. Both strains were maintained at 21 °C under continuous light of 60 µmol m^{-2} s^{-1} over the 400–700 nm waveband. Cells were maintained in mid-log exponential growth by serial transfers of one-cell inoculums to fresh medium once a fortnight. Prior to the experiments, the cultures were recloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures.

The effect of selected heavy metals was estimated in both strains [sensitive and resistant Cr(VI) cells] after 72 h exposures, by mean photosynthetic quantum yield (Φ_{PSII}). This photosynthetic response was measured using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany). Preliminary toxicity tests were performed to define the range of concentrations that included 0 and 100 % response. With these data, serial dilutions in BG-11 medium were prepared to obtain specific concentrations for each heavy metal tested. For each dose, eight replicates were inoculated with 10^4 cells mL^{-1} from mid-log exponentially growing cell cultures.

The concentration causing 50 % of Φ_{PSII} inhibition in algae was evaluated according to the *area under the curve* method prescribed by International Organization for Standardization (ISO 1982). The 72-h IC_{50}-values were determined by nonlinear regression analysis, and all the results are expressed as mean ± SD values. Data were presented as inhibition percentage of Φ_{PSII} with respect to controls. Statistical analysis was performed using the computer software package GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

Additionally, the results were analyzed using the median-effect/combination index (CI)-isobologram equation (Chou and Talalay 1984; Chou 2006), which is based on the median-effect principle (Chou 1976), that demonstrates that there is an univocal relationship between dose and effect independently of the number of substrates or products and of the mechanism of action or inhibition. This method involved plotting the dose–effect curves for each compound and their combinations in multiple diluted concentrations. These parameters were used to calculate doses of the selected heavy metals and their combinations required to produce various effect levels. CI < 1, $=1$ and >1 indicates synergism, additive effect and antagonism, respectively. The analysis was performed through in the software package CompuSyn (ComboSyn Inc., Paramus, NJ, USA).

Results

Interference due to exposure to heavy metals on the photosynthetic activity exhibited by a Cr(VI)-resistant *D. chlorelloides* strain compared to *D. chlorelloides* wild type, have been investigated. The $IC_{50(72)}$ values obtained in each one of selected heavy metal exposures are summarized in Table 1, and in both *D. chlorelloides* strains the following toxicity range was achieved: $Ag^{1+} > Co^{2+} > Hg^{2+} >> Cr^{+3} > Cu^{2+} > Zn^{2+} > Fe^{+3} > Cd^{2+}$.

Nonlinear regression analysis performed on the photosynthetic quantum yield exhibited by both *D. chlorellides* strains in each heavy metal exposure are plotted in Fig. 1. Nonlinear regression analysis performed on the photosynthetic quantum yield exhibited by both *D. chlorellides* strains in each heavy metal exposure are plotted in Fig. 1. These results demonstrated that $Dc^{Cr(VI)R25}$ keeps the same range of sensitivity exhibited by Dc^{wt} for all heavy metals tested, except for Zn^{+2}. There were extremely significant differences ($p < 0.0001$) between nonlinear regression curves from $Dc^{Cr(VI)R25}$ and Dc^{wt} exposed to Zn^{+2}.

Table 2 shows the mean combination index (CI) values correspond to combinations between Zn^{+2} and other heavy metals obtained in the range of fraction affected (Fa) 0.1–0.9. These results indicate that all binary combinations assayed induced a significant antagonist effect.

Table 1 Comparison of 72-h IC_{50} values and their associated 95 % confidence limits (CL), expressed in mg L^{-1}, correspondent to the photosynthetic quantum yield analysis from wild type (Dc^{wt}) and Cr(VI)-resistant ($Dc^{Cr(VI)R25}$) strains of *Dyctiosphaerium chlorelloides* exposed to selected heavy metals

Heavy metal	N	$IC_{50(72)}$ (CL 95 %), (mg L^{-1})	
		Dc^{wt}	$Dc^{Cr(VI)R25}$
Ag^{1+}	8	0.09 (0.08–0.09)	0.09 (0.08–0.09)
Co^{2+}	8	0.36 (0.31–0.42)	0.32 (0.27–0.37)
Hg^{2+}	8	0.37 (0.33–0.43)	0.49 (0.41–0.58)
Cr^{+3}	8	3.41 (3.18–3.65)	2.91 (2.79–3.04)
Cu^{2+}	8	4.05 (3.56–4.62)	4.82 (4.18–5.56)
Zn^{2+}	8	6.13 (4.09–9.19)	8.57 (7.34–10.01)
Fe^{3+}	8	9.82 (9.41–10.24)	9.23 (8.61–9.90)
Cd^{2+}	8	12.38 (10.08–15.21)	10.51 (7.42–14.89)

Values represent the mean of eight experiments ($n = 8$)

Discussion

The aim of our study was to analyze the degree of specificity exhibited by a Cr(VI)-resistant *D. chlorelloides* strain exposed to other heavy metals, to be used as bioelement in a whole-cell biosensor system.

Our hypothesis is based on the concept already tested by Altamirano et al. (2004) through which different microalgae genotypes render distinct sensitivities to a particular toxicant. This concept can be applied on $Dc^{Cr(VI)R25}$ strain, which used in tandem in conjunction with the wild type strain (Dc^{wt}) would allow its potential application in a dual-head biosensor system for detecting hexavalent chromium. The difference between them is their opposite sensitivity to Cr(VI). Wild type *D. chlorelloides* (Dc^{wt}) strain exhibited high sensitivity to Cr(VI) whereas $Dc^{Cr(VI)R25}$ strain exhibited high resistance to Cr(VI) oxidation state. Thus, Dc^{wt} strain would provide the biosensor sensitivity while its specificity arises from the different responses of $Dc^{Cr(VI)R25}$ strain (Fig. 2).

Beyond the practical advantages of biosensors, their measurements need to be verified and validated before being accepted. Thevenot et al. (2001) recommended several analytical parameters to characterize biosensor performance, such as sensitivity, working and linear concentration range, limits of detection and of quantification, selectivity and reliability. Some of these aspects have already been previously studied for us on Cr(VI)-resistant *D. chlorelloides* strains (Sánchez-Fortún et al. 2009; D'ors et al. 2010) and however, the toxic effect of other heavy metals on $Dc^{Cr(VI)R25}$ strain and possible detection interferences in the biosensor system caused by the presence of these heavy metals in the water samples had not yet been examined.

According to Schreiber et al. (2002) Φ_{PSII} is a good indicator of the toxic effect of aquatic contaminants in microalgae. This method not only provides kinetic

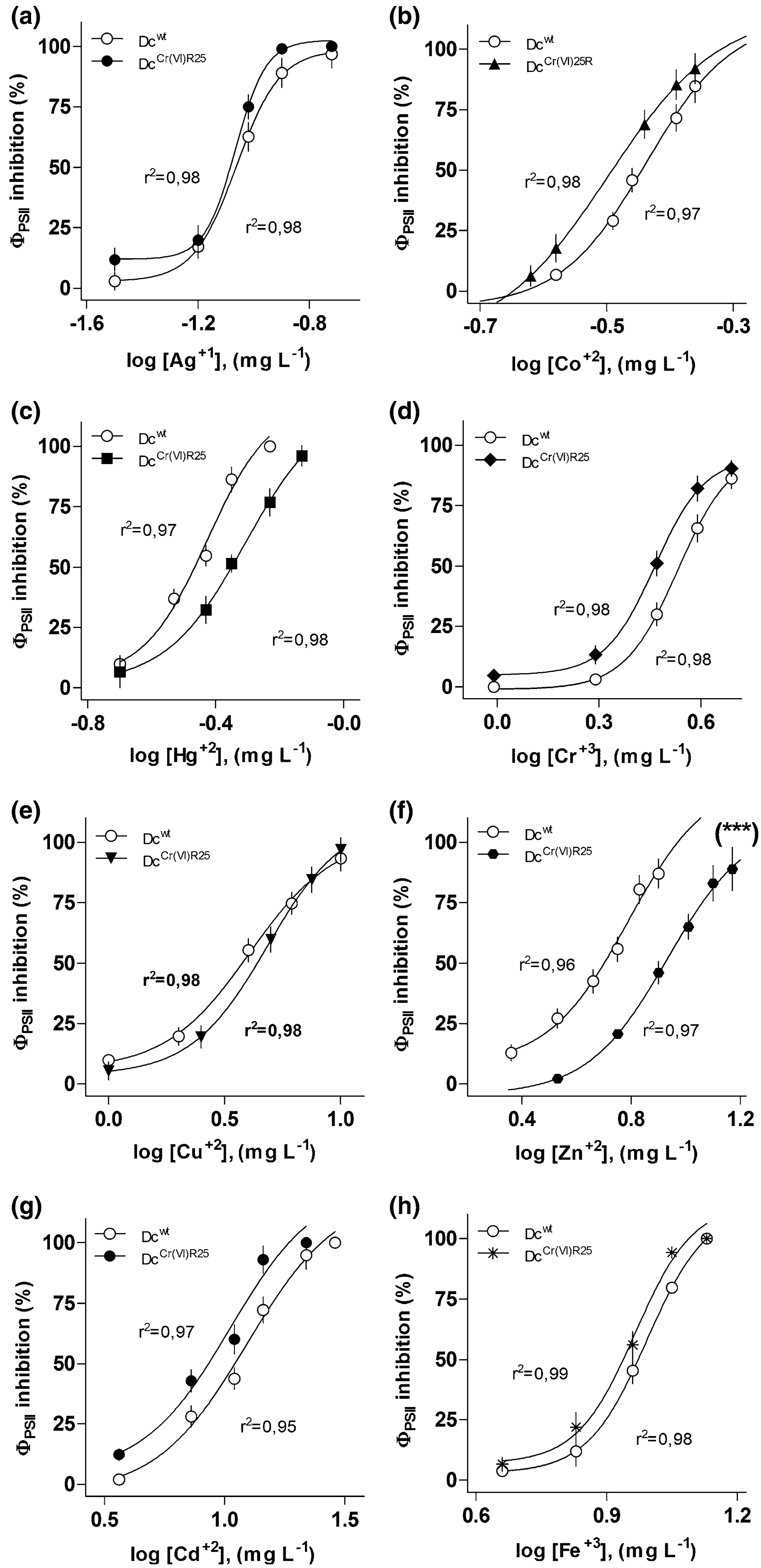

Fig. 1 Nonlinear regression analysis from $\Phi_{(PSII)}$ response induced by Ag^{+1} (**a**), Co^{+2} (**b**), Hg^{+2} (**c**), Cr^{+3} (**d**), Cu^{+2} (**e**), Zn^{+2} (**f**), Cd^{+2} (**g**) and Fe^{+3} (**h**) exposures on wild type (Dc^{wt}) and Cr(VI)-resistant ($Dc^{Cd(VI)R25}$) *D. chlorelloides* populations. Points represent means with *vertical lines* showing standard deviation ($n = 8$). (***) represents extremely significant differences ($p < 0.0001$) respect to Dc^{wt} values

Table 2 Combination index (CI) associated with affected fraction (Fa) obtained in binary combinations among Zn^{+2} and selected heavy metals

Metal	N	CI		
		Fa = 0.1	Fa = 0.5	Fa = 0.9
Ag^{+}	8	1.75 ± 0.65	1.97 ± 0.48	1.91 ± 0.87
Hg^{+2}	8	2.16 ± 0.32	2.01 ± 0.54	1.90 ± 0.91
Co^{+2}	8	2.56 ± 0.78	1.99 ± 0.41	1.65 ± 0.96
Cu^{+2}	8	2.44 ± 0.80	2.82 ± 0.67	3.34 ± 0.69
Cr^{+3}	8	2.01 ± 0.24	1.98 ± 0.58	1.95 ± 0.85
Cd^{+2}	8	2.25 ± 0.33	2.01 ± 0.88	1.81 ± 0.61
Fe^{+3}	8	1.87 ± 0.20	2.00 ± 0.80	2.18 ± 0.60

Values represent mean ± SD of four experiments ($n = 8$)

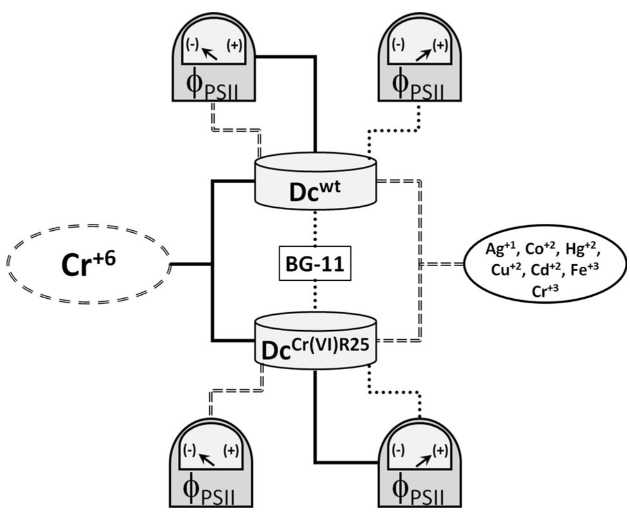

Fig. 2 Functional schematic diagram of a microalgal dual head biosensor containing Dc^{wt} and $Dc^{Cr(VI)R25}$ strains. *Dotted lines* represent negative signal, *broken lines* represent positive signal (Cr^{+6}), and the *solid lines* represent signal from other metallic ions

information on dark-light transitions, but also steady-state information on the photosynthetic performance of a sample in continuous light. As steady-state photosynthesis depends on the complex interplay of a large number of partial reactions at various levels of organization, it is affected by many more toxic substances than those inhibiting the primary reactions near to Photosystem II (PS II). So, because the specific Φ_{PSII} rate is considered a reproducible ecologically relevant response and not strongly test system specific, it was selected as an appropriate test endpoint.

The acute toxicity assays on $Dc^{Cr(VI)R25}$ and Dc^{wt} strains demonstrated a significant Φ_{PSII} decrease when they were exposed to the selected heavy metals analyzed. However, the results show that there are different sensitivity levels between heavy metals included in our study. Thus, Ag^{+1}, Co^{+2} and Hg^{+2} metal ions exhibit a very significant toxic effect on both *D. chlorelloides* strains, with $IC_{50(72)}$ values below 0.5 mg L^{-1}, whereas Cr^{+3}, Cu^{+2}, Zn^{+2} Fe^{+3} and Cd^{+2} metal ions have $IC_{50(72)}$ values between 3 and 13 mg L^{-1}. These sensitivity differences are consistent with those obtained on different phytoplankton species by other authors (Okamoto et al. 1996; Toress et al. 1997). These sensitivity differences are consistent with those obtained on different phytoplankton species by other authors, and some of them conclude that these toxicity differences between metal ions are due to some stimulating effect on cell growth associated with low concentrations of some heavy metals as zinc or cadmium (Morelli and Scarano 2001).

The similar toxic rate exhibited by both *D. chlorelloides* strains unequivocally demonstrate that $Dc^{Cr(VI)R25}$ strain has developed a specific tolerance for Cr(VI), maintaining intact the sensitive range to other metals, just as exhibited the wild-type Dc^{wt} strain. Previous studies demonstrated that the $Dc^{Cr(VI)R25}$ strain occurs within clonal cultures that have not been exposed previously to chromium (Sánchez-

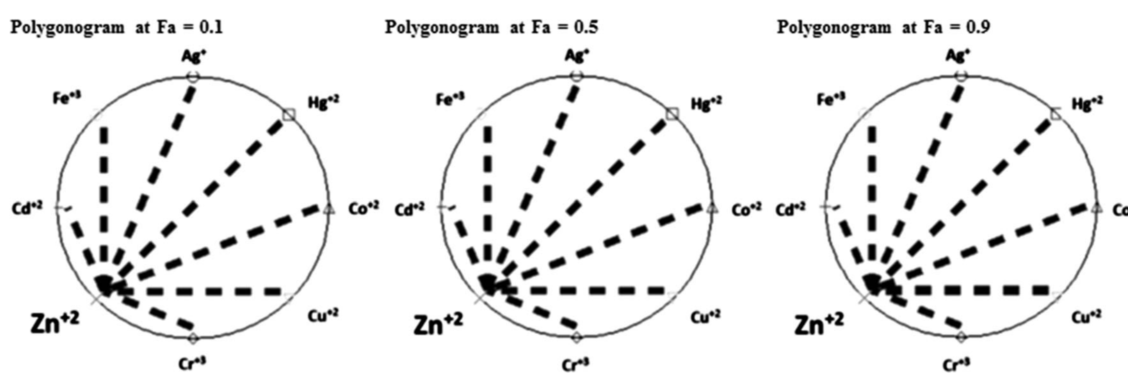

Fig. 3 Polygonal graphical representations corresponding to F = 0.1, F = 0.5 and F = 0.9, and extracted directly from the report generated by the software package Compusyn. According to the code used by this computer package, *broken lines* represent antagonistic effect among the metal ions through them connected

Fortún et al. 2009). Consequently, the presence of Cr(VI)-resistant cells in populations of *D. chlorelloides* is caused by a rare, spontaneous mutation that occurs before Cr(VI) exposure. Additionally, D'ors et al. (2010) demonstrated that $Dc^{Cr(VI)R25}$ strain continue being sensitive to Cr(III), and therefore both *D. chlorelloides* strains exhibited significant differences depending of the oxidation state of chromium compound.

Despite the similarity in response between the two strains is generalized to most studied heavy metals, non-linear regression analysis has shown statistically significant differences respect to Zn^{+2} being Cr(VI)-resistant cells are also more resistant to Zn^{+2} (Fig. 1f). Despite this difference, our results tentatively indicate that Φ_{PSII} exhibited by Dc^{wt} and $DC^{Cr(VI)R25}$ strains in presence of different heavy metals, included Cr(III), could be used to obtain a Cr(VI)-specific microalgal biosensor.

To date, there have been a considerable number of papers concerning the use of immobilized algae in the development of specific biosensors (Robinson et al. 1985; González and Bashan 2000; Abdel Hameed 2002), but unfortunately, the models performed with this technique were not capable to differ among the different heavy metals (Durrieu and Tran-Minh 2002; Banerjee et al. 2004). However, our results indicate that is possible to increase the specificity of these biosensors based on the use of a sensitive genotype (Dc^{wt}) and a resistant mutant ($Dc^{Cr(VI)R25}$) by mean the use of the differential response in photosynthetic activity. In this way, Cr(VI), Cr(III) and other heavy metals including in water samples, could be analyzed and identified by continuous monitoring.

Additionally, the combination index method (CI) was applied on the short-term toxicity results obtained from DcCr(VI)R25 exposed to selected heavy metals. The CI is a fractional approach method used in pharmacology to analyze the combined effect of drugs (Bijnsdorp et al. 2011), it was developed and mathematically derived from enzyme-substrate kinetics by Chou (2006) and their definition of additivity is essentially derived from the classical definition of isobologram.

This method is independent of any consideration on the mode of action of the substances under analysis. It can be used to analyze and quantify the degree of the deviation from additivity (synergism or antagonism) of two to n substances with similar, dissimilar or unknown mode of action at any level of effect exerted on any organism (Chou 2006).

In this work, the CI method was applied to study the interaction exhibited by several mixtures of heavy metals and possible interference on a whole-cell biosensor system containing Dc^{wt} and $Dc^{Cr(VI)R25}$ microalgal strains. As the results show that Zn^{+2} was the only metal ion capable of interfering with the process, only binary mixtures with Zn^{+2} have been studied.

In all Zn^{+2}-heavy metal mixtures analyzed, CI values between 1 and 3 were obtained. These results show that all mixtures analyzed causing a weak antagonic phenomenon (Fig. 3). However, some recently reviews about this issue state that deviations from additivity lesser than a factor of 2 should not be considered as a significant deviation from additivity for risk assessment purposes, but it is still a matter of discussion (Kortenkamp et al. 2009; Spurgeon et al. 2010; Boobis et al. 2011). For this reason, and because the low antagonistic activity is shared by all binary mixtures studied, it is likely that the presence of several heavy metals into the wastewater samples tested do not modify the interference exhibited by zinc.

Conclusions

In summary, the combined use of Dc^{wt} and $Dc^{Cr(VI)R25}$ strains as bioelements integrated in a Cr(VI) biosensor detector would have capacity to identify the presence of this oxidation state of chromium in aquatic monitoring without most other heavy metals interfere in the monitoring process. Furthermore, the ability to obtain in laboratory similar resistant *D. chlorelloides* strains to detect other heavy metals can lead the way in developing new methodologies for continuous monitoring of heavy metals in wastewater.

Acknowledgments This study has been supported by Spanish Government (CGL 2008-00652/BOS), the Madrid Community Government (S-505/AMB/0374), and the Mexican UMSNH University (PI 26.8). The technical support of Juan José Garcia and Lara de Miguel is kindly acknowledged.

Conflict of interest The authors declare that they have no conflict of interest.

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