Title: Suitability of cytotoxicity endpoints and test microalgal species to disclose the toxic effect of common aquatic pollutants

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Corresponding Author: Dr. Angeles Cid, dr.

Corresponding Author's Institution: University of A Coruña

First Author: Raquel Prado, Ph.D.

Order of Authors: Raquel Prado, Ph.D.; Rosa García; Carmen Rioboo, Ph.D.; Concepción Herrero, Ph.D.; Angeles Cid, dr.

Abstract: Pulse discharges of chemicals to aquatic environments may lead to high concentrations of them in surface waters for short periods of time, but enough to induce toxic effects on aquatic organisms; however, no many methods allow an early warning of toxicity of these agents. Acute effects of one representative chemical from each of three of the main groups of aquatic pollutants (pesticides, metals and pharmaceuticals) are studied on two green microalgal species (Chlamydomonas moewusii and Chlorella vulgaris). Flow cytometry protocols were used to detect the potential application of chlorophyll a fluorescent emission, cell viability, metabolic activity and membrane potential as cytotoxicity endpoints, besides an epifluorescence microscopy protocol for comet assay to detect genotoxicity level of cells. Obtained results confirm the suitability of them for the prospective assessment of the potential cytotoxicity of these aquatic pollutants. The two microalgal species analysed could be used as indicators in toxicity bioassays, being C. moewusii more sensitive than C. vulgaris. Among cell parameters assayed, the metabolic activity and the primary DNA damage stood out as sensitive cytotoxicity endpoints.

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Suitability of cytotoxicity endpoints and test microalgal species to disclose the toxic effect of common aquatic pollutants

Raquel Prado[†], Rosa García, Carmen Rioboo, Concepción Herrero, Ángeles Cid *

Laboratorio de Microbiología, Facultad de Ciencias, Universidad de A Coruña. Campus de A Zapateira s/n, 15008 A Coruña, Spain.

([†]) Deceased 11 January 2013

* Corresponding author. Tel.: +34 981 167 000; fax: +34 981 167 065. E-mail address: angeles.cid@udc.es

Abstract

Pulse discharges of chemicals to aquatic environments may lead to high concentrations of them in surface waters for short periods of time, but enough to induce toxic effects on aquatic organisms; however, no many methods allow an early warning of toxicity of these agents. Acute effects of one representative chemical from each of three of the main groups of aquatic pollutants (pesticides, metals and pharmaceuticals) are studied on two green microalgal species (*Chlamydomonas moewusii* and *Chlorella vulgaris*). Flow cytometry protocols were used to detect the potential application of chlorophyll *a* fluorescent emission, cell viability, metabolic activity and membrane potential as cytotoxicity endpoints, besides an epifluorescence microscopy protocol for comet assay to detect genotoxicity level of cells. Obtained results confirm the suitability of them for the prospective assessment of the potential cytotoxicity of these aquatic pollutants. The two microalgal species analysed could be used as indicators in toxicity bioassays, being *C. moewusii* more sensitive than *C. vulgaris*. Among cell parameters assayed, the metabolic activity and the primary DNA damage stood out as sensitive cytotoxicity endpoints.

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Introduction

The many ecological disturbances in aquatic ecosystems linked to anthropogenic pressures are well documented (Amado et al., 2006; Corrêa et al., 2009; Moreira-Santos et al., 2004). The widespread use of pesticides has led to these substances be ubiquitous in aquatic environments (Eullaffroy and Vernet, 2003; Kumari et al., 2007). Metals are also major pollutants of aquatic ecosystems, mainly due to disposal of industrial effluents or mining activity (Franklin et al., 2000; Pan and Wang, 2012). Pharmaceutically active substances have been recognised as an important environmental problem (Halling-Sorensen et al., 1998; Pomati et al., 2004).

The increasing concern about environmental pollution has led to the development of sensitive analytical methods to detect toxicity in water (Camacho-Muñoz et al., 2010; Infante et al., 2008; Kolpin et al., 1998; Núñez et al., 2002), but most of these techniques are expensive, time-consuming and cannot provide information of ecological relevance. Therefore, the development of convenient methods or parameters for the assessment of pollutant toxicity on aquatic organisms has become a major goal in ecotoxicological research (Lam and Gray, 2003).

Microalgae have been frequently used in ecotoxicological screening of contaminated water, and also as test microorganisms for *in vitro* toxicity bioassays. Several parameters can be measured to assess the effects of toxicants on microalgae, being growth and photosynthetic activity the most commonly monitored (Cleuvers, 2003; Shabana et al., 2001; Yang et al., 2002).

Previous studies of the toxic effects of different herbicides on microalgal physiology (Prado et al., 2011; Prado et al., 2012a, 2012b; Rioboo et al., 2009) demonstrate that flow cytometric analysis of different microalgal cell responses can be an alternative to standard algal population-based endpoints, since they allow a rapid measurement of functional responses of individual cells to stress, avoiding loss of information due to obtain average values from the simultaneous analysis of elevated cell numbers. Furthermore, measuring primary DNA damage on microalgae by means of the comet assay is a sensitive genotoxicity biomarker (Prado et al., 2009). Then, the main objective of the present study is to prove the suitability of these cytomic techniques applied on microalgal bioassays to evaluate the potential acute toxicity of other chemical pollutants in freshwater environments. The effects of a representative chemical from each of three main groups of aquatic pollutants (pesticides, metals and pharmaceuticals) are studied on two freshwater green

microalgae (*Chlamydomonas moewusii* and *Chlorella vulgaris*) by means of cytometric assays (chlorophyll *a* autofluorescence, cell viability, metabolic activity, cytoplasmic membrane potential) and the comet assay. Also, the different response level of the two microalgal species used will be compared to check which of them is more suitable for its use in toxicity bioassays.

Materials and methods

Microalgal cultures and chemical treatments

Chlamydomonas moewusii Gerloff (*Chlamydomonadaceae*) (CCAP 11/5B) and *Chlorella vulgaris* Beijerinck (*Chlorellaceae*) (CCAP 211/11B) were cultured in sterile inorganic Bristol medium (Brown et al., 1967).

All tests were carried out in 100 ml Pyrex glass bottles containing 50 ml of culture, in an incubator under controlled conditions according to that established for stock cultures: 18 ± 1 ºC, illuminated with a photon flux of 70 µmol m^{-2} s^{-1} under a dark:light cycle of 12:12 h, obtaining synchronized cultures (Altenburger, 2007). Microalgal cells in early exponential growth phase were used as inoculum for the assays. Initial cell density was adjusted to 1.5 µg chlorophyll per ml for both species assayed.

Fungicide imazalil stock solution was prepared by dissolving granulated pure pesticide in methanol, while copper sulphate and ibuprofen-Na stock solutions were prepared in distilled water. Pollutant concentrations were selected to observe their potential cytotoxic effects on cultured microalgal cells, not depending of their environmental relevance. Solutions were prepared on day prior to use in each experiment, and then diluted in the culture media to reach the final tested concentrations: ranged from 1.5 to 24 mg/L for cultures exposed to imazalil, from 1 to 30 mg/L for cultures exposed to copper; and from 25 to 180 mg/L for cultures exposed to ibuprofen-Na. To achieve these nominal concentrations of all pollutants, stock solutions volume added to the microalgal cultures never exceed 1% of final volume. Cultures without tested chemicals were included as controls; in the case of imazalil, no significant effects of the solvent were observed. All cultures were carried out in triplicate, and different analyses were done after 3 and 24 hours of exposure to the tested chemical.

Flow cytometric analysis

Flow cytometric analysis of microalgal cells were performed in a Coulter Epics XL4 flow cytometer (Beckman Coulter Inc.) equipped with an argon-ion excitation laser (488 nm), detectors of forward (FS) and side (SS) light scatter and four fluorescence detectors corresponding to different wavelength intervals: 505-550 nm (FL1), 550-600 nm (FL2), 600-645 nm (FL3) and >645 nm (FL4). Forward scatter and red chlorophyll fluorescence histograms were used to characterize the microalgal population, setting gating levels in order to exclude non-microalgal particles. For each cytometric parameter investigated, at least 10^4 gated cells were analysed per sample and fluorescence measurements were obtained in a logarithmic scale. Data were collected using listmode files and statistically analysed using the EXPO32 ADC software (Beckman Coulter Inc.). Aliquots of microalgal cultures were resuspended in phosphate buffered saline solution (PBS, pH 7.4) and analysed by FCM to study the potential alterations in the red autofluorescence (FL4), related to the chlorophyll *a* fluorescence emission, an inherent cell property on microalgae.

Besides this inherent cell property, FCM was used in combination with different fluorochromes to analyse other physiological cell parameters. Cell suspensions (2 x 10^5 cells/mL for *C. moewusii* and 1 x10^6 cells/mL for *C. vulgaris*) were incubated with the appropriate fluorochrome at room temperature and darkness for the necessary time. The lowest fluorochrome concentration and the shortest incubation time were chosen in order to obtain significant and stable staining of cells without toxicity being developed.

Cell viability was analysed by incubation of cell suspension with propidium iodide (PI) at a final concentration of 4 µM, for both microalgal species; this fluorochrome allows discriminating between live non-fluorescent cells, with an intact cellular membrane, and non-viable fluorescent cells, with permeability problems at the membrane level (Prado et al., 2009; Rioboo et al., 2009), being the orange fluorescent emission of this compound collected in the FL3 channel indicated above.

Cytoplasmic membrane potential was monitored using a slow-response potentiometric probe, the bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$); final concentration and incubation time: 1 µM 10 min for *C. moewusii*, and 5 µM 30 min for *C. vulgaris*. Cytoplasmic membrane depolarisation will be reflected in an increased intracellular anionic dye concentration,

i.e. by accumulation of dye in the cells, whilst decreased accumulation will reflect hyperpolarisation (Prado et al., 2012b). $DiBAC_4(3)$ green fluorescent emission was collected in the FL1 channel indicated above.

Metabolic activity, or cell vitality, was assessed using a fluorescein diacetate (FDA)-based cell esterase activity assay, a sensitive and rapid technique to assess phytoplankton metabolic activity (Jochem, 1999; Prado et al., 2009). A kinetic approach to the FDA assay (*in fluxo* analysis) was used in this work, recording the increase of the FDA-dependent fluorescence after FDA addition in the FL1 channel (final concentration: 0.2 µM for *C. moewusii* and 0.6 µM for *C. vulgaris*), depending on time, which allowed calculating the fluorescence generation rates in arbitrary units per minute (Prado et al., 2012b).

Comet assay

The alkaline single-cell gel electrophoresis or comet assay was applied to detect the primary DNA damage potentially induced by the exposure of microalgae to the tested chemicals. The comet assay protocol used is a modification of the original protocol (Singh et al., 1988) adapted to planktonic algae by Erbes et al. (1997), with an additional modification that is the DNA staining with SYBR Green I (Prado et al., 2009).

Two replicate slides were prepared for each treatment culture and negative control; furthermore, a positive control was also included (exposure of microalgal cultures to hydrogen peroxide at a final concentration of 0.6 mM). Slides were observed using an epifluorescence microscope Nikon Eclipse E400, with blue light as excitation light. Results are expressed as the percentage of comets vs. the total amount of nuclei analysed (at least 50 randomly chosen nuclei per slide).

Data analysis

Mean and standard deviation (S.D.) values were calculated for each treatment from two independent replicate experiments. To determine significant differences among test concentrations, data were statistically analysed by overall one-way analysis of variance (ANOVA) using SPSS 16.0 software. A p-value < 0.05 was considered statistically significant. When

significant differences were observed, means were compared using the multiple-range Duncan test.

Results and Discussion

Chlorophyll a *fluorescence*

The chlorosis state consists of a low level of residual photosynthesis, in which both photosystems gradually lose their activity, and chlorophyll are degraded to reach a residual content. The exposure of *C. moewusii* to any of the tested chemicals provoked an early effect on the natural cell autofluorescence, with the appearance of chlorotic cells (without chlorophyll fluorescent emission) after only 3 h of exposure to the highest concentrations assayed. Percentage of chlorotic cells was drastically increased after 24 h in these cultures, reaching 100% of the cell population in the case of copper and imazalil (Table 1). However, chlorotic cells in *C. vulgaris* cultures were only observed after exposure to the highest copper concentration assayed (Table 1). The measurement of *in vivo* chlorophyll *a* fluorescent emission has been found to be a sensitive tool for the rapid detection of compounds and environmental conditions that exhibit harmful effects on photosynthetic organisms, and a reduction of chlorophyll a fluorescent emission was observed for plant cells exposed to diverse pollutants in fluorescence microscope studies (di Toppi et al., 2005; Hjorth et al., 2006; Nancharaiah et al., 2007) and also by flow cytometry (Franqueira et al., 2000; González-Barreiro et al., 2004; Prado et al., 2011). Previous studies of copper toxicity on a marine diatom showed the sensitivity of chlorophyll *a* fluorescence detected by flow cytometry, being affected by copper concentrations higher than 0.25 mg/L after 24 hours of exposure, whereas at this time the viable cell percentage remained near to 97% at the highest copper concentration assayed, 1 mg/L (Cid et al. 1997).

Cell viability and membrane potential

Cell viability and cytoplasmic membrane potential analysis showed profound alterations of chlorotic cells at plasma membrane level, with a disruption of the membrane integrity (they are PI-positive, non-viable cells) and, in accordance with this, a totally depolarised plasma membrane (they showed a drastic increase of $DiBAC_4(3)$-derived fluorescence, as a consequence of the

influx of the fluorochrome), both for *C. moewusii* and *C. vulgaris* and independently of the chemical nature of the chlorosis-inductor agent, as could be observed in figure 1 for the highest copper concentration assayed. These features had already been observed for herbicide-induced chlorosis in *C. moewusii* cells (Prado et al., 2012b), so it may be considered a general biomarker of the irreversible damage of the cell induced by the presence of pollutants (Pouneva, 1997).

A separate analysis of non-chlorotic cells also showed a reduced percentage of viable cells in *C. moewusii* cultures exposed to high concentrations of any of the tested chemicals (Fig. 2A, B, C), while cell viability in *C. vulgaris* cultures was maintained above 99% for imazalil and ibuprofen (Fig. 2D, F), although cultures of this microalgal species exposed to the highest copper concentration certainly showed a reduced percentage of viable non-chlorotic cells (Fig. 2E). A parallelism was observed between the percentage of viable cells (PI-negative) and the percentage of cells capable of maintaining a polarisation of their plasma membrane for the different cultures (Fig. 2), although in the case of *C. vulgaris* after 3 h of exposure to 30 mg/L of copper, the percentage of non-chlorotic polarised cells was notably lower than that of non-chlorotic viable cells (Fig. 2E.1.), which may indicate that the induced damage in the plasma membrane start with changes in the permeability properties (depolarisation) before the disruption of the membrane integrity occurs. The membrane potential probe $DiBAC_4(3)$ has been frequently used to study cell viability (Ben Amor et al., 2002; Jepras et al., 1995; Lloyd and Hayes, 1995; Papadimitriou et al., 2006), but there are also studies pointing out that cells without a membrane potential are not necessarily non-viable (Breeuwer and Abee, 2000; Prado et al., 2012a).

Then, chlorosis is an indicator of cell mortality but membrane damages could occur before this drastic pigment degradation, according to Veldhuis et al. (2001).

As mentioned above, cells with a totally depolarised plasma membrane showed a massive influx of the fluorochrome and, as a consequence, a drastic increase of the derived fluorescence, but may be of interest analysing variations of $DiBAC_4(3)$ fluorescence for non-chlorotic cells maintaining at least a certain membrane polarisation, limiting more or less the influx of the fluorochrome, to study possible cytoplasmic membrane potential alterations before a total depolarisation that reflects the cell is not viable. *C. moewusii* sensitivity was exhibited once more at this level, showing a deep reduction of the membrane potential (Fig. 3A, B, C). For *C. moewusii*

exposed to ibuprofen, the cytoplasmic membrane potential was significantly affected at low concentrations while the highest concentrations did not show differences with respect to control (Fig. 3C), which may be due to only in some way more resistant cells were analysed in these cultures since the rest of them showed a total depolarisation of their membrane. This parameter was of special interest in the case of *C. vulgaris*, whose cell viability was not affected after the exposure to any of the tested chemicals, except in cultures exposed to the highest copper concentration, and thus a total depolarisation of the plasma membrane was not observed, but a reduction of the cytoplasmic membrane potential (increasing depolarisation level reflected by increased $DiBAC_4(3)$-derived fluorescence) was certainly observed in response to any of the substances, generally in a concentration and exposure time dependent manner (Fig. 3D, E, F).

The marine diatom species, *Phaeodactylum tricornutum*, showed an important increase in cellular membrane potential after 96 h of exposure to copper concentrations higher than 0.1 mg/L, non-detectable after only 24 hours (Cid et al. 1996).

Metabolic activity

Metabolic activity, assessed as esterase activity, was shown to be a sensitive cytotoxicity endpoint since the two microalgal species showed early alterations of this physiological parameter after exposure to low concentrations of any of the tested chemicals (Fig. 4). For esterase activity analysis only metabolically active cells (capable of turn FDA into fluorescein) with an intact plasma membrane (capable of retain fluorescein) were taken into account, i.e., cells for which a FDA-derived fluorescein fluorescence increase was not observed over time have been excluded (chlorotic cells and a certain percentage of non-chlorotic cells whose membrane was disrupted, as shown by PI assay).

A significant reduction of esterase activity was observed after the exposure of both microalgal species to any of the ibuprofen concentrations assayed with respect to control cultures, although this reduction was more pronounced at first, after only 3 h of exposure, and a slight recuperation was observed later, specially in cultures exposed to lower concentrations while those exposed to high concentrations hardly showed differences between the two time points (Fig. 4C, F). This may be related to an attempt of microalgae to adapt to adverse conditions, making

possible this slight activity recuperation, although this would not be occur at higher concentrations of the toxic agent.

Copper and imazalil also affect to esterase activity of both microalgae, generally by reduction of this enzymatic activity in a concentration manner, but not always in a linear way (Fig. 4A, B, D, E). However, a stimulation was observed at first for *C. vulgaris* exposed to the lowest copper concentration assayed (Fig. 4E), according to several studies where a stimulation of the FDA hydrolysis-derived fluorescence generation was observed for other microalgal species in response to low copper concentrations and/or short-term exposures to this metal, suggesting this effect was probably not associated to an increase of esterase activity but to cell membrane and intracellular pH modifications (Franklin et al., 2001; Hadjoudja et al., 2009; Yu et al., 2007). This effect was also observed in *C. moewusii* cultures exposed to high concentrations of imazalil and copper (Fig. 4A, B), which might be related to only more resistant, metabolically active, cells were analysed in these cultures, and this resistance may be due to the activation and upregulation of detoxification processes in which esterases may take part (Jamers et al., 2009).

Comet assay

Primary DNA damage in microalgae detected by the comet assay has shown to be a very sensitive biomarker, proving the genotoxicity of each of the assayed compounds to both microalgal species, although *C. moewusii* showed again a higher sensitivity (Table 1). DNA damage was detected after only 3 h of exposure at low concentrations of the toxic agent. The difference between the percentages of comet-nuclei observed after 3 and after 24 h of exposure was reduced as the pollutant concentration increased, which may be due to a severe DNA damage induced by the longer exposure time so that a high degree of DNA fragmentation would led to the loss of these fragments during electrophoresis (Devaux et al., 1997), resulting in underestimation of the number of affected cells that are actually present in cultures exposed to high concentrations of the toxic compound. The detection of comets was even impossible in some cases when analysing cultures exposed to high concentrations after 24 h (Table 1).

The assessment of DNA in individual cells following exposure to pollutants has been used as a valuable ecotoxicological tool concerning molecular genotoxicity biomarkers (Akcha et al.,

2008; Ali and Kumar, 2008; Mitchelmore et al., 1998). DNA damage detected by the comet assay has been regarded as a marker of oxidative stress damage (Watanabe and Suzuki, 2002), since reactive oxygen species has been shown to be active genotoxic agents, the hydroxyl radical being the main genotoxic species (Gaivao et al., 1999). Oxidative stress damage can be also involved in membrane alterations (Prado et al. 2011), that can be correlated with membrane potential alterations and chlorophyll degradation observed in this study.

Conclusion

Results obtained in this work confirm the potential use of the assayed protocols for the prospective assessment of the potential cytotoxicity of different chemical aquatic pollutants, with the advantage that means to detect a group of complex reactions that only may be possible in intact cells. However, the high concentrations required to provoke alterations of the assayed parameters in a 24 hours period could be a problem for transferring this methodology to the field for environment monitoring programmes. Among cell parameters assayed, the esterase activity (as a reflection of cell metabolic activity) and the primary DNA damage stood out as sensitive cytotoxicity endpoints. Chlorophyll *a* fluorescence would be also a good biomarker, especially in the case of *C. moewusii*, with the added advantage that no commercial fluorochrome is necessary to analyse this microalgal cell inherent property, conferring this microalgal species a higher sensitivity to pollutants.

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Figure and table captions

Figure 1. Biparametric histograms showing the PI-derived fluorescence, as an indicator of cell viability (A, B, E, F), and $DiBAC_4(3)$-derived fluorescence, as an indicator of cytoplasmic membrane potential (C, D, G, H), *vs.* the chlorophyll *a* fluorescence, for *C. moewusii* (A, B, C, D) and *C. vulgaris* (E, F, G, H) control cultures (A, C, E, G) and cultures exposed to 30 mg/L copper after 24 h (B, D, F, H).

Figure 2. Comparison of the percentages of viable cells (PI-) and polarised cells, obtained with respect to non-chlorotic cells, for *C. moewusii* (A, B, C) and *C. vulgaris* (D, E, F) cultures after 3 (1) and 24 h (2) exposed to different concentrations of imazalil (A, D), copper (B, E) and ibuprofen (C, F). Significant differences with respect to control were indicated by (*) ($p < 0.05$).

Figure 3. Cytoplasmic membrane potential variations, indicated by $DiBAC_4(3)$-derived fluorescence expressed as percentage with respect to control (for which a value of 100 is assigned, indicated by the dashed line), for non-chlorotic polarised cells in *C. moewusii* (A, B, C) and *C. vulgaris* (D, E, F) cultures after 3 and 24 h of exposure to different concentrations of imazalil (A, D), copper (B, E) and ibuprofen (C, F). Significant differences with respect to control were indicated by (*) ($p < 0.05$).

Figure 4. Cell metabolic activity variations, indicated by the esterase activity expressed as percentage with respect to control (for which a value of 100 is assigned, indicated by the dashed line), for non-chlorotic metabolically active cells in *C. moewusii* (A, B, C) and *C. vulgaris* (D, E, F) cultures after 3 and 24 h of exposure to different concentrations of imazalil (A, D), copper (B, E) and ibuprofen (C, F). Significant differences with respect to control were indicated by (*) ($p < 0.05$).

Table 1. Effects of the exposure to different concentrations of imazalil, copper and ibuprofen on chlorophyll *a* fluorescence, expressed as percentage of chlorotic cells, and DNA integrity, expressed as percentage of comet-nuclei, for *C. moewusii* and *C. vulgaris*. Significant differences with respect to control were indicated by (*) ($p < 0.05$); n.d.: non-detectable.

Table 1

			Chlorotic cells (%)		Comet nuclei (%)	
			3 h	24 h	3 h	24 h
C. moewusii	Imazalil (mg l^{-1})	0	0	0	14.9 ± 1.2	18.7 ± 1.8
		1.5	0	0	34.4 ± 2.7*	56.5 ± 1.8*
		3	0	0	62.8 ± 3.6*	72.5 ± 3.1*
		6	0	0	75.5 ± 4.9*	87.9 ± 0.6*
		12	0	26.3 ± 2.3	81.5 ± 3.1*	n. d.
		24	16.0 ± 1.3	100 ± 0.0	95.7 ± 0.9*	n. d.
	Copper (mg l^{-1})	0	0	0	15.2 ± 1.1	18.2 ± 0.5
		1	0	0	33.2 ± 3.4*	70.3 ± 4.4*
		5	0	22.1 ± 0.1	76.7 ± 3.6*	n. d.
		10	4.6 ± 0.0	56.2 ± 1.4	88.3 ± 0.6*	n. d.
		20	8.3 ± 0.4	91.8 ± 0.2	92.7 ± 0.7*	n. d.
		30	28.1 ± 0.1	100 ± 0.0	95.1 ± 0.6*	n. d.
	Ibuprofen (mg l^{-1})	0	0	0	13.6 ± 1.1	15.5 ± 0.7
		25	0	0	15.6 ± 0.4	17.4 ± 0.7
		50	0	0	18.5 ± 1.6*	23.6 ± 2.8*
		90	0	0	50.0 ± 3.1*	50.8 ± 3.3*
		140	12.3 ± 0.2	14.3 ± 0.9	63.3 ± 4.0*	67.3 ± 2.0*
		180	33.8 ± 0.8	67.4 ± 1.9	86.8 ± 1.7*	n. d.
C. vulgaris	Imazalil (mg l^{-1})	0	0	0	16.2 ± 1.3	15.4 ± 0.2
		1.5	0	0	29.4 ± 1.6*	40.7 ± 2.5*
		3	0	0	42.3 ± 3.2*	51.5 ± 4.0*
		6	0	0	62.1 ± 2.4*	71.1 ± 3.5*
		12	0	0	78.9 ± 2.1*	87.5 ± 0.8*
		24	0	0	86.0 ± 0.9*	n. d.
	Copper (mg l^{-1})	0	0	0	15.1 ± 0.5	14.6 ± 0.7
		1	0	0	17.0 ± 1.8	17.6 ± 0.2
		5	0	0	17.9 ± 1.1	22.1 ± 1.6*
		10	0	0	18.2 ± 1.8	24.5 ± 3.0*
		20	0	0	20.6 ± 1.8*	24.8 ± 2.5*
		30	24.1 ± 3.3	46.9 ± 0.9	49.2 ± 4.2*	n. d.
	Ibuprofen (mg l^{-1})	0	0	0	16.3 ± 0.5	17.1 ± 0.6
		25	0	0	18.3 ± 0.6	19.2 ± 1.2
		50	0	0	19.7 ± 1.2	23.1 ± 0.7*
		90	0	0	28.5 ± 1.4*	44.3 ± 1.7*
		140	0	0	41.8 ± 3.3*	59.5 ± 1.6*
		180	0	0	63.8 ± 2.7*	75.7 ± 4.4*

Figure 1

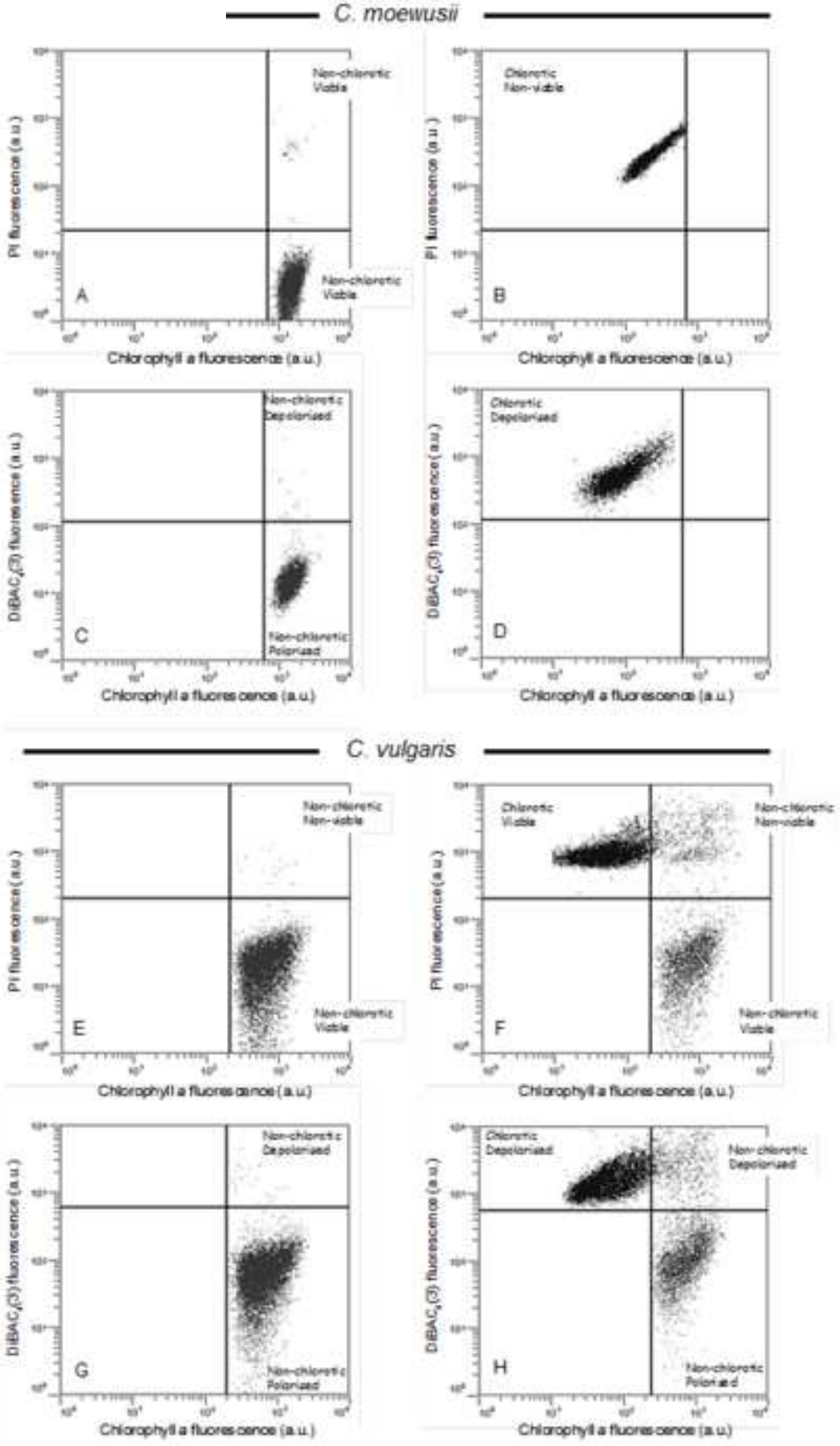

C. moewusii
Non-chlorotic Viable
PI fluorescence (a.u.)
A
Non-chlorotic Viable
Chlorophyll a fluorescence (a.u.)
Chlorotic Non-viable
PI fluorescence (a.u.)
B
Chlorophyll a fluorescence (a.u.)
Non-chlorotic Depolarised
DiBAC4(3) fluorescence (a.u.)
C
Non-chlorotic Polarised
Chlorophyll a fluorescence (a.u.)
Chlorotic Depolarised
DiBAC4(3) fluorescence (a.u.)
D
Chlorophyll a fluorescence (a.u.)
C. vulgaris
Non-chlorotic Non-viable
PI fluorescence (a.u.)
E
Non-chlorotic Viable
Chlorophyll a fluorescence (a.u.)
Chlorotic Viable
Non-chlorotic Non-viable
PI fluorescence (a.u.)
F
Non-chlorotic Viable
Chlorophyll a fluorescence (a.u.)
Non-chlorotic Depolarised
DiBAC4(3) fluorescence (a.u.)
G
Non-chlorotic Polarised
Chlorophyll a fluorescence (a.u.)
Chlorotic Depolarised
Non-chlorotic Depolarised
DiBAC4(3) fluorescence (a.u.)
H
Non-chlorotic Polarised
Chlorophyll a fluorescence (a.u.)

Figure 2

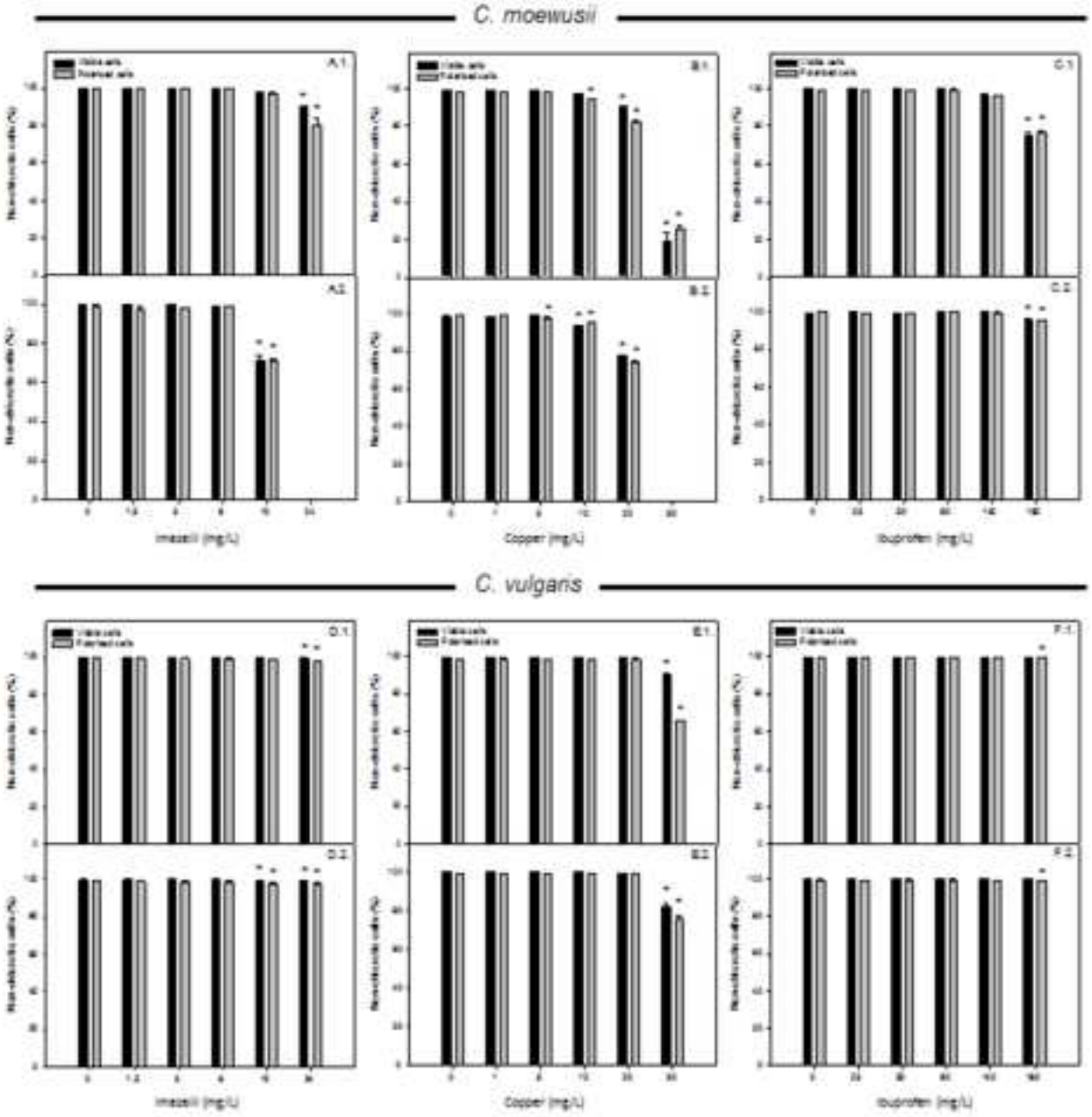


Figure 3

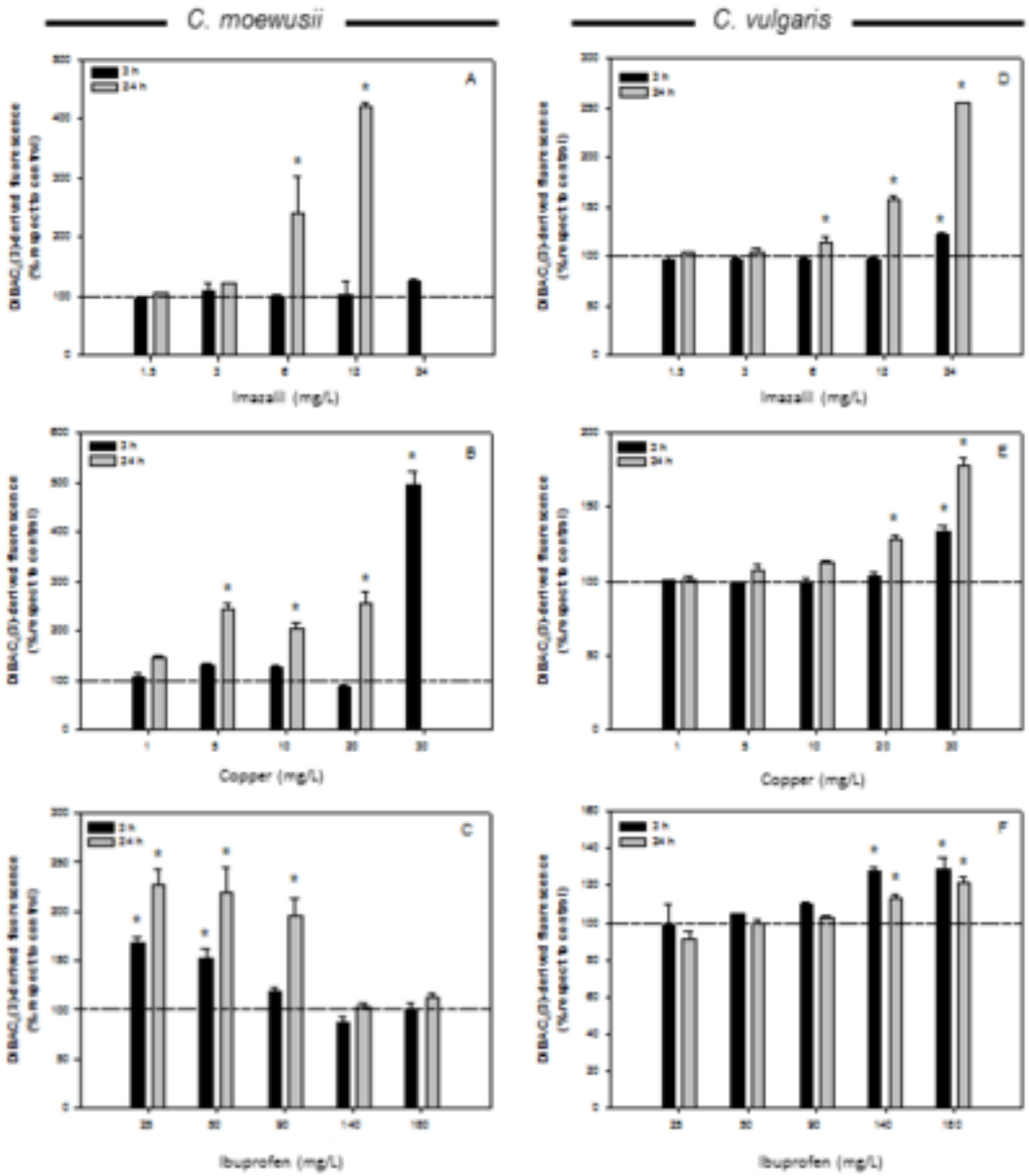
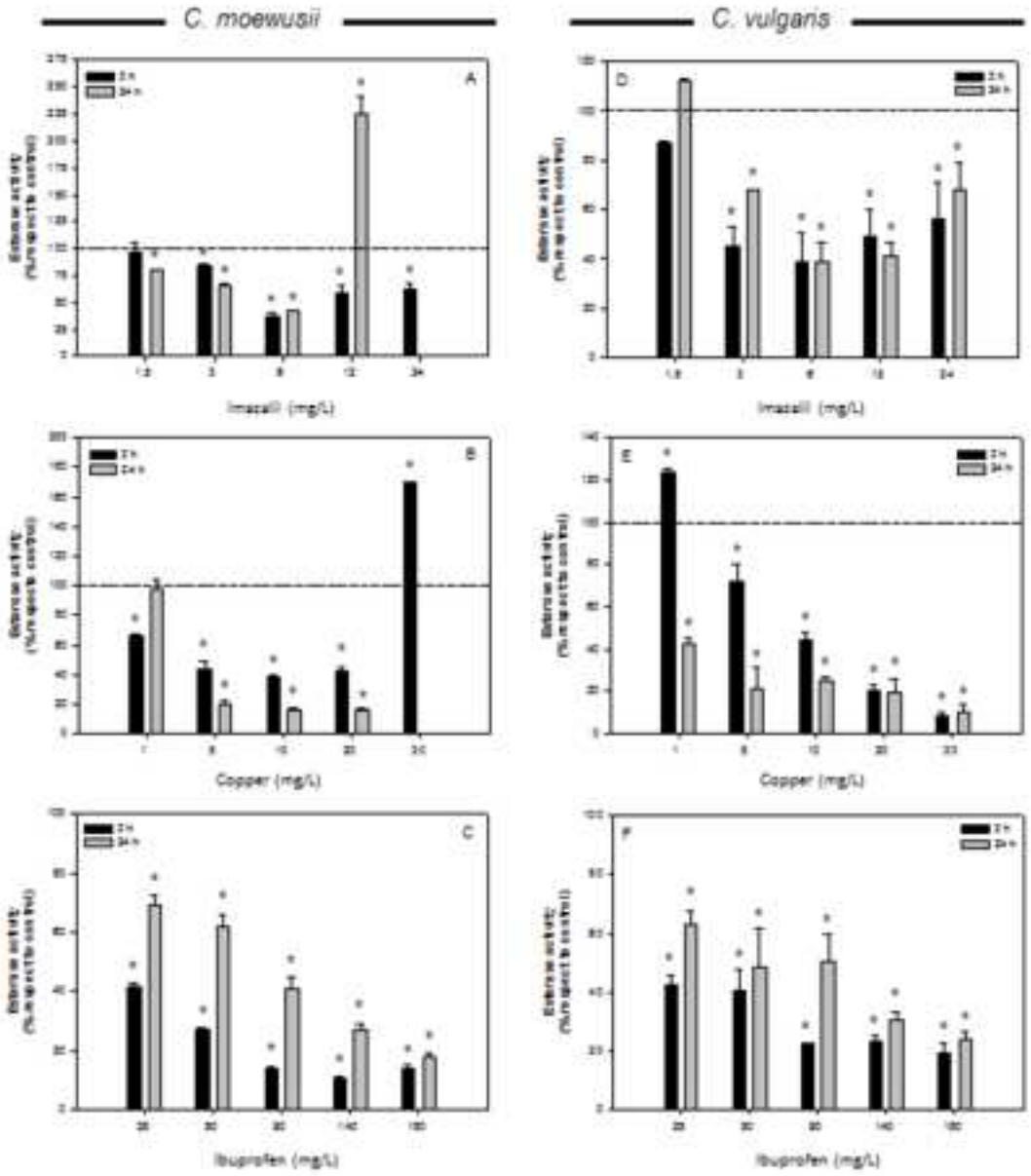

C. moewusii
C. vulgaris
A
B
C
D
E
F
Imazalil (mg/L)
Copper (mg/L)
Ibuprofen (mg/L)

Figure 4


C. moewusii
C. vulgaris
A
D
B
E
C
F
Imazalil (mg/L)
Copper (mg/L)
Ibuprofen (mg/L)