Development of a short-term assay based on the evaluation of the plasma membrane integrity of the alga *Pseudokirchneriella subcapitata*

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Abstract Membrane integrity has been used as a criterion for the definition of cell viability. In the present work, staining conditions (time and dye concentration) for the evaluation of membrane integrity in a fluorescence microplate reader, using the membrane-impermeant nucleic-acid dye SYTOX Green, were optimized. Incubating *Pseudokirchneriella subcapitata* algal cells with 0.5 μmol/l SYTOX Green for 40 min allowed a clear discrimination between live (intact plasma membrane) and dead cells (with compromised plasma membrane). Algal cell suspensions, labelled with SYTOX Green, exhibited a green fluorescence proportional to the fraction of the cells with a permeabilized plasma membrane. The optimized staining conditions were used to assess the toxicity of 1-pentanol on *P. subcapitata* in a short-term exposure (6 h) assay. The loss of membrane integrity in the cell population increased with the concentration of 1-pentanol. The 6-h EC_{10} and EC_{50} values were 7,617 mg/l 1-pentanol (95 % confidence limits 4,670–9,327) and 12,818 mg/l 1-pentanol (95 % confidence limits 10,929–15,183), respectively. The developed microplate-based short-term assay can be useful in the high-throughput screening of toxics or environmental samples using the alga *P. subcapitata*.

Keywords Membrane integrity · Microplate assay · *Selenastrum capricornutum* · 1-Pentanol · SYTOX Green · Acute toxicity

Introduction

Algae are key functional organisms because they are the dominant primary producers and thus constitute the basic link in aquatic food chains; disrupting this production base may cause effects at higher trophic levels. For these reasons, algae are routinely used in toxicity tests as representatives of trophic level 1. Within algae, the chlorophyta (green alga) *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and also as *Raphidocelis subcapitata*) has been used in the methods of international agencies such as the U.S. EPA (U.S.-EPA 2002) or OECD (OECD 1984) and in many national standards.

The cellular membrane can be a critical target of the action of toxics since it is one of the first structures with which they come into contact. In addition, membrane integrity is particularly important to the normal functioning of the cell and has been used as a criterion for defining cell viability. Cells with a compromised membrane cannot maintain an electrochemical gradient and are usually classified as dead cells (Vives-Rego et al. 2000). Several fluorescent-based protocols have been proposed to evaluate plasma membrane integrity. Usually, nucleic acid dyes such as ethidium bromide (EB) and propidium iodide (PI), which are excluded from live cells, are employed (Haugland 2005). However, the use of EB or PI with *P. subcapitata* algal cells is not adequate because both exhibit an orange

fluorescence (EB and PI emission fluorescences are 605 and 617 nm, respectively) which can overlap and therefore be confused with the autofluorescence of the algal cells. *P. subcapitata* alga cells exhibit a red autofluorescence (emission at 680 nm) due to their photosynthetic pigments (chlorophyll *a*). In this context, SYTOX Green seems to be a good alternative since it is excluded from live cells (with intact plasma membrane) and exhibits a bright green fluorescence upon binding with nucleic acid in dead cells (with permeable plasma membrane) (Fig. 1). SYTOX Green has been used in evaluating the viability of bacteria, yeast cells (Haugland 2005) and phytoplankton, such as in cyanobacteria, diatoms, dinoflagellates and green algae (Sato et al. 2004; Ribalet et al. 2007; Timmermans et al. 2007; Segovia and Berges 2009; Chang et al. 2011; Peperzak and Brussaard 2011). The use of SYTOX Green in the evaluation of plasma membrane integrity of *P. subcapitata* algal cells has been weakly exploited. In fact, the use of this dye, by flow cytometry, has only recently been reported in evaluating the viability of *P. subcapitata* cells exposed to nanoparticulate CeO_2 (Rogers et al. 2010) or rice paddy herbicides (Nagai et al. 2011). However, the use of SYTOX Green with *P. subcapitata* algal cells in microplate assays has never been performed. Microplate readers are cheaper instruments than flow cytometers and allow for the performance, simultaneously, of several replicas of the assay using a small sample volume.

In the present work, we describe the optimization of staining conditions for evaluating the plasma membrane integrity of the alga *P. subcapitata,* using SYTOX Green, in a 96-well microplate format. The applicability of the optimized staining conditions was tested by evaluating the toxicity of 1-pentanol using *P. subcapitata* algal cells in a short-term (6 h) assay. 1-Pentanol was chosen as the toxicant because it is used as a solvent and is likely to be found in effluents from several industries such as: plastics, synthetic resins and some elastomers; synthetic rubber; cleaning, polishing and sanitation preparations; industrial organic chemicals; and petroleum refining (PubChem Compound, USA—National Centre for Biotechnology Information, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=6276&loc=ec_rcs#x321).

Materials and methods

Strain, media and culture conditions

In this work, the freshwater green alga *P. subcapitata* (strain 278/4) was used. The original strain was obtained from the Culture Collection of Algae and Protozoa (CCAP), UK.

The alga was maintained in an OECD algal test medium (OECD 1984) with 20 g/l agar (Merck), in the dark, at 4 °C. Medium stock solutions were prepared, sterilized (by autoclaving or by membrane filtration, pore size 0.45 μm) and stored (in the dark at 4 °C) according to OECD guidelines (OECD 1984). Algal stock cultures were obtained from cultures in the exponential phase of growth (2 days), which were centrifuged (2,500×*g*, 5 min) and resuspended in fresh medium.

The starter cultures were prepared weekly by inoculating a loop of algal cells (from agar slant) in 20 ml OECD medium in 100-ml Erlenmeyer flasks. The cells were incubated for 2 days, at 25 °C, on an orbital shaker at 100 rpm, under continuous "cool white" fluorescent light (fluorescent lamps with a colour temperature of 4,300 K) at an intensity of 4,000 lux at the surface of the flask, verified using an illumination meter (HI 97500, Hanna Instruments). The pre-cultures were prepared by inoculating 100 ml of OECD medium in 250-ml Erlenmeyer flasks, with an initial cell concentration of ~5×10^4 cells/ml from the starter culture. Cells were incubated for 2 days under the conditions described above for the starter cultures. The cultures were prepared by inoculating 100 or 400 ml of OECD medium in 250-ml or 1-l Erlenmeyer flasks, respectively, with an initial cell concentration of ~5×10^4 cells/ml from the pre-culture. The purity of the cultures was verified by microscopic examination of a subsample.

Evaluation of cell concentration

The algal cell concentration was measured by direct cell counting using either a microscope and a counting chamber or an automated cell counter (TC10, Bio-Rad). Additionally, the algal concentration was evaluated, indirectly, by spectrophotometrically measuring the absorbance at 750 nm

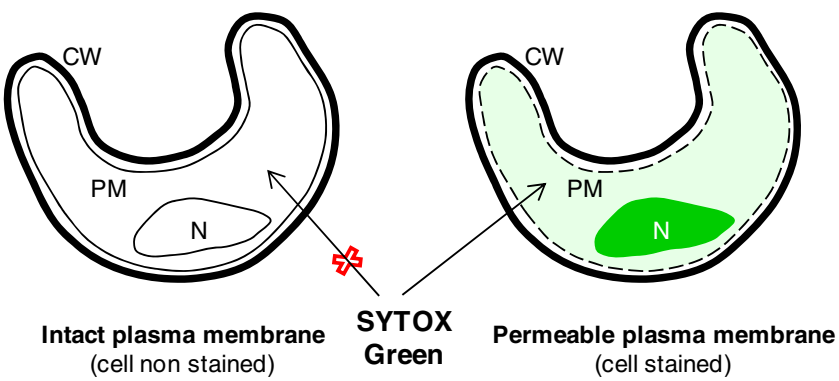

Fig. 1 Diagrammatic representation of mode of action of SYTOX Green stain. The fluorescent probe is excluded from live algal cells with an intact plasma membrane but penetrates cells with a damaged plasma membrane, binding to nucleic acids and enhancing the fluorescence emission. *CW* cell wall, *N* nuclei, *PM* plasma membrane

(U.S.-EPA 2002); for the latter, a calibration curve (number of cells versus absorbance) was first constructed.

Algal cell staining

Algal cells in the exponential phase of growth (2 days) were harvested by centrifugation (2,500×*g*, 5 min) and suspended in fresh culture medium at 2×10^6 cells/ml. Cell suspensions (100 µl), in a concentration range of 0.1–10×10^5 cells/ml (final concentration), were placed in a black 96-well microplate (OptiPlate-96 F, PerkinElmer) and mixed with aliquots (100 µl) of SYTOX Green (Molecular Probes, Life Technologies), at a final concentration of 0.1–1.0 µmol/l, from working solutions of 0.2–2.0 µmol/l. Total volume per well was 200 µl. Cells were incubated in the dark at 25 °C. The stock solution of SYTOX Green (5 mmol/l) was provided in dimethyl sulphoxide (DMSO) and stored at −20 °C. Intermediary (250 µmol/l) and working SYTOX Green solutions were prepared, before use, by diluting the stock solution in OECD medium. In the assay, the final concentration of DMSO was≤0.02 % (*v*/*v*).

As positive control, algal cells were heat-treated in order to permeabilize the plasma membrane. Thus, algal cells (suspended in OECD culture medium) were placed in a glass tube and heat-treated (at 65 °C for 1 h) in a water bath. The treated suspension was then cooled to room temperature and stained as described above. Microscopic observation confirmed that, using this procedure, all heat-treated cell populations displayed a permeabilized plasma membrane (Fig. S1 in "Electronic supplementary material").

Fluorescence intensity (in relative fluorescent units, RFU) was measured in a PerkinElmer (Victor3) microplate reader at a fluorescence excitation wavelength of 485/14 nm and an emission wavelength of 535/25 nm. Fluorescence was corrected by subtracting cell, culture medium and dye autofluorescence and was normalized, when appropriate, considering cell concentration.

The percentage of cells with a permeabilized membrane (% CPM) was calculated using the following equation:

$$\%\ \mathrm{CPM} = (F_a/F_{max}) \times 100 \qquad (1)$$

where F_a is the fluorescence in the assay and F_{max} is the mean fluorescence in the sample where all the cells presented a permeabilized membrane (heat-treated cells−positive control).

Fluorescence microscopy

Cells were also examined using epifluorescence microscopy. Algal cells were incubated with 0.5 µmol/l SYTOX Green for 40 min, at 25 °C, in the dark, and were observed using a Leica DLMB epifluorescence microscope, equipped with a HBO-100 mercury lamp and filter set GFP from Leica (filter set GFP: excitation filter (band-pass filter, BP) BP 470/40, dichromatic mirror 500 and suppression filter BP 525/50). Images were acquired with a Leica DC 300 F camera and processed using Leica IM 50-Image Manager software.

The percentage of cells with permeabilized membrane (dead cells) was also determined microscopically. Thus, different proportions of live and heat-treated cells were mixed (at a final concentration of 1×10^6 cells/ml) and stained as described above. For each mixture, at least three samples of 200 cells (total of 600 cells for each mixture) were scored in randomly selected microscope fields.

Evaluation of 1-pentanol toxicity

Algal cells were exposed to the toxicant in a similar way to that described by Blaise and Vasseur (2005) in an algal microplate growth toxicity test. Briefly, algal cells in exponential phase of growth (2 days) were harvested by centrifugation (2,500×*g*, 5 min) and suspended at 5.5×10^6 cells/ml in a concentrated culture medium. Ten concentrations of 1-pentanol (Riedel-deHaen) (in a concentration range of 2,400 to 32,000 mg/l) were prepared by geometrical dilution of the toxicant in deionized water. The assays were carried out in black 96-well microplates and sterilized with ultraviolet light for 15 to 20 min prior to use. The test solutions were dispensed in the microplate in a predetermined pattern. Each well received 200 µl of the appropriate dilution of 1-pentanol (in quintuplicate) or deionized water as a negative control (tenfold) and 20 µl of cell suspension in a culture medium concentrated 11 times (final cell concentration, 5×10^5 cells/ml). Additionally, as positive control, 20 µl of a heat-treated cell suspension (65 °C, 1 h) was used in the same conditions as the negative control (tenfold). The microplates were covered with a low-evaporation membrane (Breathe-easy) and incubated at 25 °C, under continuous "cool white" fluorescent light (as described earlier) for 6 h.

Staining was carried out by adding 10 µl of a working solution of SYTOX Green 11.5 µmol/l (prepared in OECD medium before use) followed by incubation in the dark at 25 °C; final dye concentration was 0.5 µmol/l. After 40 min of incubation, fluorescence intensity was measured, as described earlier. Fluorescence was corrected by subtracting cell, culture medium, dye and 1-pentanol autofluorescence and normalized, when appropriate, considering cell concentration. The percentage of cells with permeabilized membrane was calculated using as reference the mean fluorescence in the sample where all the cells presented a permeabilized membrane.

Reproducibility of the results and statistical analysis

All experiments were carried out in quintuplicate and repeated three to four times. Although absolute data for the fluorescence measurements were not comparable for the experiments performed on different days, the observed trends were fully

consistent among the independent experiments and a typical example is shown. Fluorescence data were expressed as the mean±standard deviation (SD) of quintuplicate measurements, calculated with 95 % confidence limits.

1-Pentanol toxicity was expressed as EC_{10} and EC_{50} values, which represent the concentration of the toxicant that induced a loss of membrane integrity in 10 and 50 % of the cell population, respectively. The EC values were calculated considering that the concentration–response relationship can be described by the probit function; EC values were obtained using weighted linear regression analysis on probit-transformed data (TOXCALC version 5.0.32, Tidepool Scientific Software).

Results

Optimization of algal cell staining

The evaluation of loss of membrane integrity (cell viability) was based on the exclusion of SYTOX Green dye by intact plasma membranes (live cells). Cells with a damaged plasma membrane (dead cells) were penetrated by the fluorescent probe which displayed ~1,000-fold green fluorescent enhancement (absorbance maxima, 504 nm; emission maxima, 523 nm) upon binding with nucleic acid (Haugland 2005).

The microplate assay was optimized as a function of SYTOX Green concentration (0.1–1.0 μmol/l) and the incubation time (up to 120 min). Suspensions containing 1×10^6 cells/ml of live or heat-permeabilized *P. subcapitata* cells were stained with SYTOX Green. The evolution of the staining of the algal cells was monitored by measuring the emission of fluorescence at 535 nm upon excitation at 485 nm. As can be seen in Fig. 2, heat-treated cells (dead cells) increased their fluorescence with time and reached a plateau at around 20–40 min. For the SYTOX Green concentrations tested, after 5 and 10 min, the fluorescence signals were 55–75 and 75–85 %, respectively, of those observed after 120 min; after 40 min, the fluorescence signals were>95 % of those observed after 120 min. The fluorescent signals remained stable until 120 min. Although a high fluorescence signal was observed after 5–10 min, an incubation time of 40 min was selected in order to obtain a stronger signal. Heat-treated algal cells incubated with 0.7 or 1.0 μmol/l SYTOX Green displayed a similar fluorescence pattern. With the aim of obtaining a strong signal with a low dye concentration (to decrease the costs associated with the fluorescent probe), 0.5 μmol/l SYTOX Green, 40 min, was selected as algal cell staining condition. This condition corresponds to >75 % of the maximum fluorescence signal observed. Remarkably, live cells remained unstained even though they remained in the presence of 1.0 μmol/l SYTOX Green for 120 min; in this condition, live algal cells exhibited a fluorescence<2.5 % of the maximum fluorescence observed (Fig. 2). The fluorescence of heat-treated algal cells

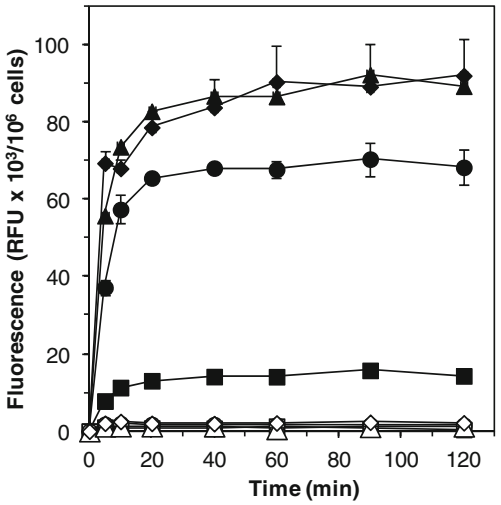

Fig. 2 Kinetics of the staining of the alga *P. subcapitata*. Cells (1×10^6 cells/ml) were incubated with 0.1 (*squares*), 0.5 (*circles*), 0.7 (*triangles*) and 1.0 (*diamonds*) μmol/l SYTOX Green at 25 °C. Live cells (*open symbols*) and dead cells (*closed symbols*) were used. This is a typical example of an experiment performed three times. Each point represents the mean of five replicates (*n=5*); standard deviations are presented with 95 % confidence limits (*vertical error bars*). Where no error bars are shown, they are within the points

(dead cells) increased linearly with cell concentration (0.5 to 10×10^5 cells/ml), while the fluorescence of live cells (with intact plasma membrane) remained very low (Fig. S2 in "Electronic supplementary material").

The exclusion of SYTOX Green by live cells of *P. subcapitata* was confirmed by epifluorescence microscopy. Live cells of *P. subcapitata* remained unstained (Fig. 3a, b), while dead cells displayed a considerable green fluorescence (Fig. 3d, e, g, h). In the case of live cells, a 4.7-fold increase in the shutter time used for dead cells only allowed a very weak green fluorescence to be observed (Fig. 3b). This means that no significant surface binding of the dye occurred in live *P. subcapitata* algal cells. The large difference in fluorescence emission between live (with intact membrane) and dead cells (with compromised plasma membrane) (Fig. 2; Fig. S2 in "Electronic supplementary material") allowed the two populations to be discriminated easily from each other (Fig. 3j, k). Using fluorescence microscopy, a clear distinction between live cells and cells with a compromised membrane was observed after 10 min of incubation with 0.5 μmol/l SYTOX Green.

Assessment of the percentage of cells with permeabilized membrane (dead cells)

In order to validate the staining conditions, cell suspensions of intact and heat-treated algal cells were combined in different proportions (at a final cell concentration of 1×10^6 cells/ml) and stained using the conditions selected above. Considering that the maximum fluorescence presented by the

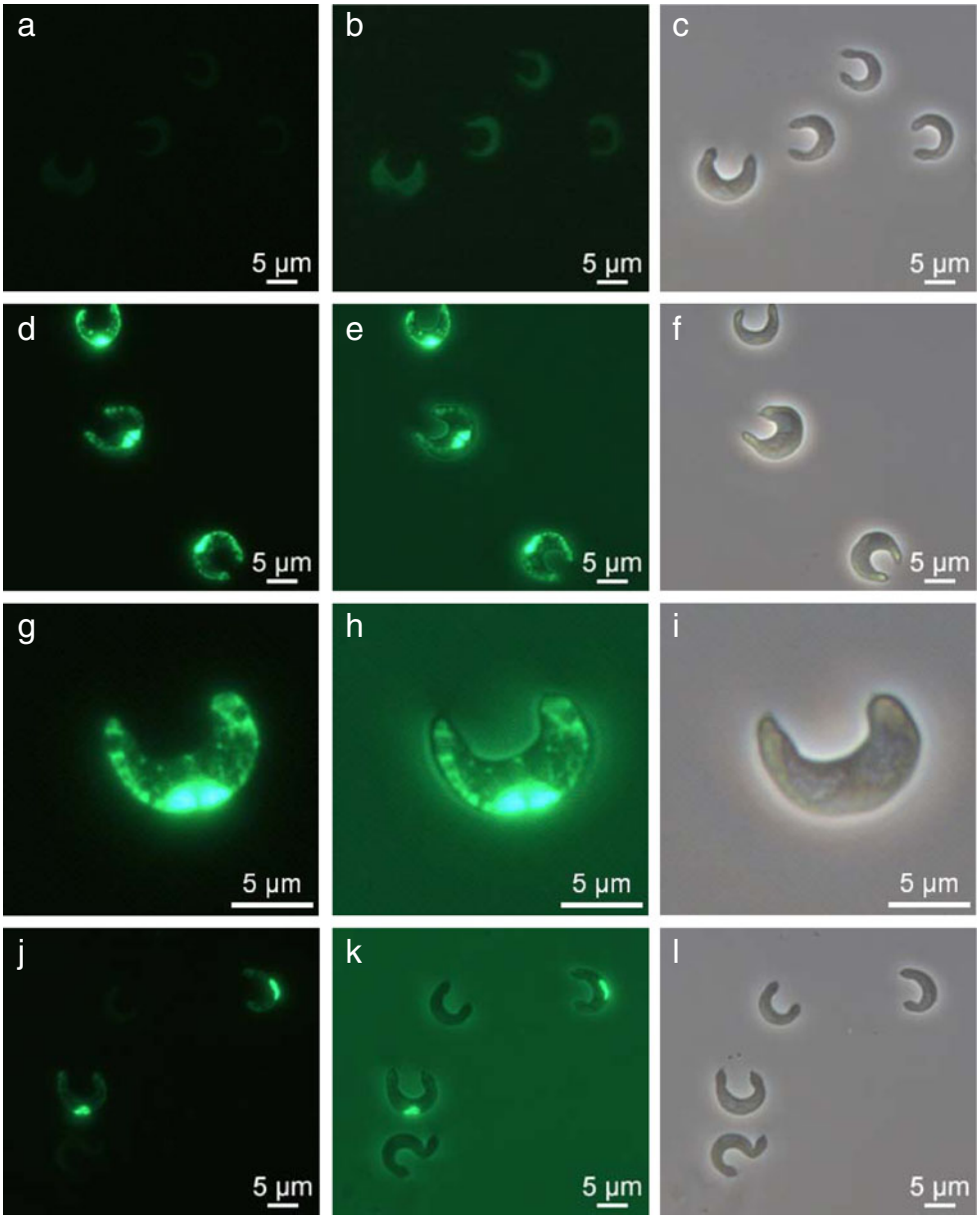

Fig. 3 Visualization of live and dead cells of the alga *P. subcapitata* stained with SYTOX Green. Cells were incubated with 0.5 µmol/l SYTOX Gren for 40 min at 25 °C. Live cells are not stained (**a** and **b**). Dead cells display a green fluorescence (**d**, **e**, **g** and **h**). Cell population constituted of 50 % live and dead cells (**j** and **k**). Fluorescence micrographs (**a**, **b**, **d**, **g** and **j**); fluorescence plus phase-contrast micrographs (**e**, **h** and **k**); phase-contrast micrographs of the same cells of fluorescent images (**c**, **f**, **i** and **l**). In **b**, live cells were shot with 4.7× the shutter time used in **a**

population constituted of only heat-treated cells, it was possible to calculate the percentage of cells with a permeabilized membrane in a given cell suspension (Fig. 4). The percentage of cells with a permeabilized membrane (dead cells) was also determined by fluorescence microscopy. The percentage of stained algal cells was linearly related to the fraction of heat-treated cells in the sample. A good correlation between the percentage of cells with a permeabilized membrane (determined by both methods) and the percentage of heat-treated cells in the sample was obtained (Fig. 4).

Evaluation of 1-pentanol toxicity in a short-term bioassay

An evaluation of the effect of 1-pentanol on *P. subcapitata* plasma membrane integrity was carried out using a microplate assay in order to test the applicability of the selected staining conditions.

Cells of *P. subcapitata*, in the exponential phase of growth, were exposed to 1-pentanol in a concentration range of 2,400 to 32,000 mg/l, for 6 h, under continuous light. After this time, cells were incubated with 0.5 µmol/l SYTOX Green for 40 min and the percentage of cells with a permeabilized membrane was determined considering the mean of the higher signal, which corresponded to samples where all cells were dead. A dose–response curve was obtained by plotting the percentage of algal cells with a permeabilized plasma membrane (dead) against the concentration range of 1-pentanol (Fig. 5). The EC_{10} and EC_{50} values, which represent the concentrations of the toxicant that induced the loss of membrane integrity in 10 or 50 % of the cell population, respectively, were also determined (Table 1). It was

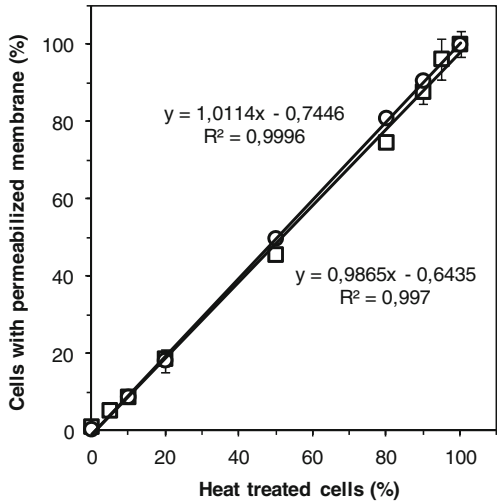

Fig. 4 Comparison of use of microscopy and microplate reader for assessing the percentage of *P. subcapitata* algal cells with a permeabilized membrane. Different proportions of live and heat-treated cells of *P. subcapitata*, at a final concentration of 1×10^6 cells/ml, were incubated with 0.5 μmol/l SYTOX Green for 40 min at 25 °C. The percentage of cells with a permeabilized membrane was determined by fluorescence microscopy (*circles*) or calculated (*squares*) considering the maximum fluorescence exhibited by the population that was constituted exclusively of heat-treated cells, as described in "Materials and methods". This is a typical example of an experiment performed three times. In each experiment, the fluorescence was measured (using a microplate reader) in five replicates for each mixture ($n=5$); for epifluorescence microscopy, at least three samples of 200 cells (total of 600 cells for each mixture) were scored in randomly selected microscope fields ($n=3$). Standard deviations are presented with 95 % confidence limits (*vertical error bars*). Where no error bars are shown, they are within the points

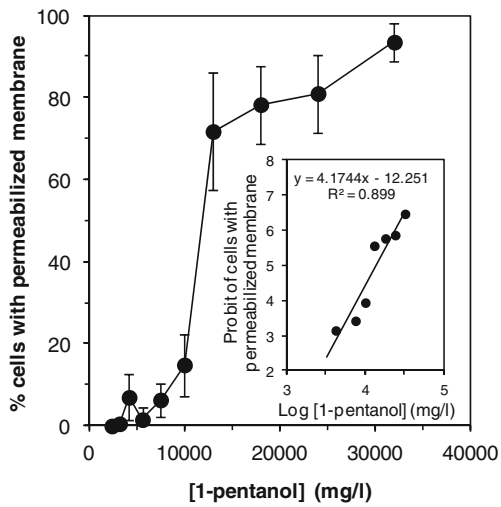

Fig. 5 Dose–response plot of *P. subcapitata* cells exposed to 1-pentanol. Cells were incubated with different 1-pentanol concentrations for 6 h and subsequently incubated with 0.5 μmol/l SYTOX Green, for 40 min, at 25 °C. *Inset*, probit of cells with permeabilized membrane versus log of 1-pentanol concentration. Each point represents the mean obtained from three independent experiences performed in quintuplicate ($n=15$); standard deviations are presented with 95 % confidence limits (*vertical error bars*)

possible to determine that 10 or 50 % of the cell population of *P. subcapitata* lost membrane integrity when exposed over 6 h to 7,617 mg/l 1-pentanol (95 % confidence limits 4,670–9,327) or 12,818 mg/l 1-pentanol (95 % confidence limits 10,929–15,183), respectively.

During the time test (6 h), parallel assays using clear microplates allowed a small growth to be confirmed in cells exposed to lower concentrations or included in the negative control (cells without toxicant). No growth was observed for high 1-pentanol concentrations. The dose–response curve obtained with normalized fluorescence (taking into account the number of cells) or without normalized fluorescence (assuming that no growth occurred) gave similar EC values.

Discussion

The use of cell-based fluorescence technologies has been shown to be advantageous in comparison with non-fluorescent methodologies in drug discovery and safety pharmacology (Fritzsche and Mandenius 2010).

A fluorescent probe to be used as an indicator of plasma membrane integrity should ideally be selective (only penetrate cells with permeabilized plasma membranes) and display a marked enhancement of fluorescence once inside the cells. In the present work, a microplate assay was developed based on an evaluation of plasma membrane integrity in the alga *P. subcapitata.* The staining of algal cells is rapid (incubation time of 40 min) and can be carried out with a low dye concentration (0.5 μmol/l) (Fig. 2). According to the manufacturer's protocols (Molecular Probes, Life Technologies), the recommended conditions for staining bacteria and yeast cells with SYTOX Green are: 0.5–5 μmol/l (bacteria) and 1–50 μmol/l (yeast), for >5 min (bacteria) and >10 min (yeast). In the case of phytoplankton, a range of concentrations for 0.1 to 5 μmol/l SYTOX Green has been described in the literature (Veldhuis et al. 2001; Franklin and Berges 2004; Regel et al. 2004; Sato et al. 2004; Binet and Stauber 2006; Daly et al. 2007; Ribalet et al. 2007; Timmermans et al. 2007; Lin et al. 2009; Chang et al. 2011; Peperzak and Brussaard 2011); the staining times described in these works also vary between 5 and 60 min. In the present work, a SYTOX Green concentration closest to the lowest values was used. Even though a high fluorescence was observed after 5–10 min of incubation with the dye (55–85 % of the maximum for a given concentration), an incubation time of 40 min was selected in order to obtain a stronger signal with a lower dye concentration. In these conditions, a signal >75 % of the maximum fluorescence was observed.

SYTOX Green is completely excluded from the live cells of *P. subcapitata* (Fig. 3a–c) and penetrated cells with a compromised plasma membrane (Fig. 3d–i). Once inside the algal cells, the probe exhibited a strong fluorescence which allowed

Table 1 Effect of 1-pentanol on membrane integrity of *P. subcapitata* evaluated using SYTOX Green

Effect parameter	Time of exposure to 1-pentanol (h)	EC (mg/l)[a]	
		10	50
Loss of membrane integrity	6	7,617 (4,670–9,327)	12,818 (10,929–15,183)

EC effect concentrations

[a] Values in parenthesis are 95 % confidence intervals. The mean values and confidence intervals were obtained from three experiences performed in quintuplicate ($n=15$)

live and heat-permeabilized *P. subcapitata* cells to be clearly separated (Figs. 2 and 3j–l).

The microplate assay, as well as the direct microscopic observation of the algal cells, is very simple and does not require a wash step after incubation of the cells with a fluorescent probe. In the case of microscopic observation of the algal cells, due to its characteristic autofluorescence, it is desirable to use a band-pass (BP) suppression filter instead of the more common long-pass (LP) suppression filter. In the first case, only the green fluorescence can be observed; using LP suppression filters, the red-autofluorescence can be observed, which can make the observation of faintly green cells difficult.

The disruption of plasma membrane integrity by the action of chemical or physical treatments leads to cell death. The classical viability test requires algal cell growth on solid media and takes 1 week to produce colonies that are discernible to the naked eye due to the slow growth of *P. subcapitata* algal cells. In this classical test, the concept of viable cells is associated with the ability to divide and give rise to colonies. Conversely, the assay using SYTOX Green is based on the capacity of viable cells to exclude the fluorescent probe. Cells may be unable to proliferate but nonetheless retain membrane integrity. The classical plate count assay will not detect these living but non-culturable algal cells, while the use of the SYTOX Green assay allows them to be detected.

Using a positive control (heat-treated cells), it was possible to calculate the percentage of algal cells with a permeabilized membrane (Fig. 4). The loss of membrane permeability can be used as an end-point in ecotoxicity studies and a dose–response curve can be obtained (Fig. 5) by calculating the respective 6-h EC values (Table 1). In the short-term assay described in the present work, the small growth observed in the negative control (without toxicant) and in the lower 1-pentanol concentrations did not affect the dose–response curve. Thus, similar results were obtained with normalized fluorescence (taking into account the number of cells) or without normalized fluorescence (assuming no growth during the 6-h assay). However, if an expansion of the test time (to 24 or 72 h) is desired, then it is recommended to normalize the fluorescence values, taking into account the number of cells after a given time.

In the conventional algal inhibition test (OECD 1984), toxicity is assessed based on the inhibition of algal growth (algistatic effect) over 72 h, i.e. the assay measures chronic toxicity. This classical assay only allows a slow response (after 3 days); additionally, during the duration of the assay, contaminants can be lost to test containers (Franklin et al. 2005). In order to avoid these drawbacks, short (1–27 h) toxicity tests based on enzyme inhibition (esterases) by the toxicants have been developed (Arsenault et al. 1993; Snell et al. 1996; Blaise and Menard 1998; Franklin et al. 2001). Another possibility for rapid (acute) toxicity assessment is to evaluate the impact of toxicants on membrane integrity using SYTOX Green. In this case, the algicidal effect and the mode of action (membrane integrity) of the toxicants are evaluated.

The microplate-based assay developed can be used as a first-screening toxicity bioassay. This new method is fast (6 h of incubation with the toxicant plus 40 min of incubation with the fluorescent probe) and easy to perform (it does not require any washing step). It is carried out in 96-well microplates and quantification of fluorescence is performed automatically (avoiding cell counting using an epifluorescence microscope, which is time and labour costly), using a very common device for fluorescence quantification.

In conclusion, in this study, a microplate-based assay was developed, using SYTOX Green, for the quantitative evaluation of disruption of cell membranes of the alga *P. subcapitata* (algicidal effect) by physical or chemical treatments. The staining process was optimized, revealing that the incubation of algal cells with 0.5 μmol/l SYTOX Green, over 40 min, allowed a clear discrimination between live (with intact membrane) and dead cells (with compromised membrane). Based on the optimized conditions for algal staining, a new (acute) short-term (6 h) bioassay was proposed; it is a simple assay wherein no centrifugation or washing steps are required. After exposing the algal cells to the toxicant in the microplate, cells were incubated with SYTOX Green and fluorescence was measured. The miniaturization of the assay, coupled with the automatic fluorescence reading, gives it potential as an important tool for high-throughput screening of environmental pollutants using *P. subcapitata* algal cells.

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