Assessment of cellular reduced glutathione content in *Pseudokirchneriella subcapitata* using monochlorobimane

Manuela D. Machado · Eduardo V. Soares

Abstract The green alga *Pseudokirchneriella subcapitata* has been extensively used for the assessment of adverse impacts of pollutants. Glutathione is involved in antioxidant defence and drug detoxification. Intracellular reduced glutathione (GSH) concentration can be used as an indicator of the health of cells. This work describes a simple and fast fluorescent cell-based assay for the evaluation of intracellular GSH in the alga *P. subcapitata,* using monochlorobimane (mBCl). Metabolically active algal cells incubated with 50 μmol L^{-1} mBCl form fluorescent bimane–glutathione (B-SG) adducts that can be measured fluorometrically. The distribution of GSH (B-SG adducts) in whole cells can be observed by epifluorescence microscopy, in the form of blue fluorescent spots. Depletion of cellular GSH with iodoacetamide, inhibition of glutathione S-transferase with ethacrynic acid or heat-induced death of the cells inhibited the formation of fluorescent adducts in the presence of mBCl. The fluorometric assay, using the 96-well microplate format, was able to detect GSH depletion in algal cells. This cell-based assay can be used to evaluate decreases in GSH content due to exposure to toxicants. This assay is amenable to automation and may be useful in high-throughput toxicity screening using the alga *P. subcapitata*.

Keywords Fluorescence microscopy · Glutathione · Microplate assay · Monochlorobimane · *Selenastrum capricornutum* · Toxicity assay

Introduction

Reduced glutathione (GSH), a tripeptide (composed of γ-L-glutamate, L-cysteine and glycine), is the major free thiol in most living cells and is involved in cellular antioxidant defence and drug detoxification (Sies 1999).

The depletion in GSH content in *Scenedesmus bijugatus* and *Chlamydomonas reinhardtii* algal cells exposed to copper has been described (Nagalakshmi and Prasad 2001; Stoiber et al. 2007). Similarly, the GSH content of the green alga *Scenedesmus acutus* showed a downward trend with an increase in the Cr(VI) concentration in a sulphate-free buffer (Gorbi et al. 2006). Recently, it has been described that the exposure of the marine unicellular alga *Phaeodactylum tricornutum* to ethyl 2-methyl acetoacetate leads to a reduction in GSH content (Yang et al. 2011); in the same way, the GSH content in *Pseudokirchneriella subcapitata* declined when exposed to nonylphenol (Gao and Tam 2011). It is probable that GSH was consumed in the defence against agents that impose oxidative stress.

Intracellular GSH concentration appears to be a sensitive indicator of the health of a cell. Therefore, GSH depletion can be used as an early toxicity marker (Schoonen et al. 2005). Intracellular measures of GSH in toxicity screening have been described in human and Chinese hamster cell lines (Schoonen

et al. 2005). In a similar way, a decrease in GSH content upon exposing *P. subcapitata* cells to chemical species can be used in toxicity screening.

Currently, the methods available for GSH measurement in algal cells require several manipulations with different reagents and are quite time-consuming. Usually these techniques involve a cell lysis step followed by glutathione quantification using enzymatic or chromatographic methods (ion-exchange chromatography or high-performance liquid chromatography; Anderson 1985; Le Faucheur et al. 2005; Lei et al. 2006; Yang et al. 2011). These procedures are labour-intensive and expensive. The development of cell-based assays, using fluorescent probes, for toxicity testing of environmental pollutants (pharmaceuticals and other organic compounds as well as metals), have attracted increasing interest (Fritzsche and Mandenius 2010).

Monochlorobimane (mBCl) is a non-fluorescent cell-permeant probe. Once inside the cell, it reacts with GSH to form fluorescent bimane–glutathione (B-SG) adducts, in a reaction catalyzed by glutathione S-transferase (GST; Fig. 1; Haugland 2005). Comparative studies have shown that GSH determination with mBCl or using glutathione reductase gave similar results (Cook et al. 1991). Additionally HPLC analysis has shown that GSH is the only thiol that reacts with mBCl (Fernández-Checa and Kaplowitz 1990). Due to its specificity for GSH, mBCl has been extensively used for the assessment of GSH in tissue homogenates (by adding both mBCl and GST) by a fluorometric method (Kamencic et al. 2000) or in whole cells of mammalians (Nair et al. 1991; Barhoumi et al. 1995; Stevenson et al. 2002; Sebastia et al. 2003; Franco et al. 2007), plants (Hartmann et al. 2003) and yeasts (Li et al. 1996), by confocal laser-scanning microscopy, flow cytometry or using a fluorescence microplate reader. In this context, mBCl has emerged as a good candidate to assess GSH content in cells of the alga *P. subcapitata*. As far as we know the use of mBCl with *P. subcapitata* cells in a fluorometric assay or by epifluorescence microscopy has never been described.

The aim of the present work was to optimise a simple fluorescent assay to assess changes in GSH in whole cells of the alga *P. subcapitata* using the 96-well microplate format. The monitoring of the sub-cellular distribution of GSH (B-SG adduct localisation) by epifluorescence microscopy was optimised. The in situ GSH quantification in *P. subcapitata* cells is also described. In addition, the utility of the fluorescent assay for GSH monitoring in high-throughput screening of toxic substances is discussed.

Material and methods

The freshwater green alga *Pseudokirchneriella subcapitata* (strain 278/4) was obtained from the Culture Collection of Algae and Protozoa (CCAP), UK. The alga was maintained in OECD algal test medium (OECD 1984) with 20 gL^{-1} agar (Merck, Germany), in the dark, at 4°C. Medium stock solutions were prepared, sterilised (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*, 15 min), washed, resuspended in fresh medium and stored in the dark at 4°C. These stock cultures were transferred to fresh medium, at least monthly, to prepare new pre-cultures.

The pre-cultures were prepared by inoculating 40 mL OECD medium, in 100 mL Erlenmeyer flasks, with an initial cell concentration of ~5×10^4 cells mL^{-1}. 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), with an intensity of 4,000 lux (~54 μmol photons m^{-2} s^{-1}) at the surface of the flask, verified using an illumination meter (HI 97500, Hanna Instruments).

The 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^{-1} from the pre-culture after 2 days of growth. Cells were incubated under the conditions described above for the pre-cultures. The purity of the cultures was verified, on a weekly basis, by a microscopic examination of a subsample.

The algal cell concentration was measured by direct cell counting using a microscope and a counting chamber or using an automated cell counter (TC10, Bio-Rad). Additionally, the

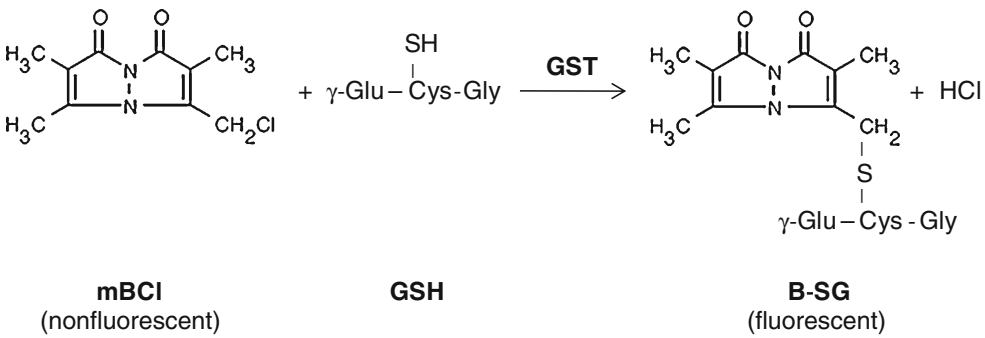

Fig. 1 Molecular structure of non-fluorescent monochlorobimane (mBCl) and its reaction with glutathione (GSH) to form a fluorescent bimane–glutathione (B-SG) adduct, in a reaction catalyzed by glutathione S-transferase (GST)

algal concentration was evaluated, indirectly, by spectrophotometrically measuring the absorbance at 750 nm (U.S.-EPA 2002); in the latter, a calibration curve (number of cells versus absorbance) was first constructed.

Treatment of algal cells

For the depletion of glutathione, algal cells in the exponential phase of growth (2 days) were harvested by centrifugation (2,500×*g*, 15 min) and resuspended in fresh culture medium at 1×10^6 cells mL^{-1}. Then, cells were incubated with 1.0×10^{-3}–1.0 mmol L^{-1} iodoacetamide (Sigma-Aldrich), in the dark, at 25°C for 1 h, with shaking (100 rpm). The stock solution of iodoacetamide consisted of 10 mmol L^{-1} of the compound in deionised water. After treatment with iodoacetamide, cells were washed once with deionised water and suspended in OECD culture medium. The washing step, with deionised water, did not provoke cells burst, evaluated by direct microscopic observation.

The inhibition of GST was carried out by incubating the algal cells with ethacrynic acid. Thus, cells in the exponential phase of growth (2 days) were harvested and resuspended in fresh culture medium at 1×10^6 cells mL^{-1}, as described for the treatment with iodoacetamide. Subsequently cells were incubated with 0.1 mmol L^{-1} ethacrynic acid (Sigma-Aldrich), at 25°C for 1 h, with shaking (100 rpm). The stock solution of ethacrynic acid consisted of 100 mmol L^{-1} of the compound in ethanol. The final concentration of ethanol in the assay was 0.1 % (*v*/*v*).

Heat-treated cells were obtained by placing algal cells in a water bath at 65°C for 1 h. Subsequently cells were harvested and resuspended in fresh culture medium.

Evaluation of lethality induced by heat treatment

Cell suspension composed by heat-treated cells was adjusted to 2×10^6 cells mL^{-1}. Four replicates of 200 μL of the cell suspension were plated on OECD agar medium and incubated at 25°C, under continuous light for 2 weeks.

Optimisation of algal cell staining

Algal cells were harvested in the exponential phase of growth by centrifugation (2,500×*g*, 15 min) and suspended in fresh culture medium at 1×10^6 cells mL^{-1}. Monochlorobimane (mBCl, Sigma-Aldrich) was added to the cells at a final concentration of 20–100 μmol L^{-1} from a working solution of 1 mmol L^{-1}. The stock solution of mBCl (50 mmol L^{-1}) was prepared in dimethyl sulphoxide (DMSO) and stored at −20°C; the working solution was prepared before use by diluting the stock solution in 0.1 mol L^{-1} PBS buffer (pH 7.0). In the assay the final concentration of DMSO was below 0.2% (*v*/*v*). The cell suspensions were incubated with mBCl in the dark at 25°C. At defined time intervals reported in the figures, fluorescence intensity (in relative fluorescent units, RFU) was measured in a PerkinElmer (Victor3) microplate reader at a fluorescence excitation wavelength of 355/40 nm and an emission wavelength of 460/25 nm using black 96-well microplates (OptiPlate-96 F, PerkinElmer). The mixing of the cells with mBCl was carried out in an Eppendorf tube and aliquots of 200 μL were dispensed in 96-well microplates. Similar results were obtained when the mixture was carried out in the microplates (data not shown). Fluorescence was corrected by subtracting cell, culture medium, and dye autofluorescence and normalised considering cell concentration.

Cells were also examined using a Leica DLMB epifluorescence microscope (Leica Microsystems, Wetzlar GmbH, Germany) equipped with a HBO-100 mercury lamp and filter set A (for the visualisation of the formation of glutathione-monochlorobimane adducts) or filter set I3 (for autofluorescence observation) from Leica. Filter set A: excitation filter (band-pass filter, BP) BP 340–380, dichromatic mirror 400 and suppression filter (long-pass filter, LP) LP 425; filter set I3: excitation filter BP 450–490, dichromatic mirror 510 and suppression filter LP 515. Images were acquired with a Leica DC 300F camera (Leica Microsystems, Switzerland) and processed using Leica IM 50-Image manager software.

Quantification of GSH

GSH concentration in whole cells was also determined. For this assay, algal cells at 1×10^7 cells mL^{-1} (1×10^6 cells per well) were incubated with 50 μmol L^{-1} mBCl at 25°C in the dark, during 2 h. Fluorescence was corrected by subtracting cell, culture medium, and dye autofluorescence and normalised considering cell concentration. Algal GSH concentrations were calculated using a GSH calibration curve. The GSH standard curve in a range of 0.25–4.0 nmol, i.e. 2.5–40 μmol L^{-1} (final concentration) was obtained by combining convenient volumes of GSH standard solutions with 1 U mL^{-1} glutathione S-transferase–GST (final concentration) and 50 μmol L^{-1} mBCl (final concentration), in OECD culture medium. A blank was performed by mixing OECD medium, GST and mBCl. The mixtures were incubated at 25°C in the dark for 2 h. Under these conditions, the detection limit (DL) was 0.6 nmol (6 μmol L^{-1}) GSH. The DL was calculated with the help of a section of the calibration curve close to the origin and using both the slope and the standard deviation of *y* residual ($S_{y/x}$; Miller and Miller 2005).

L-Glutathione reduced (Sigma-Aldrich, G4251) stock solution (100 mmol L^{-1}) was prepared in OECD culture medium and stored at −20°C. GSH standard solutions (0.5 or 1 mmol L^{-1}) were prepared before use (to minimize oxidation by atmospheric oxygen) by diluting the stock solution in the same medium. GST (Sigma-Aldrich, G6511) stock solution

(10 U mL^{-1}) was prepared in OECD culture medium and stored at −20°C.

The assay (samples and calibration curve) was conducted in a black 96-well microplate. The total volume in each well was 100 µL.

The GSH standard curve was carried out in triplicate and the assay with algal cells in quintuplicate. Fluorescence intensity was measured, in a microplate reader, as described above.

Reproducibility of the results

All experiments were repeated two to four times. Although, for the fluorescence measurements absolute data were not comparable in 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.

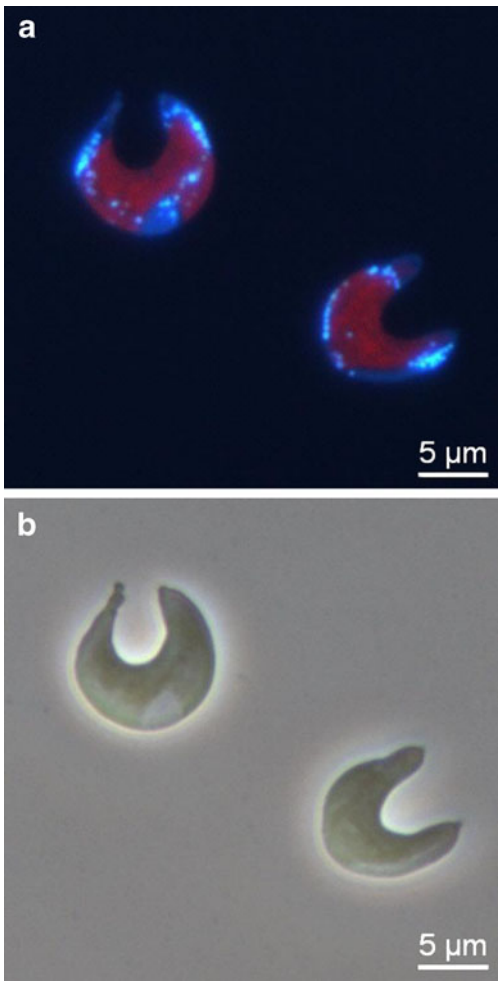

Fig. 2 Visualisation of bimane–glutathione (B-SG) adducts in the alga *P. subcapitata.* Cells were incubated with 100 µmol L^{-1} monochlorobimane for 90 min at 25°C. Live cells display *bright blue fluorescent spots* due to the formation of B-SG adducts. **a** Fluorescence micrograph. **b** Phase-contrast micrograph of the same cells

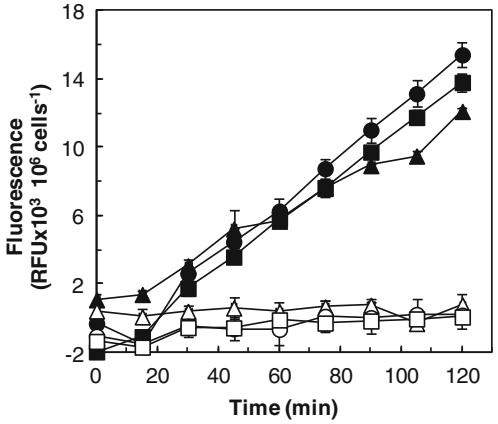

Fig. 3 Kinetics of the formation of bimane-glutathione adducts in the alga *P. subcapitata.* 1×10^6 cells mL^{-1} were incubated with 20 (*triangles*), 50 (*circles*) or 100 (*squares*) µmol L^{-1} monochlorobimane at 25°C. Live cells (*closed symbols*) and glutathione-depleted cells due to the treatment with 1.0 mmol L^{-1} iodoacetamide for 1 h (*open symbols*) were used. This is a typical example of an experiment performed twice. *Each point* represents the mean of five fluorescence readings±SD

Results

Optimisation of algal cell staining for fluorescence microscopy

Cells of the alga *P. subcapitata*, in the exponential phase of growth, were exposed to several mBCl concentrations, for

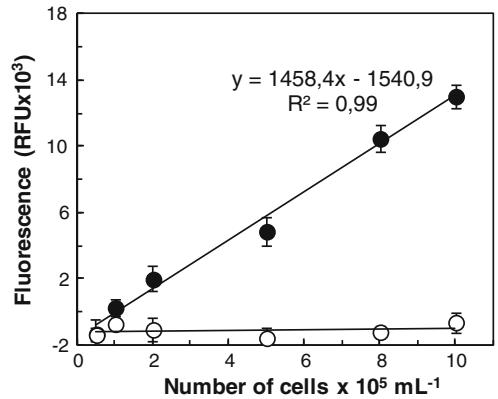

Fig. 4 The linear fluorescence response to increasing numbers of *P. subcapitata* cells containing glutathione. Cells were incubated with 50 µmol L^{-1} monochlorobimane for 90 minutes at 25°C. Metabolically active cells with glutathione (GSH; *closed circles*) and GSH-depleted cells due to the treatment with 1.0 mmol L^{-1} iodoacetamide for 1 h (*open circles*) were used. This is a typical example of an experiment performed twice. *Each point* represents the mean of five fluorescence readings±SD

30–120 min in order to localise GSH distribution as B-SG adducts. B-SG adducts can be observed by fluorescence microscopy in the form of blue fluorescent spots (Fig. 2a).

After incubation with 20 μmol L^{-1} mBCl for 120 min, it was possible to observe only very small B-SG spots which disappeared very quickly upon exposure to the excitation wavelength (photobleaching). Incubation with 50 μmol L^{-1} mBCl for 120 min produced fluorescent spots of a bigger size than those observed with 20 μmol L^{-1} (data not shown). However, satisfactory results were only observed with 100 μmol L^{-1} mBCl; with this probe concentration, small B-SG adducts were observed after 30 min and were clearly observed after 60–90 min with little photobleaching (Fig. 2a). Extending the contact time to 120 min did not produce bigger B-SG spots and did not improve image quality (data not shown).

Optimisation of the microplate assay

The assay was optimised as a function of the probe concentration (20, 50 and 100 μmol L^{-1} mBCl), the incubation time of the algal cells with the probe and the cell concentration (0.5 to 10×10^5 cells mL^{-1}). The formation of B-SG adducts in algal cells was monitored by measuring the emission of fluorescence at 460 nm using a 96-well microplate reader.

Metabolically active cells displayed a strong fluorescence signal even at the lowest mBCl concentration tested (20 μmol L^{-1}). Similar RFU were obtained for 50 and 100 μmol L^{-1} mBCl. Thus, 50 μmol L^{-1} mBCl was selected since it corresponded to the lowest concentration that gave a high signal (Fig. 3). A fluorescence plateau was not reached with these mBCl concentrations with an incubation time of 120 min. Taking into account the data reported above, in microscopic observations, an incubation time of 90 minutes was selected.

The fluorescence due to the formation of B-SG adducts increased linearly with the cell concentration in metabolically active algal cells with GSH. On the contrary, in GSH-depleted cells the fluorescence remained very low at a constant value (Fig. 4). The fluorescence exhibited by live cells with GSH (2×10^5 cells mL^{-1}) was more than 2,000 times higher than with the same number of GSH-depleted cells. In the case of 10×10^5 cells mL^{-1} the fluorescence was more than 13,000 times higher than the corresponding number of GSH-depleted cells (Fig. 4).

In summary, the optimal staining conditions of the alga *P. subcapitata* were: mBCl at a final concentration of 50 μmol L^{-1} for the fluorometric assay and 100 μmol L^{-1} for fluorescence microscopy; algal cell suspension in OECD culture medium at a

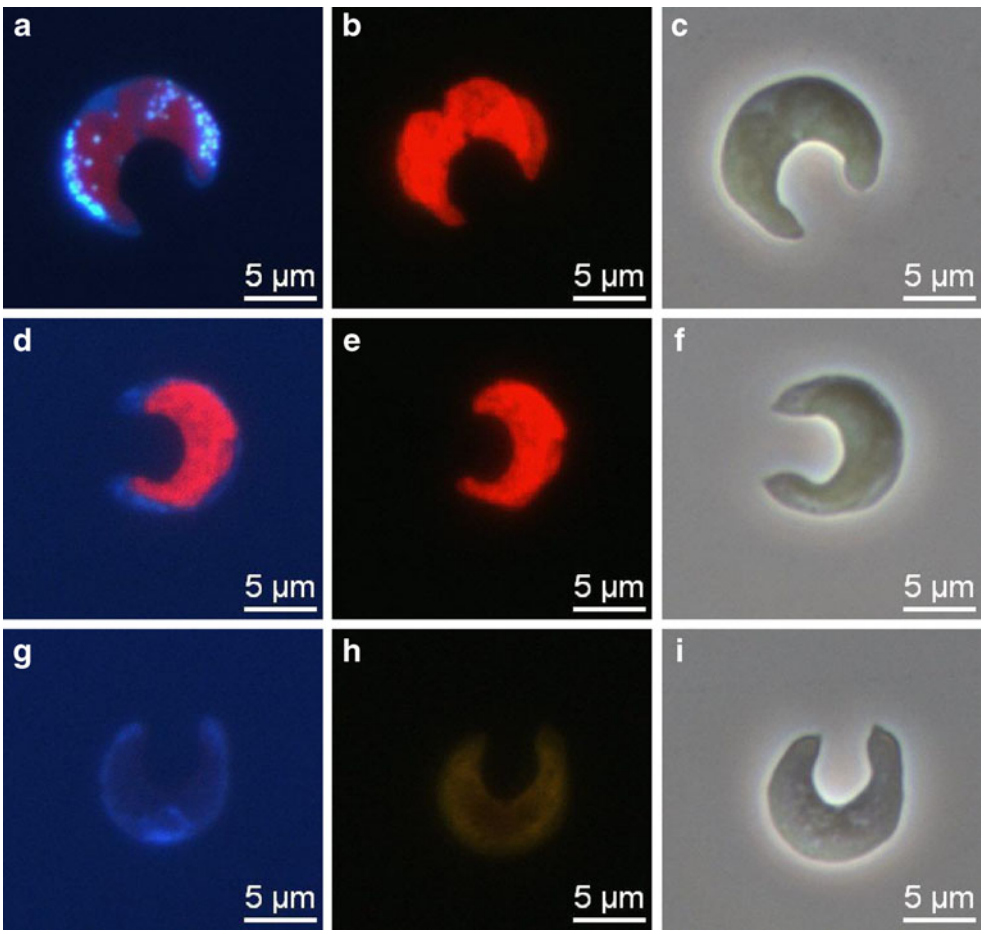

Fig. 5 Influence of the presence of glutathione and the metabolic state of cells of the alga *P. subcapitata* in the formation of bimane–glutathione (B-SG) adducts. Cells were incubated with 100 μmol L^{-1} monochlorobimane for 90 min at 25°C. Live cells (**a**) display *bright fluorescent spots* (B-SG adducts), which are absent in glutathione (GSH)-depleted cells (by treatment with 1.0 mmol L^{-1} iodoacetamide for 1 h; **d**) or in heat-treated (65°C, for 1 h) cells (**g**). Metabolically active cells and cells treated with iodoacetamide exhibit autofluorescence (**b**, **e**), which is absent in dead cells (**h**). Fluorescence micrographs (**a**, **b**, **d**, **e**, **g**, **h**); phase-contrast micrographs of the same cells (**c**, **f**, **i**). Fluorescent images of GSH-depleted cells (**d**) and heat-treated cells (**g**) were shot with 2× the shutter time used in image (**a**); similarly, the autofluorescence image of heat-treated cells (**h**) was shot with 2× the shutter time used in images (**b**) and (**e**)

final cell concentration from 2 to 10×10^5 cells mL^{-1} (which corresponds to 4×10^4–2×10^5 cells per well) for the fluorometric assay and 10×10^5 cells mL^{-1} for microscopy; staining for 90 minutes in the dark at 25°C.

Validation of the staining protocol

The cell GSH content of the alga was depleted by treating the cells with iodoacetamide in order to confirm if the emission of fluorescence was attributable to GSH. Cells were subsequently washed and stained with mBCl as described above. Iodoacetamide is an alkylating agent which binds covalently to thiol groups (Hansen and Winther 2009). Iodoacetamide has been used to cause depletion of GSH in different cell models such as yeast (Millard et al. 1997) and mammalian cells (Liu et al. 1996; Schmidt and Dringen 2009). As it can be seen in Fig. 3, the treatment of algal cells with 1.0 mmol L^{-1} iodoacetamide for 1 h hampered the emission of fluorescence at 460 nm. This result was confirmed by fluorescence microscopy. GSH-depleted cells, although metabolically active (they displayed orange autofluorescence; Fig. 5e), did not demonstrate fluorescent spots when incubated with 100 μmol L^{-1} mBCl for 90 min (Fig. 5d). Increasing the shutter time used for non-treated cells by twofold revealed only a faint blue cell background staining (Fig. 5d).

Ethacrynic acid has been used as an inhibitor of GST (Phillips and Mantle 1991; Hoffman et al. 1995; Maeda et al. 2006). The incubation of algal cells with 0.1 mmol L^{-1} ethacrynic acid for 1 h inhibited the emission of fluorescence at 460 nm (data not shown). Microscopic examination of cells treated with ethacrynic acid and subsequently stained with mBCl confirmed the absence of fluorescent spots and originated similar images to iodoacetamide treated cells (Fig. 5d). This result suggests the involvement of GST in the formation of B-SG adducts in *P. subcapitata* cells.

Algal cells were also heat-treated at 65°C for 1 h; this treatment provoked the loss of viability (>99.99%) of the cell population, evaluated by the classical plate count assay in OECD-agar medium. This treatment also originated the disappearance of the typical orange autofluorescence (Fig. 5h). Heat-killed cells incubated with 100 μmol L^{-1} mBCl for 90 min were not able to form fluorescent spots (B-SG adducts; Fig. 5g). The addition of GST (1 UmL^{-1}) to these algal cells did not promote the formation of B-SG adducts; most likely algal cells loss the GSH pool during heat treatment. B-SG adducts can only be observed in metabolically active cells with GSH (Fig. 5a).

Evaluation of cellular GSH variation

The applicability of mBCl in the assessment of GSH content variation in algal cells was tested by manipulating cellular GSH. For this purpose, cells of *P. subcapitata* were exposed to different concentrations of iodoacetamide for 1 h. As can be seen in Fig. 6, the increase in the iodoacetamide concentration provoked a decrease in fluorescence. A concentration of 0.0010 mol L^{-1} iodoacetamide resulted in a small (~10%) decrease in fluorescence, while 1.0 mol L^{-1} abolished fluorescence (Fig. 6a). Therefore, the optimised assay allowed for the evaluation of GSH content variation in algal cells. Considering the fluorescence emitted by non-treated cells (control), it was possible to determine the reduction in fluorescence and consequently the reduction in cellular GSH content (Fig. 6b).

In situ GSH quantification

GSH content, in whole cells, was determined from the GSH standard curve by linear regression analysis. The cellular GSH content in *P. subcapitata* algal cells in exponential

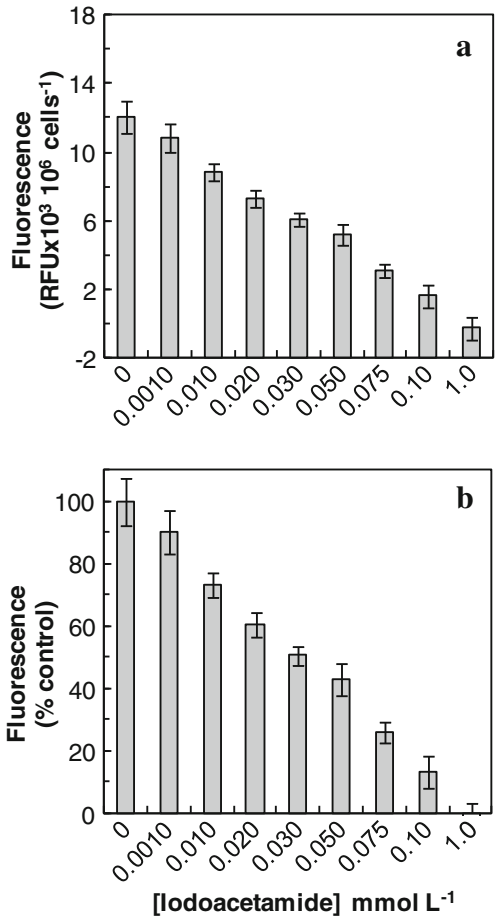

Fig. 6 Variation in glutathione content in the alga *P. subcapitata*; 1×10^6 cells mL^{-1} were exposed to different iodoacetamide concentrations for 1 h. Subsequently, cells were washed and exposed to 50 μmol L^{-1} mBCl for 90 min at 25°C. Fluorescence (presented as relative fluorescence units; RFU) exhibited by cell suspensions (**a**); percentage of the fluorescence relative to the control (cells not treated with iodoacetamide; **b**). This is a typical example of an experiment performed four times. *Each point* represents the mean of five fluorescence readings±SD

phase of growth was 982±57 amol cell^{-1}; the mean and the standard deviation were calculated from two experiments carried out in quintuplicate (n=10). The coefficient of variation was <6%.

Discussion

An ideal stain for GSH in live cells should present several characteristics: (a) it should readily penetrate the membrane, (b) be non-toxic to cells, (c) specific and (d) the fluorescent product should be stable and well-retained by the cells (Stevenson et al. 2002). Monochlorobimane is a lipophilic probe that passively diffuses across the cell membrane. Once in the cytoplasm it reacts specifically with GSH, leading to the formation of B-SG adducts (Puchalski et al. 1991). The reaction of mBCl with GSH is very specific because it is catalysed by glutathione S-transferase (GST; Puchalski et al. 1991). For this reason mBCl is the most specific of the currently available GSH probes (Haugland 2005). The present study shows that mBCl can also be used in *P. subcapitata* algal cells. As with other cell models (mammalian, plant and yeast cells), the formation of blue fluorescent spots only occurs in metabolically active algal cells with GSH. Depletion of cellular GSH with iodoacetamide, inhibition of GST with ethacrynic acid or heat-treatment of algal cells inhibited the formation of blue fluorescent spots. With the assayed concentrations of mBCl (20–100 μmol L^{-1}) weak unspecific binding of the dye was observed.

In the protocol optimised in the present work algal cells were incubated with mBCl in OECD culture medium at 25°C. These conditions were selected for two reasons: (a) simplicity and (b) these correspond to the growth conditions of the alga; under these conditions, GSH content can be evaluated without any treatment (centrifugation or washing step). With this protocol no sample preparation, cell disruption, or extract filtration steps were required.

Manipulation of the GSH content by incubating the algal cells with different concentrations of iodoacetamide showed that the present assay enables monitoring of GSH depletion of the alga. This procedure can be useful in the evaluation of the redox environment of the cells in GSH redox cycle research. It is also possible to obtain a dose–response curve and calculate the different EC values. This can be done by plotting the % of fluorescence (relative to the control–untreated cells) against the concentrations range of the toxic under evaluation, in a similar way as with iodoacetamide.

The in situ GSH quantification in *P. subcapitata* cells in exponential phase of growth was carried out (982±57 amol cell^{-1}). This value is of same order of magnitude of the GSH concentration (384±89 amol cell^{-1}) reported by Lavoie et al. (2009) for *P. subcapitata* cells in stationary phase of growth. Nevertheless, Stoiber et al. (2007) described a lower total GSH concentration (14–37 amol cell^{-1}) for *P. subcapitata* in stationary phase of growth.

Microplate readers are the most commonly used devices for fluorescence quantification (Fritzsche and Mandenius 2010). The monitoring of GSH depletion due to exposure to toxicants can be easily carried out in 96-well microplates. The automation and the fluorescence reading speed of such devices confers the possibility of high-throughput screening of GSH content changes in *P. subcapitata* as a consequence of exposure to a large number of toxicants. The developed assay may be used to advance our understanding of the cellular effects of these toxicants. In addition, direct visualisation of the presence or absence of fluorescent spots by fluorescent microscopy allows the individual examination of the cell population.

To conclude, mBCl can be used in the assessment of glutathione content in the alga *P. subcapitata* in a rapid and easy (without sample preparation) cell-based assay. The procedure can be carried out using simple instrumentation (a microplate reader). Only metabolically active cells with GSH are able to form adducts with mBCl. Cellular localisation of these adducts can be visualised in the form of blue fluorescent spots by microscopy. The assay described in the present work may be a useful tool in the high-throughput screening of *P. subcapitata* GSH depletion due to the toxic effects of different chemical species.

Acknowledgements The authors thank the Fundação para a Ciência e a Tecnologia (FCT) through the Portuguese Government for their financial support of this work through the grant PEST-OE/EQB/LA0023/2011 to IBB. Manuela D. Machado gratefully acknowledges the post-doctoral grant from FCT (SFRH/BPD/72816/2010).

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