Thermoluminescence as a complementary technique for the toxicological evaluation of chemicals in photosynthetic organisms

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Abbreviations: B-band: TL band due to $S_{2/3}Q_B^-$ recombination; BrB: bromobenzene; C-band: TL band due to $D^+Q_A^-$ recombination; CQ: chloroquine; D: tyrosine D of photosystem II; DEA: diethanolamine; EC_{50}: concentration of test chemical that modified the biomarker by 50% in comparison with appropriate untreated control; HTL: high temperature thermoluminescence; $InNO_3$: indium nitrate; PpG: propyl gallate; PSII: photosystem II; Q-band: TL band due to $S_2Q_A^-$ recombination; Q_A and Q_B: primary and secondary quinonic electron acceptors of PSII; S_2 and S_3: oxidized states of the manganese oxygen evolving

complex of PSII; SMFA, sodium monofluoroacetate; STL: standard thermoluminescence; TL: thermoluminescence; t_{max}: temperature of the maximum intensity of a TL band.

Abstract

Thermoluminescence is a simple non-invasive technique very useful for study electron transfer reactions on photosystem II (standard thermoluminescence) or the level of lipid peroxidation in membranes (high temperature thermoluminescence) in photosynthetic organisms. Both techniques were used to investigate the effects produced on *Chlorella vulgaris* cells by six compounds: the chemical intermediates bromobenzene and diethanolamine, the antioxidant propyl gallate, the semiconductor indium nitrate, the pesticide sodium monofluoroacetate and the antimalarian drug chloroquine. Electron transfer activity of the photosystem II significantly decreased after the exposure of *Chlorella* cells to all the six chemicals used. Lipid peroxidation was slightly decreased by the antioxidant propyl gallate, not changed by indium nitrate and very potently stimulated by diethanolamine, chloroquine, sodium monofluoroacetate and bromobenzene. For five of the chemicals studied (not bromobencene) there is a very good correlation between the cytotoxic effects in *Chlorella* cells measured by the algae growth inhibition test, and the inhibition of photosystem II activity. The results suggest that one very important effect of these chemicals in *Chlorella* cells is the inhibition of photosynthetic metabolism by the blocking of photosystem II functionality. In the case of sodium monofluoroacetate, diethanolamine and chloroquine this inhibition seems to be related with the induction of high level of lipid peroxidation in cells that may alter the stability of photosystem II. The results obtained by both techniques supply information that can be used as a supplement to the growth inhibition test and allows a more complete assessment of the effects of a chemical in photosynthetic organisms of aquatic ecosystems.

1. Introduction

Model systems and bioassays are used in environmental toxicology to provide information for risk assessment of chemicals. To improve the efficiency in evaluating the hazard of a large number of chemicals, providing mechanistic information, it would be helpful to carry out batteries of rapid and mode of action based screening tests (Katsumata et al., 2009; Repetto, 2013).

Standard (STL) and high temperature thermoluminescence (HTL) measurements are useful techniques for the study of the effects of stress conditions in photosynthetic organisms (Ducruet, 2003; Havaux, 2003). Its use in aquatic toxicology has been however very limited. Luminescence is a general term which indicates a light emitting process when the molecules in an electronically excited state decay to the stable ground state. Luminescence from organic or inorganic material can be also thermally induced and enhanced by heating the sample in the dark. This process is called thermoluminescence and describes the emission of light at characteristic temperatures from samples containing chemiluminescence active species, radical pair states or electron hole pairs (Ducruet, 2003; Havaux, 2003).

STL is normally used to study the photochemical activity of photosystem II (PSII). PSII is a large pigment-protein complex which uses light energy to water splitting producing molecular oxygen as by-product and providing an unlimited supply of reducing power needed to convert CO_2 into the organic molecules of life (for a review see Diner and Babcock, 1996). The reaction centre (RC) of PSII is composed of an heterodimer formed by the D1 and D2 membrane proteins that bears all the cofactors needed for charges separation and stabilization: six chlorophylls (Chl) (including four of the Chl centre, P680), two pheophytins, two plastoquinones Q_A and Q_B, one non-heme iron atom, one tetranuclear manganese cluster (Mn_4CaO_5) and two redox active tyrosines. Photons are captured by the light-harvesting pigments and driven as excitation energy to P680 forming an excited state called P680*. The

P680* state is highly reducing, and an electron is rapidly transferred to the terminal plastoquinone (PQ) electron acceptor bound to the Q_B site within the D1 protein. On receiving two electrons, the PQ is protonated to plastoquinol (PQH_2) and leaving the Q_B site in the PSII complex. The P680 radical cation ($P680^+$) formed in primary charge separation has a very high redox potential, estimated to be approx. +1.3 V. This high-oxidation state is required to drive the water-splitting reaction that occurs at a catalytic site, the manganese cluster (for a review see Rappaport et al., 2002; Diner and Rappaport, 2002).

The photochemical activity of PSII complex can be measured by using the STL technique. Photosynthetic luminescence is a weak light emission originating from recombination of positive and negative charge pairs generated by a prior illumination in PSII, some of these leading to the recreation at a low yield of an exciton in the chlorophyll antenna, with a probability to deactivate as fluorescence. Luminescence decay phases can be better resolved by TL emission technique, which consists in illuminating the sample at a temperature sufficiently low to make negligibly small the recombination rate of the charge pairs under investigation, then to reveal them successively as TL bands by a progressive warming (for a review see Vass and Inoue, 1992; Ducruet, 2003; Sane, 2004).

After a sequence of short flashes, a so-called B-band of TL, located between 15 and 40 °C depending on species and conditions, is observed in healthy dark-adapted photosynthetic material, such as leaves and algae. This emission is due to the recombination of $S_2/S_3Q_B^-$ pairs, Q_B being the secondary quinonic electron acceptor and S_2/S_3, the states of the oxygen-evolving complex storing positive charges (Rutherford et al., 1982; Demeter and Vass, 1984). The changes on the intensity and temperature maximum of this band reflect alterations in the functionality of PSII complex. Herbicide treatment (diuron, atrazine), which blocks the Q_A to Q_B electron transfer, induces the appearance of a Q-band peaking at about 5 °C, due to the $S_2Q_A^-$ recombination. A C-band at about 55 °C can also be detected in particular conditions.

This TL band is due to $D^+Q_A^-$ recombination (Demeter et al., 1993, Johnson et al., 1994), D^+ being the oxidized form of tyrosine D on the inactive branch of PSII.

STL measurements are useful for the study of the effects of stress conditions on PSII electron transport in intact leaves, in algal and cyanobacterial cells, as well as in thylakoids and isolated membrane complexes (Rutherford and Inoue, 1984; Rutherford et al., 1984a; Zurita et al., 2005c; Ducruet and Vass, 2009). The technique is simple, inexpensive and powerful in monitoring several aspects of the PSII electron transport. The fact that alterations in the peaks of the TL curves may be interpreted as changes in the redox potentials of donors and acceptors on PSII makes this technique a useful tool for understanding the process of adaptation, responses to abiotic and biotic stresses, and structural modifications in the PSII polypeptides.

HTL is a useful technique for the study of lipid peroxidation processes in photosynthethic organisms (Havaux, 2003). Lipid peroxidation is a complex process known to occur in both plants and animals. This process involves a chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical, a rearrangement of the double bonds in unsaturated lipids and the formation and propagation of lipid radicals. As a result of these reactions takes place an eventual destruction of membrane lipids with the production of a variety of breakdown products, including alcohols, ketones, alkanes, aldehydes and ethers (Dianzani and Barrera, 2008). In stress situations the reactive oxygen and nitrogen species are generated at higher than normal rates, and as a consequence, lipid peroxidation occurs. In photosynthetic organisms a large amount of oxygen is formed in thylakoids in the vicinity of powerful oxidation-reduction systems that readily reduce oxygen to dangerous superoxide and produce harmful singlet oxygen which could initiate lipid peroxidation processes (Dalton, 1995; Repetto et al., 2012).

The lipid peroxidation is commonly detected and quantified by measuring by-products of the reaction, such as ethane, conjugated diens, malondialdehyde, and other carbonyl compounds (Heath and Packer, 1968; Pryor and Castle, 1984; Chatterjee and Agarwal, 1988; Havaux and Niyogi, 1999). The most commonly used test is called TBARS Assay (thiobarbituric acid reactive substances assay) where the concentration of malondialdehyde is determined by reaction with thiobarbituric acid to yield a fluorescent product. However, there are other sources of malondialdehyde, so this test is not completely specific for lipid peroxidation. In recent years, the development of immunochemical detection of 4-hydroxynonenal histidine adducts has opened more advanced methodological possibilities for qualitative and quantitative detection of lipid peroxidation in various human and animal tissues (Weber et al., 2013). These chemical methods cannot be however routinely used for the assessment of lipid oxidation in phytoplankton because the analysis requires high amounts of biological material, and they are often time-consuming and, therefore, not adequate for high throughput screening. The second problem is the difficulty of separating the peroxidative reactions that take place in phytoplankton from the reactions occurring in no phytoplankton materials.

Recently, a new TL-based technique has been proposed as a useful method to detect lipid peroxidation in photosynthetic organisms that may solve some of the aforementioned problems (Vavilin and Ducruet, 1998; Havaux, 2003; Ducruet and Vass, 2009). When chlorophyll-containing material is gradually heated to temperatures above 70 °C, a rise of chlorophyll luminescence emission is often observed. This increase in the luminescence intensity at high temperature was named HTL (Matorin et al., 1989; Venediktov et al., 1989). A broad HTL band peaking around 135 °C (HTL2 band) is generated in the reaction of thermal decomposition of peroxidized lipids that, in turn, leads to the formation of carbonyls in triplet state followed by migration of excitation energy toward chlorophylls (Venediktov et

al., 1989; Vavilin et al., 1991; Vavilin and Ducruet, 1998). Its amplitude has been correlated with the accumulation of malodialdehyde, an indicator of lipid peroxidation in standard chemical tests (Vavilin and Ducruet, 1998). Consequently, HTL2 band measurements have been used to study *in vivo* lipid peroxidation in plants subjected to various oxidative stress conditions (Merzlyak et al., 1992; Havaux and Niyogi, 1999; Ducruet, 2003; Baroli et al., 2004; Havaux, 2003). In a future perspective HTL might be applied as a fast screening tool before more sophisticated methods like mass spectrometry are applied to identify the most sensitive molecular target in a given tissue.

STL and HTL techniques have been applied in several *in vitro* toxicological studies, mainly using thylakoids and cell cultures (Ducruet et al., 2007). Using thylakoids isolated from leaves of greenhouse grown peas (*Pisum sativum L.*), Mohanty et al. (1989) suggested that Cu (II) does not block electron flow between the primary and secondary quinonic electron acceptor but modifies the secondary quinonic acceptor site in such a way that it becomes unsuitable for further PSII photochemistry. Horváth et al. (1998) reported that the amplitude of the B-band was gradually decreased, which corresponds to the Cu (II) induced inactivation of Tyrosine Z. The simultaneous stepwise shift in the peak position of the B-band indicated, however, that $S_3Q_B^-$ charge recombination is more resistant to Cu (II). Nitrite, but not formate, fluoride or nitrate anions, inhibited PSII in spinach thylakoids, probably at the donor side (Sahay et al., 2006).

Toxicological applications of HTL have been reported by several authors. A rise of thermoluminescence at 120-130 °C was detected when cultures of the unicellular green alga *Chlorella (C.) pyrenoidosa* were exposed for 5 h to 10 µM of the oxidative stress promoter methyl viologen (Vavilin and Ducruet, 1998). Hörcsik et al. (2007) found photodestruction of D1 protein in *C. pyrenoidosa* caused by Cr (VI) due to enhanced oxidative stress and lipid peroxidation, as indicated by the appearance of a HTL2 band. Schumann et al. (2009) have

developed a rapid and easy to handle TL based technique for evaluation of carbon tetrachloride-induced oxidative stress on rat hepatocytes.

The aim of this work has been the application of two simple, non-invasive and inexpensive TL techniques (STL and HTL) to study the alterations produced on the fresh-water green alga *C. vulgari*s by six chemicals. This green alga was selected as a representative of primary producers. It is a genus of single-cell green algae, widely distributed in freshwater and seawater. It has a short cycle of growth, which makes it ideal for aquatic ecotoxicity studies (Wong et al., 1997). The chemical intermediates bromobenzene and diethanolamine, the antioxidant propyl gallate, the semiconductor indium nitrate, the pesticide sodium monofluoroacetate and the antimalarian drug chloroquine were selected as representative of several chemical groups according to their use. The results have been compared with those obtained by our group by using the algae growth inhibition test (Zurita et al., 2005a,b; Zurita et al., 2007a,b,c,d).

2 Material and Methods

2.1 Cultures

The unicellular green algae *Chlorella vulgaris* var. *viridis,* kindly provided by Dr. Muñoz-Reoyo (CISA, Spain), were grown as previously described (Ramos et al., 1996) in Bold's Basal Medium (BB medium) (Culture Collection of Algae and Protozoa, Cambridge, UK; http://www.ccap.ac.uk/media/recipes.htm) under a water-saturated sterile atmosphere containing 5% CO_2 at 22 °C, constant agitation and a light dark cycle of 16:8 h (white light of 40 $\mu E\ m^{-2}\ s^{-1}$).

2.2 Chemicals

Stock solutions of bromobenzene (CAS number 108-86-1), indium nitrate (CAS number 13770-61-1), sodium monofluoroacetate (CAS number 62-74-8), diethanolamine (CAS number 111-42-2) and chloroquine (CAS number 54-05-7) were prepared daily in deionised water. Propyl gallate (CAS number 121-79-9) was solubilised in dimethyl sulfoxide. All the chemicals were from Sigma-Aldrich (USA), unless sodium monofluoroacetate (Honeywell´s Riedel-de Haën). They were sonicated for 30 min and sterilized by filtration through a 0.22 μm Millipore filter. The concentrations of the chemicals used in this work were chosen taking into account the EC_{50} values obtained for growth inhibition (biomarker) of *C. vulgaris* cells after 24 h exposure to the different chemicals, as previously published (Zurita et al., 2005a,b; Zurita et al., 2007a,b,c,d). The EC_{50} value is the concentration of the chemical that modified the biomarker (growth inhibition, for *C. vulgaris*) by 50% in comparison with appropriate untreated control. Two different concentrations of chemicals were used, one below EC_{50} values and the second one above or close to the EC_{50} values. The two concentration used and the EC_{50} values for the six chemicals are shown in Table 1. STL and HTL experiments were carried out using cultures of *C. vulgaris* in exponential phase of growth (with about 10^6 cell ml^{-1}). The cultures were exposed in BB medium to the two different concentrations of chemicals selected in 80 ml flasks for 24 h at 20 °C using constant agitation at 40 r.p.m., under a continuous cold light source of 110 μE m^{-2} s^{-1}. The suspensions were then centrifuged at 2000 r.p.m for 10 min, quantified at 665 nm and adjusted to 0.1 mg chlorophyll ml^{-1} by using BB medium. Control experiments in the absent of the chemicals were also treated as above described.

2.3 Thermoluminescence measurements

TL glow curves of *Chlorella* cell suspensions were obtained using two similar home-built devices designed by Dr. Ducruet (France) for luminescence detection from 0 ºC to 80 ºC (STL) and from 10 ºC to 160 ºC (HTL). A detailed description of these systems can be obtained elsewhere (Ducruet, 2003; Zurita et al., 2005c; Guerrero et al., 2014). Briefly, temperature regulation, signal recording and flash sequences were driven by a computer through a National Instrument DAQ-Pad1200 interface, using dedicated software. Temperature regulation of the sample cuvette was provided by a double-stage Marlow thermoelectric Peltier plate (model DT 1089-14; Marlow Industries, USA) cooled by a temperature-controlled bath. Luminescence emission was detected by a H5701-50 Hamamatsu photomultiplier module. Single turn-over flashes were provided by a xenon white light (Walz XST-103). Typically, for STL measurements 50 μl *Chlorella* cell suspensions were dark-incubated for 2 min at 20 ^{o}C, then cooled to 1 ^{o}C for 1 min and illuminated at the end of this period with different numbers of saturating single turn-over flashes (separated by 1 s). Luminescence emission was recorded while warming samples from 0 ^{o}C to 80 ^{o}C at a heating rate of 0.5 ^{o}C per s. For HTL measurements 50 μl *Chlorella* cell suspensions were adsorbed by filtration on a piece of filter paper (0.45 μm, Whatman) that was pressed against the copper film, dark-incubated for 10 min at 20 ^{o}C, and cooled to 10 ^{o}C for 1 min. Luminescence emission was then recorded while warming samples from 10 ºC to 160 ºC at a heating rate of 0.1 ºC per s. N_2 gas was flushed on the sample during HTL experiments in order to desiccate samples and prevents any oxidation induced by high temperatures. The instruments were driven by a PC computer, with a specially developed acquisition program (Ducruet 2003 and references therein). Data acquisition, signal analysis and graphical simulation were performed as previously described (Zurita et al., 2005c; Ducruet and Miranda, 1992; Ducruet et al., 2011).

3 Results

3.1 Characterization of light-induced thermoluminescence bands in non-treated Chlorella vulgaris cells

Before the TL analysis of the effects of chemicals on *Chlorella* cells, the TL bands "*in vivo"* using healthy cells from cultures of this green alga were characterized by using STL technique. Excitation of dark-adapted non-treated *Chlorella* cell suspensions with a series of saturating single turn-over flashes at 1 ºC induced the appearance of TL glow curves with important differences in the total signal intensity. TL curves induced by 1, 2 and 3 flashes are shown in Figure 1 as examples. The light emission curve obtained after illumination with two flashes was the best of the series and showed a t_{max} at about 15 ºC and a small band around 58-60 ºC (Figure 1, 2F). The signal peaking at about 15 ºC could be assigned to the TL B-band, due to the recombination of positive charges stored on S_2 and S_3 states of the oxygen evolving complex with an electron stabilized as Q_B^- on the secondary quinonic acceptor of PSII (Rutherford et al., 1982). The intensity of B-band exhibited a typical four-oscillation period with maximum after the 2nd and 6th flashes (data not shown). This pattern suggests that in dark-adapted *Chlorella* cells the ratio S_0:S_1 and Q_B:Q_B^- are about 25:75% and 50:50%, respectively (Inoue, 1996). Thus, after one single flash, the S_1Q_B centers will go to the luminescence-emitting state $S_2Q_B^-$, generating a B-band peaking at about 25 °C (Figure 1, 1F), generally identified as the B_2-band component. Two flashes induced the best glow curve because generate an important amount of PSII centers in both $S_2Q_B^-$ and $S_3Q_B^-$ luminescence states (Rutherford and Inoue, 1984). Besides, the yield from the latter recombination is higher than that from the former by a factor of 1.7-2.0 (Rutherford et al., 1984b). The much higher contribution of the second component of B-band (B_1-band) after two flashes, induces the appearance of a TL glow curve significantly shifted to lower temperatures and broadened.

A second small component of the signal peaking around 58-60 ºC appears in all the TL glow curves obtained (Figure 1). We assigned this component to the C-band, originated from the recombination reaction of the positive charges stored on tyrosine D residue and electron stabilized as Q_A^- on the primary quinonic acceptor of PSII (Rutherford et al., 1982). The appearance of this band suggests that in healthy *Chlorella* cells there was a small PSII population with the electron transfer blocked both on donor and acceptor side.

3.2 Effects of chemicals on light-induced thermoluminescence bands in Chlorella vulgaris cells

STL technique was used to determine the effects of the six environmental chemicals on PSII electron transfer activity of *Chlorella* cells. In order to compare the effects at doses non-cytotoxic and cytotoxic of the chemicals compound studied in this work, TL experiments were carried out using one concentration below and other close to the EC_{50} values, respectively (Table 1). Algae cultures in exponential phase of growth were exposed for 24 h to two concentrations of each chemical. TL emissions were then recorded between 0 ºC and 80 ºC after the illumination of cells suspensions at 1 ºC with two flashes (Figure 2).

The exposure of *Chlorella* cells for 24 h to bromobenzene did not significantly change the properties of the light-induced electron transfer on PSII (Figure 2, BrB). The incubation with 1 mM bromobenzene did not alter the TL signal assigned to B-band regarding control sample, while 2 mM bromobenzene decreased the signal intensity only by about 13%. Propyl gallate and indium nitrate caused however a moderate decrease on TL B-band of *Chlorella* cells (Figure 2, PpG and $InNO_3$, respectively). The intensity of the TL band was decreased by about 10% and 40% after incubation with the lower and higher concentrations of both chemicals, respectively.

The total intensity of TL emission of the B-band was not significantly modified by 5 mM of sodium monofluoroacetate (Figure 2, SMFA), but the exposure of the cells to 20 mM concentration induced a decrease of about 30% of the signal. Both concentrations of sodium monofluoroacetate induced the appearance of a more broadened TL glow curve with a significant up-shift of the temperature of the t_{max}. TL emissions were also particularly sensitive to the exposure to chloroquine (Figure 2, CQ), showing 50% and 80% reduction of the intensity of B-band with 0.1 and 0.25 mM concentration, respectively.

The incubation of *Chlorella* cells with diethanolamine markedly reduced the electron transfer activity of PSII (Figure 2, DEA). No signal was detected after the treatment with 30 mM of diethanolamine. However, 15 mM of this chemical lowered about 15% the intensity of the B-band and induced the appearance of a more broadened TL glow curve with a shoulder peaking at about 46 ºC (Figure 2, DEA). This component appearing at higher temperatures was tentatively assigned to the afterglow (AG) emission band usually induced by far red illumination (Miranda and Ducruet, 1995).

To characterize this band, TL glow curves of *Chlorella* cells suspension were obtained after illumination with only one flash (Figure 3). Under these conditions, the AG-band may be better studied as the B-band has a lower intensity. Thus, in dark-adapted *Chlorella* cell suspensions incubated with 15 mM diethanolamine, the excitation with one saturating single turn-over flash at 1 ºC induced the appearance of a more broadened TL glow curve compared with the control in the absence of diethanolamine (Figure 3, DEA 1). A very clear shoulder peaking at about 46 ºC was observed in these treated cells which does not appear in the control cells where only one band appears at 25 °C corresponding to B_2-band. This TL curve could be well simulated by two components, one with t_{max} of 25 ºC originating from the B_2-band and a clear second component with t_{max} of 45 ºC that was assigned to AG-band. The mathematical analysis of the two components allowed estimate a contribution of 48% and

52% to the total signal intensity for the B and AG bands, respectively. No TL emission signal due to B-band was obtained in cells incubated with the higher concentration of diethenolamine (30 mM). However, a small band of unknown origin peaking at about 70 ºC could be observed. This band also appeared in TL curves induced by two flashes in cells incubated by 30 mM diethanolamine (Figure 2, DEA).

3.3 Effects of chemicals on the high temperature thermoluminescence emissions in Chlorella vulgaris cells

The effects of the six chemicals studied on the lipids of the photosynthetic membrane of *Chlorella* have been studied by using HTL technique. *Chlorella* cells in exponential phase of growth were exposed for 24 h to two different concentrations of each of the six chemicals. HTL emissions were then recorded in a large temperature range (10 ºC-160 °C) at a heating rate of 0.1 °C/s as described in Materials and Methods section. Figure 4 shows that a clear HTL2 band with a t_{max} at about 130-150 ºC was present in most control and chemical treated samples. Analysis of HTL2 bands obtained, clearly suggested the existence of a high level of lipid peroxidation in photosynthetic membranes of *Chlorella* after the incubation with four of the chemicals investigated: bromobencene, sodium monofluoroacetate, diethanolamine and chloroquine. *Chlorella* cells treated with 30 mM diethanolamine showed the most important increase in the intensity of the HTL2 band. SMFA at the higher concentration and CQ at the two concentration tested induced also important increase of the HTL2 band. The level of lipid peroxidation increased slightly with 2 mM bromobenzene, in comparison with control sample, and was not modified by either indium nitrate or propyl gallate. Interestingly, this last chemical seemed to slightly reduce the basal level of lipid peroxidation detected in non-treated cells.

4 Discussion

Thermoluminescence is a technique very simple, inexpensive and powerful to study some of the processes occurring in photosynthetic organisms. The effects produced by six chemical compounds on the photosynthetic activity of PSII and lipid peroxidation in *Chlorella vulgaris* cells were studied by using STL and HTL techniques, respectively. The results have been compared with those obtained previously by our group (Zurita et al., 2005a,b; Zurita et al., 2007a,b,c,d) in algae growth inhibition test studies. The additional data obtained allow a more complete assessment of the toxicological effects of the chemicals evaluated.

Bromobenzene is a colourless liquid used as solvent for crystallization, intermediate in organic synthesis and additive to motor oils. It can be present in atmospheric, terrestrial and aquatic environments, being biologically non-degradable. However, there is limited available information about the toxic hazard of bromobenzene in the environment. It is known that causes centrilobular cytoplasmic alteration and hepatocyte hypertrophy in mammals (Dodd et al., 2012). Bromobenzene is not a particularly potent phytotoxic compound showing low inhibition activity to *Chlorella* cells growth (EC_{50}= 2.35 mM), which is less sensitive than other environmental receptors, including *Vibrio fischeri* bacteria, the cladocera *Daphnia magna* or fish cell lines (Zurita et al., 2007a). A very slight decrease in PSII functionality was detected after the treatment of cells with a chemical concentration close to the EC_{50} (Table 1 and Figure 2, BrB).

The 1.4 fold increase of lipid peroxidation by 2 mM bromobenzene observed in *Chlorella* cells, according to the intensity of HTL2 band (Figure 4, BrB), is in agreement with the reduction in glutathione levels in several fish cell lines exposed to concentrations higher than 1 mM bromobenzene and with the increase of reactive oxygen species from 3.2 mM reported by Schoonen et al. (2005). In addition, Zurita et al. (2007a) observed a maximum

stimulation on the antioxidant enzyme glucose-6-phosfate dehydrogenase in the PLHC-1 fish cell line after a treatment with 1.8 mM bromobenzene, confirming the situation of oxidative stress. This significant increase in lipid peroxidation induced by bromobenzene in *Chlorella* cells can be also related to the accelerated membrane phospholipid degradation described by Lamb et al. (1984). The decrease in the B-band observed after the treatment with the highest concentration of bromobenzene (Figure 2, BrB) can also be explained taking into account the effects on the PSII activity of the phospholipid degradation processes. Thus, it seems that the main cytotoxic effects of BrB seem not to be related with the inhibition of PSII activity. Other effects on the *Chlorella* metabolism induced by BrB (as lipid peroxidation) must be responsible for the growth inhibition measured in previous work (Zurita et al., 2007a).

Propyl gallate is an antioxidant widely used to preserve and stabilize the freshness, nutritional value, flavour and colour of foods, fats and oils, cosmetics and medicinal preparations (Van der Heijden et al., 1986). Propyl gallate is not very toxic to *Chlorella*, showing much more toxicity in other systems (Zurita et al., 2007c). A consistent decrease in PSII functionality (B-band) was detected in parallel with cytotoxicity (Table 1 and Figure 2, PpG). Propyl gallate uncouple oxidative phosphorylation by increasing the permeabillty of mitochondrial inner membranes to protons (Fusi et al., 1991). A similar effect on chloroplast may explain the inhibition of TL B-band of PSII.

Although there are many studies that demonstrate the antioxidant capacity of propyl gallate (Karthikeyan et al., 2005), other authors have reported oxidant properties (Kobayashi et al., 2004; Kawanishi et al., 2005). The mitochondria alternative oxidase, that catalyzes a cyanide-resistant reduction of oxygen to water without translocation of protons, is specifically inhibited by propyl gallate, stimulating H_2O_2 production in plant mitochondria (Josse et al., 2003). High concentrations of propyl gallate have been reported to produce loss of mitochondrial membrane potential and to be oxidant in non-vegetal systems due to depletion

in glutathione, stimulating glucose-6-phosphate dehydrogenase activity (Zurita et al., 2007c; Han and Park, 2010).

Therefore, in order to clarify this discrepancy, the protective and toxic effects of propyl gallate, we have investigated the possible lipid peroxidation induced by this chemical by using HTL technique (Figure 4, PpG). Our results clearly support the antioxidant properties of propyl gallate. The observed slight reduction on lipid peroxidation (Figure 4, PpG), in comparison to the control, is in agreement with its proposed antioxidant capacity (Kawanishi et al., 2005). In fact it has been recently demonstrated in PSII membranes isolated from spinach leaves, that the scavenging capacity of radicals by propyl gallate significantly suppressed $^1O_2$ formed by oxidation of organic molecules (lipids and proteins) (Yadav and Pospíšil, 2012).

Indium nitrate is used in the industry of batteries, to prevent build-up of hydrogen gas within sealed battery casings, plating and as semiconductor in other chemical applications. It is also used for the treatment of Hodgkin and non-Hodgkin's lymphoma and for the management of hypercalcemia (Lipcamon and Sahl, 2002). However, there are no reports on its phytotoxicity. The proliferation of *Chlorella* cells is very sensitive to indium nitrate exposure, showing a parallel inhibition of PSII functionality as shown by the significant decrease of B-band (Figure 2, $InNO_3$). It has been reported that treatment of rats with indium trichloride produced changes in heme biosynthesis (Woods et al., 1979). A similar effect on *Chlorella* could explain the decrease on PSII activity.

The two concentrations of indium nitrate tested in this work did not affect the lipid peroxidation level in *Chlorella* cells in comparison with control conditions (Figure 4). These results are not in agreement with the reported induction by indium of lipid peroxidation in mice liver (Verstraeten et al., 1997; Repetto and del Peso, 2012). Indium promotes membrane rigidification and lateral phase separation; both parameters are well correlated with the ability

of the ions to stimulate Fe^{2+}-initiated lipid peroxidation. According to Zurita el al. (2007d), toxicity to fish cells at higher concentrations (5-7 mM) than those investigated with HTL in alga seems to be mediated by oxidative stress, and particularly by NADPH-dependent lipid peroxidation. Thus, taking into account our results, in *Chlorella* cells the toxic effect of indium nitrate seems not to be related with the lipid peroxidation process.

Sodium monofluoroacetate is one of the most potent pesticides. It is mainly used as a rodenticide and as a vertebrate pest control agent. At least since the 1940s, it is known as one of the most toxic substances around the world, retaining the restricted use classification imposed by the USEPA in 1978. Leaf chlorosis, necrosis and suppression of seeding growth were reported in the plant *Helianthus annus* exposed to 6.5 mM sodium monofluoroacetate (Cooke, 1976). From the soil organisms evaluated by O'Halloran et al. (2005), including the earthworm *Eisenia fetida* and microorganisms, lettuce (*Lactuca sativa*) was more sensitive than oats (*Avena sativa*). Lettuce seedling emergence and seedling shoot growth were adversely affected at a soil concentration of 7 mg per Kg of sodium monofluoroacetate. Zurita et al. (2007b) reported the lowest non observed adverse effect level in *C. vulgaris* for sodium monofluoroacetate (0.1 mM) in comparison to *D. magna, V. fischeri* and fish cell lines.

Sodium monofluoroacetate triggers its main toxic action by blocking the Krebs cycle. The compound is incorporated into fluoroacetyl coenzyme A, which is condensed with oxalacetate to form fluorocitrate, a process dubbed as ''lethal synthesis'' (Buffa and Peters, 1950). The intensity of HTL2 band was increased in *Chlorella* more than four-fold by sodium monofluoroacetate, reflecting a status of oxidative stress probably generated by the metabolic blockage. In fact, Dojcinovic et al. (2005) demonstrated that inhibition of the mitochondrial electron transport chain of the tricarboxylic acid cycle by sodium monofluoroacetate causes increased expression of nucleus-encoded alternative oxidase genes in plants. In addition, Zurita et al. (2007b) reported that glucose-6-phosphate dehydrogenase activity was the only

biomarker stimulated in RTG-2 fish cells exposed to sodium monofluoroacetate. Taking into account the protection of fluoroacetate toxicity observed in PLHC-1 cells pre-treated with sulphydryl reagents, another mechanism of action of sodium monofluoroacetate seems to be the binding to sulphydryl groups, including those of glutathione. The 30% reduction on the intensity of B-band of TL with the highest concentration of the chemical may be also related to the oxidative stress processes induced by sodium monofluoroacetate. However, this chemical induced another major change in the characteristics of the TL emission signal. Whereas a single TL band is generally observed in untreated *Chlorella* cells, sodium monofluoroacetate produced a more broadened TL curve with a significant upshift of the t_{max}. These results suggest that sodium monofluoroacetate could also induce major changes in the ratio among the two components responsible for the emission of B-band: S_2/S_3 states of the manganese cluster and/or the reduced state of Q_B (Vass and Inoue, 1992).

Diethanolamine (DEA) is a viscous liquid widely used as chemical intermediate, anticorrosion agent in metalworking fluids and surface-active agent in cosmetic formulations, pharmaceuticals and agricultural products. It presents low cytotoxicity to *Chlorella* cell proliferation (Zurita et al., 2005b), while the algae *Skeletonema costatum* and *Phaeodactylum tricornutum* have been reported to be more sensitive in a recent ecotoxicological evaluation (Libralato et al., 2010). However, the TL emission bands in *Chlorella* were very sensitive and dose dependent. At concentrations around the EC_{50} for cell proliferation, the intensity of B-band showed a reduction of 15%, and disappeared when the concentration was doubled (30 mM). In adition, at this last concentration, another major change was a potent stimulation in lipid peroxidation, with an approximately 100-fold increase.

Diethanolamine probably inhibits the mitochondrial fatty acid beta-oxidation. Zurita et al. (2005b) have reported a marked steatosis in PLHC-1 cells exposed to diethanolamine. Toxicity studies on vertebrates have shown that diethanolamine inhibits cellular choline

uptake, causing a wide range of indirect effects, including increased susceptibility to oxidative damage, negative effects on phospholipid metabolism, altered mitochondrial function and structure, DNA methylation, altered transcription of genes involved in cell growth regulation and tumour development (Hansen et al., 2010). In the marine copepod *Calanus finmarchicus,* diethanolamine caused the up-regulation of the transcription of genes involved in protein catabolism, metal ion homeostasis, defence against oxidative stress, accompanied by altered expression of fatty acid derivates, amino acids and cholines (Hansen et al., 2010). All these metabolic alterations, including the observed important lost in the PSII activity, can be related to the interference of phospholipids accumulation in the metabolism. It has been reported that phospholipid accumulation produces a loss of mitochondrial integrity (Barbee and Hartung, 1979), a process that can also occur in chloroplasts. The HTL experiments of this work showed enormous levels of lipid peroxidation in *Chlorella* cells treated with diethanolamine (Figure 4, DEA). This suggests that this chemical besides inducing an accumulation of lipids in the chloroplasts, also promotes its degradation by peroxidation processes.

Interestingly, the lower concentration (15 mM) of diethanolamine induced the appearance of a more broadened TL glow curve with a shoulder peaking at about 46 ºC (Figure 2, DEA and Figure 3). We tentatively assigned this component appearing at higher temperatures to the AG emission band usually induced by far red illumination (Miranda and Ducruet, 1995). A similar AG-band has been observed in *Chlamydomonas* cells excited by white light (Ducruet et al., 2011). The presence of an AG-band in TL curves recorded in cells excited by white light flashes reflects the amount of PSII centres in the $S_{2/3}Q_B$ non-radiative state immediately after preillumination, in which the arrival of an electron transferred from stroma along cyclic/chlororespiratory pathway(s) produces the radiative state $S_{2/3}Q_B^-$ that emits luminescence (Ducruet el al., 2005). This back electron transfer can be induced by warming that activates the cyclic pathway(s) leading to an AG emission as $S_{2/3}Q_B$ centers are

converted to $S_{2/3}Q_B^-$ by electrons arriving from an unknown stroma reductant (Ducruet el al., 2005). In *Chlorella* cells grown in the presence of diethanolamine, phospholipids can provide additional NADPH in the stroma through the respiratory pathway (Alric, 2010), thus stimulating the cyclic versus linear electron transfer pathway to maintain the ATP/NADPH ratio required by the Benson-Calvin cycle. This stimulation of the cyclic/chlororespiratory pathway can explain the appearance of the AG-band in TL emissions induced by white light. In *Chlorella* cells incubated in the presence of 30 mM diethanolamine there is not photosynthetic electron transfer on PSII (see Figure 2, DEA). However, the growth inhibition test has shown that at this concentration the percentage of *Chlorella* living cells was still about 40% (Zurira et al., 2005b). So, diethanolamine should activate alternative photosynthetic metabolism, without the participation of PSII (chlororespiration, cyclic electron transport around PSI, etc.) or not photosynthetic metabolism processes that maintain the cells viability.

According to Foster (1994), the lysosomotropic drug chloroquine is probably the second or third most widely consumed drug in the world. It is still the main pharmaceutical used for the prophylaxis and treatment of malaria, and is also used for treatment of autoimmune diseases, collagen vascular and dermatological disorders and extraintestinal amebiasis. In addition, CQ has been proposed as a promising inexpensive drug in the management of HIV disease (Romanelli et al., 2004). It is the most potent compound evaluated according to the inhibition of the proliferation of *Chlorella* cells, with an eight-fold increase in toxicity when the exposure time period was increased from 24 to 72 h (Zurita et al., 2005a). Jjemba (2002) demonstrated that the growth of soybean plants was decreased by increasing concentrations of chloroquine, while the number of bacteria and protozoa in soil was either unchanged or increased. Although the inhibition of bioluminescence in *V. fischeri*

is less sensitive, the crustacean *D. magna* is even more sensitive than *Chlorella* (Zurita et al., 2005a).

Thermoluminescence measurements in *Chlorella* cells were particularly sensitive to chloroquine, showing important reductions on B-band intensity (Figure 2, CQ), while HTL2 intensity was increased 5.5-fold by 0.25 mM chloroquine, in comparison to control (Figure 4, CQ). These results agree with the previously reported oxidative stress in rats induced by chloroquine, with the consequence of lipid peroxidation as mechanism of the nephrotoxicity (Murugavel and Pari, 2004). According to Sugioka et al. (1987), the ferriprotoporphyrin IX-chloroquine complex remarkably promotes peroxidative cleavage of unsaturated phospholipids. The significant decrease of the activity of PSII can be related to lipid peroxidation on chloroplast and also with the possible binding of chloroquine to porphyrin groups of the PSII cofactors.

In conclusion, for five of the chemicals studied (not BrB) there is a very good correlation between the cytotoxic effects in *Chlorella* cells measured by the algae growth inhibition test (Zurita et al., 2005a,b; Zurita et al., 2007a,b,c,d), and the inhibition of PSII activity measured by STL. Thus, we may propose that one very important effect of these chemicals in *Chlorella* cells is the inhibition of photosynthetic metabolism by the blocking of PSII functionality. In the case of sodium monofluoroacetate, diethanolamine and chloroquine this inhibition seems to be related with the induction of high level of lipid peroxidation in cells that may alter the stability of PSII. Other toxic mechanisms different from lipid peroxidation must be however responsible for the inhibition of PSII induced by PpG and $InNO_3$. The inhibition of growth in *Chlorella* cells induce by BrB seems not to be related by the blocking of photosynthetic electron transfer activity on PSII.

Consequently, although no single biomarker is viable in gaining a comprehensive understanding of xenobiotic stress (Brain and Cedergreen, 2009), the quantification of TL

emissions is a useful technique to study specific effects of environmental chemicals on photosynthetic organisms. STL and HTL techniques provide specific information on photosynthetic electron transport and on lipid peroxidation induced by oxidative stress, respectively. This information is complementary to that obtained by the growth inhibition test and allows a more complete assessment of the effects of a chemical in organisms of aquatic ecosystems, and about the mechanisms of the toxicity.

Conflict of interest

None of the authors had any conflicts to declare.

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Figure legends

Figure 1. Thermoluminescence glow curves from non-treated *Chlorella vulgaris* cells recorded after a series of single turn-over flashes (indicated on each curve). 50 µl of cell suspensions from *Chlorella* culture at exponential growth phase were dark-adapted for 2 min at 20 ºC and subsequently cooled to 1 ºC during 1 min. Then, 1, 2 or 3 saturating single turn-over flashes (separated by 1 s) were given at the end of this period. Luminescence emission was then recorded while warming samples from -5 ºC to 80 ºC at a heating rate of 0.5 ºC per s. For other technical details see Material and Methods section.

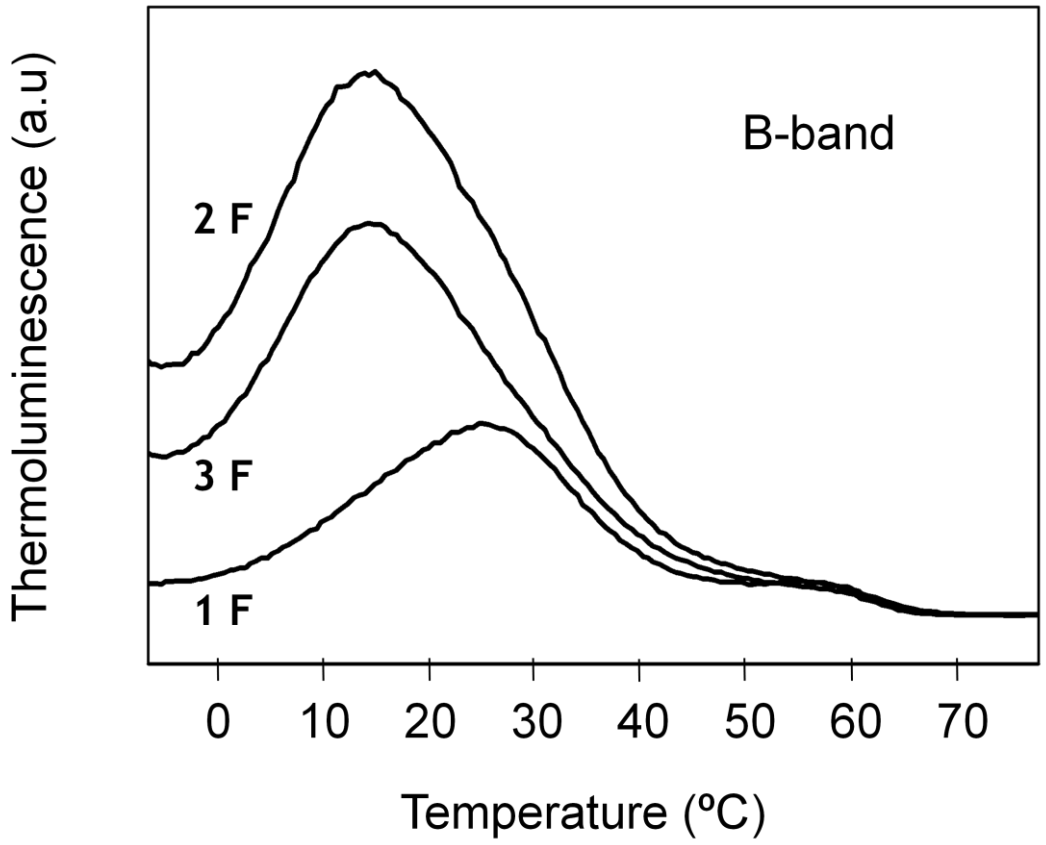


Figure 2. Thermoluminescence glow curves from *Chlorella vulgaris* cells exposed for 24 h to six different chemicals. Bromobenzene (BrB), propyl gallate (PpG), indium nitrate ($InNO_3$), sodium monofluoroacetate (SMFA), diethanolamine (DEA) or chloroquine (CQ). *Chlorella* cells suspensions were exposed to two different concentrations of each chemical as described in Material and Methods section. After the treatment, 50 μl of cells suspension were dark-adapted for 2 min at 20 ºC and subsequently cooled to 1 ºC during 1 min. Two saturating single turn-over flashes (separated by 1 s) were given at the end of this period. Luminescence emission was then recorded while warming samples from 0 ^{o}C to 80 ^{o}C at a heating rate of 0.5 ^{o}C per s. For other technical details see Material and Methods section. Symbols: (●) Control non-treated samples; (○) *Chlorella* cells exposed to the lower concentration of the chemical; (Δ) *Chlorella* cells exposed to the higher concentration of the chemical. Lower and higher concentrations of the chemicals, respectively: BrB, 1 and 2 mM; PpG, 0.5 and 1 mM; $InNO_3$, 0.3 and 0.7 mM; SMFA, 5 and 20 mM; DEA, 15 and 30 mM; CQ, 0.1 and 0.25 mM.

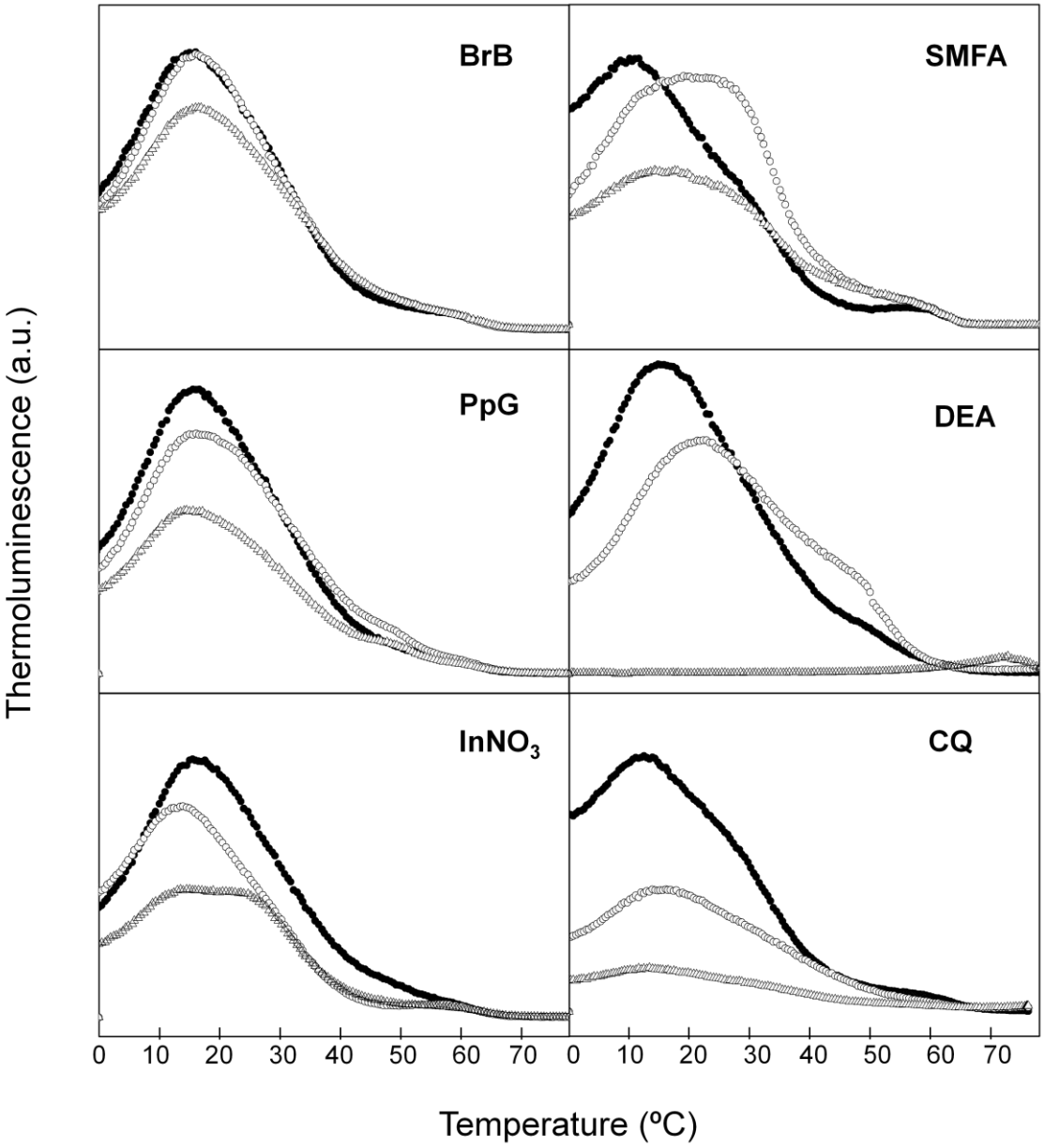

BrB
SMFA
PpG
DEA
InNO3
CQ
Thermoluminescence (a.u.)
0 10 20 30 40 50 60 70
0 10 20 30 40 50 60 70
Temperature (ºC)

Figure 3. Thermoluminescence glow curves from *Chlorella vulgaris* cells exposed for 24 h to diethanolamine (DEA). *Chlorella* cells suspensions were exposed to two different concentrations of diethanolamine (15 and 30 mM) as described in Material and Methods section. After the treatment, 50 μl of cells suspension were dark-adapted for 2 min at 20 ºC and subsequently cooled to 1 ºC during 1 min. One saturating single turn-over flash was given at the end of this period. Luminescence emission was then recorded while warming samples from 0 ^{o}C to 80 ^{o}C at a heating rate of 0.5 ^{o}C per s. For other technical details see Material and Methods section. Symbols: Control, non-treated samples; DEA 1, *Chlorella* cells exposed to 15 mM diethanolamine; DEA 2, *Chlorella* cells exposed to 30 mM diethanolamine. The B and the AG-bands resulting from the decomposition of the TL curve induced in *Chlorella* cells exposed to the lower concentration of the chemical are shown in dots.

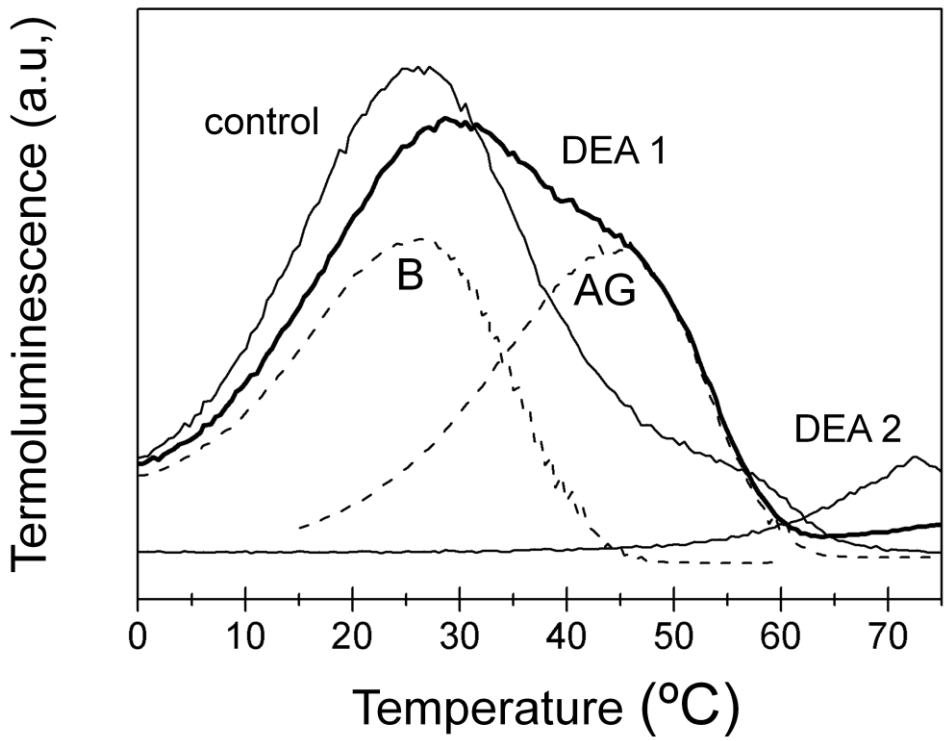
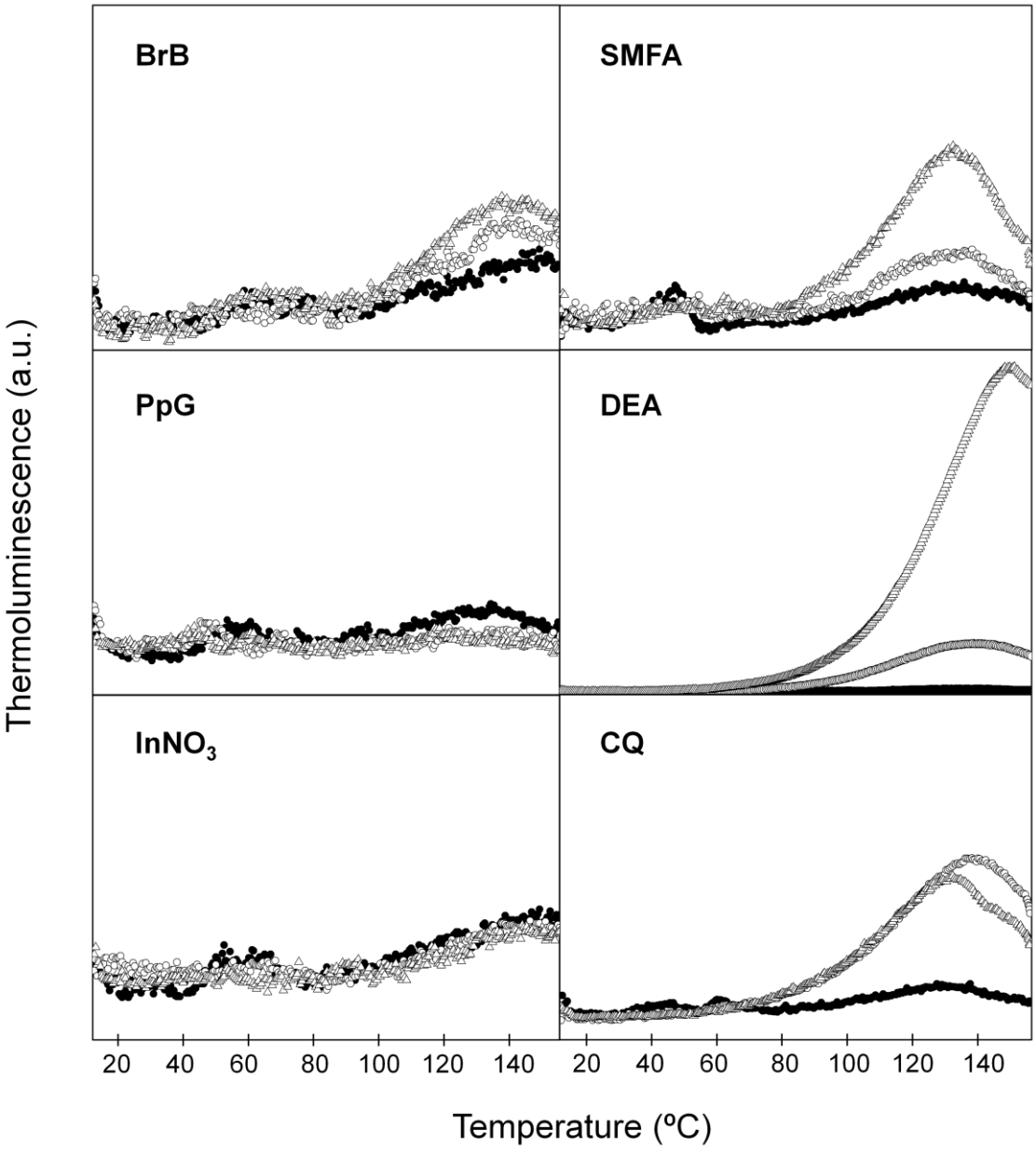

Termoluminescence (a.u,)
control
DEA 1
B
AG
DEA 2
0
10
20
30
40
50
60
70
Temperature (ºC)

Figure 4. High temperature thermoluminescence glow curves from *Chlorella vulgaris* cells exposed for 24 h to six different chemicals. Bromobenzene (BrB), propyl gallate (PpG), indium nitrate ($InNO_3$), sodium monofluoroacetate (SMFA), diethanolamine (DEA) or chloroquine (CQ). *Chlorella* cells were exposed to two different concentrations of the chemicals as described in Material and Methods section. After the treatment, 50 μl of cells suspension were adsorbed by filtration on a piece of filter paper, dark adapted for 10 min at 20 ºC and cooled to 1 ºC for 1 min. Luminescence emission was then recorded without flash excitation while warming samples from 10 ºC to 160 ºC at a heating rate of 0.1 ^{o}C per s. In order to desiccate the samples and prevents any oxidation induced by high temperature, N_2 was flushed on the samples during HTL experiments. For other technical details see Material and Methods section. Symbols: (●) Control non-treated samples; (○) *Chlorella* cells exposed to the lower concentration of the chemical; (Δ) *Chlorella* cells exposed to the higher concentration of the chemical. Lower and higher concentrations of the chemicals, respectively: BrB, 1 and 2 mM; PpG, 0.5 and 1 mM; $InNO_3$, 0.3 and 0.7 mM; SMFA, 5 and 20 mM; DEA, 15 and 30 mM; CQ, 0.1 and 0.25 mM.


BrB
SMFA
PpG
DEA
InNO3
CQ
Thermoluminescence (a.u.)
20
40
60
80
100
120
140
Temperature (ºC)