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Ascorbate and glutathione contents in duckweed, *Lemna minor*, as biomarkers of the stress generated by copper, folpet and diuron

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Glutathione and ascorbate are essential components of the general antioxidative strategy to overcome oxidative stress due to environmental constraints such as pollution. The variation of glutathione and ascorbate contents in duckweed (*Lemna minor*) was investigated after a 48 h exposure to copper, diuron and folpet under laboratory conditions in order to determine whether changes in their level could serve as suitable and early biomarkers of pollution. One could observe that diuron and folpet caused the glutathione level to increase, its redox status remaining unchanged, while copper led to a depletion of this antioxidant and to an increase in its oxidation rate. When duckweed was contaminated by folpet and the metal, an increase of the ascorbate pool size occurred from concentrations as low as 1 mg l^{-1} and 50 µg l^{-1} respectively. While the ascorbate pool became more oxidized because of exposure to copper concentrations ≤ 200 µg l^{-1}, folpet caused an increase in its reduction rate. Diuron was responsible for depletion of ascorbate, the redox status of which remained unchanged. Because it is an adaptation to stress and a defence process, the increase in the antioxidant pool size was proposed as a biomarker of exposure to an unsafe environment. Since depletion of antioxidant and an increase in its oxidation rate weakened cellular defences and indicated a precarious state, they could constitute early indicators of toxicity. So they were proposed as potential biomarkers of toxicity. It was concluded that the antioxidant content in duckweed might serve as a useful biomarker for monitoring water quality.

Keywords: ascorbate, biomarker, duckweed, glutathione, pesticide.

Abbreviations: AsA, ascorbic acid; DAsA, dehydroascorbic acid; DHAR, dehydroascorbate reductase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiotreithol; EDTA, ethylene diamine tetraacetic acid; FW, fresh weight; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; ROS, reactive oxygen species.

Introduction

Visible injuries and significant decrease of growth and vitality of plants usually become apparent after a long exposure or, when duration of contamination was shorter, with high levels of toxicants. It has already been proposed that the effects of pollutants on biochemical pathways might be detected earlier since the appearance of symptoms generally resulted from long term alteration to the metabolism (Koricheva *et al*. 1997). Among metabolic changes, the antioxidative defence mechanisms of plants were of particular interest. Indeed, it has often been

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reported that a common effect of many pollutants is to generate free radicals and reduced forms of oxygen that may damage cellular components such as lipids, proteins or DNA (McKersie and Leshem 1994, Foyer *et al*. 1997). Induction of these defence mechanisms has commonly been reported in plants exposed to pollution. Glutathione and ascorbate are essential parts of the general strategy required to overcome oxidative stress imposed by these environmental constraints.

Glutathione (γ–Glu–Cys–Gly) is an essential plant metabolite and an important regulator of numerous cellular metabolisms (Alsher 1989). It is the main non-protein reduced sulphur component of the cell and it plays a key role in the antioxidative defence mechanisms of plants through the sulphydryl group of its cystein either directly or indirectly (Foyer *et al*. 1997). It is able to quench singlet oxygen, superoxide and hydroxyl radicals, thus deactivating these harmful ROS (Gille and Sigler 1995). It can also break the propagation of the radical chain responsible for lipoperoxidation by removing acyl peroxides directly or indirectly when it is used as a substrate by glutathione peroxidase (Drotar *et al*. 1985). Glutathione is one of the main reducing agents in the cell and it recycles ascorbic acid from its oxidized to its reduced form. Furthermore it is involved in the detoxification of xenobiotics as a substrate for the enzyme glutathione S-transferase (Marrs 1996) and lastly it also participates in the protection against heavy metals as a precursor of the phytochelatins which are metal-binding peptides in plants (Rauser 1995).

Ascorbate is found in great quantities in the plants in which it exhibits many antioxidative properties (Foyer 1993). It can directly scavenge ROS (including hydroxyl and superoxide radicals and hydrogen peroxide) either non-enzymatically or enzymatically (McKersie and Leshem 1994). In the latter case, it is used as a substrate for the H_2O_2-splitting enzyme ascorbate peroxidase (Nakano and Asada 1981). It can also indirectly act as an antioxidant by regenerating the membrane-bound α–tocopherol which is involved in the scavenging of peroxyl radicals and singlet oxygen (Schraudner *et al*. 1997).

The objective of the present study was to determine whether changes of glutathione and ascorbate content of *Lemna minor* could serve as suitable and early indicators of pollution in the absence of visible symptoms. So we investigated the variation of these contents during exposure to three contaminants of Champagne's vineyard, namely copper, folpet and diuron. Copper and folpet are two fungicides widely used in grape to control mildew and other fungal diseases (Buchenauer 1990). They are generously sprayed in vineyards in Champagne and are among the most widely used agrochemicals in those fields. Diuron is a phenylurea herbicide, widely used in pre-emergence programmes to control germinating grass and dicotyledonous weeds in grapevines and other crops such as vegetables, fruits and cereals (Tomlin 1997).

In this work, these three compounds were used as 'model' toxicants which are likely to modulate antioxidant content and we investigated the glutathione and ascorbate response after a 48 h exposure of *L. minor* to each of these pollutants.

Materials and methods

Chemicals

Diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea > 99 % purity) and copper (as $CuSO_4.5\ H_2O$) were purchased from Riedel-de-Haën and Sigma respectively (both Saint Quentin Fallavier, France). Folpet (*N*-[(trichloromethylthio)phtalimide]) formulated as Folpan 500® and blank formulation without active

ingredient were obtained from Makhteshim Agan France. Dehydroascorbic acid and 2-vinylpyridine were purchased from Aldrich (Saint Quentin Fallavier, France). All other chemicals were from Sigma (Saint Quentin Fallavier, France).

Plant material, growth conditions and treatment procedures

Lemna minor L. (duckweed) was collected from an artificial pond of the University campus. The plants were disinfected by immersing the fronds in 0.01 M NaOCl for 20 s and rinsing with distilled water. The stock cultures were maintained in 2 l plastic (PVC) aquariums containing 500 ml of inorganic growth medium (Chollet 1993) that consisted of KNO_3: 202 mg l^{-1}; KH_2PO_4: 50.3 mg l^{-1}; K_2HPO_4: 27.8 mg l^{-1}; K_2SO_4: 17.4 mg l^{-1}; $MgSO_4.7H_2O$: 49.6 mg l^{-1}; $CaCl_2$: 11.1 mg l^{-1}; Na_2-EDTA: 10 mg l^{-1}; $FeSO_4.7H_2O$: 6 mg l^{-1}; H_3BO_3: 5.72 mg l^{-1}; $MnCl_2.4H_2O$: 2.82 mg l^{-1}; $ZnSO_4$: 0.6 mg l^{-1}; $(NH_4)Mo_7O_{24}.4H_2O$: 43 µg l^{-1}; $CuCl_2.2H_2O$: 8 µg l^{-1}; $CoCl_2.6H_2O$: 54 µg l^{-1}. Before the medium was autoclaved its pH was adjusted to 6.5. The aquariums were placed in a controlled environment room at 25 ± 1°C under continuous illumination provided by cool white fluorescent lamps (Sylvania® Grolux F 36 W) with a light intensity of 2500 ± 100 lux (*c.* 40 µmol m^{-2} s^{-1}) and plants were subcultured twice a week.

For all experiments, approximately 30 double-fronded colonies of *L. minor* (200–250 mg) were taken from the stock cultures and exposed for 48 h to concentrations of copper ranging from 0 to 1000 µg l^{-1}, diuron ranging from 0 to 100 µg l^{-1} or folpet ranging from 0 to 10 mg l^{-1} according to the procedure described in Teisseire *et al.* (1998). Briefly, the toxic compound was dissolved in growth medium (50 ml) in crystallizing dishes (Ø 11 cm) before the plants were added. Controls consisted of toxicant-free medium. Experiments including copper were conducted using EDTA-free medium since EDTA is a chelating agent that reduces the bioavailability of metals. For experiments carried out with folpet, in which a formulated compound was used, blank formulation, without active ingredient, was added to the controls. Then, the crystallizing dishes were placed in a controlled environment room as above for 48 h.

Extraction of antioxidants

At the end of the 48 h exposure the fronds were patted dry between two layers of filter paper and weighed. They were homogenized in 1.5 ml of cold 5% (w/v) metaphosphoric acid with a Potter homogenizer (Servodyne mixer head, Cole Parmer, Niles, IL, USA). The homogenate was then centrifuged for 30 min at 19000 *g* and the clear supernatant was used for the determination of both glutathione and ascorbate.

Determination of glutathione

Glutathione content (GSH and GSSG) was determined enzymatically using the method of Griffith (1980), slightly modified. This method is based on the specificity of glutathione reductase. Total glutathione and GSSG contents were determined directly and GSH after subtraction of GSSG from total glutathione.

To determine GSSG, GSH of the sample was derivatized by adding 8 µl of 2-vinylpyridine to 200 µl of metaphosphoric extract that had been neutralized with 72 µl of 1 M triethanolamine. After vortexing for 30 s the mixture was allowed to incubate for 1 h at 25 °C.

An aliquot of the extract (40 µl of metaphosphoric extract and 150 µl of the 2-vinylpyridine derivatized extract for total glutathione and GSSG determination, respectively) was added to the reaction medium consisting of phosphate buffer (50 mM, pH 7.5), EDTA (2.5 mM), DTNB (1 mM), GR (0.5 unit, baker yeast type III, Sigma), NADPH (0.1 M) in a final volume of 1 ml at 25 °C. Reaction was started with the addition of the NADPH and the increase in absorbance at 412 nm was monitored for 3 min at 25 °C. Calibration curves were carried out using standards of GSH (1.6–80 µM) and GSSG (0.8–40 µM) prepared in 5% (w/v) metaphosphoric acid.

Determination of ascorbate

Both reduced (AsA) and oxidized (DAsA) ascorbate were determined as described by Knörzer *et al.* (1996), adapted from the bipyridyl method of Okamura (1980). In this method AsA is determined directly and DAsA after reduction and subtraction of AsA from total ascorbate.

For AsA determination, the metaphosphoric acid extract was neutralized with 25 µl of 1.5 M triethanolamine, vortexed and potassium phosphate buffer (150 µl, 0.15 M, pH 7.4) and H_2O (150 µl) were added.

Ten percent (w/v) trichloroacetic acid (300 µl), 44 % (v/v) phosphoric acid (300 µl), 4% (w/v) 2,2´-dipyridyl (300 µl in 70 % ethanol), and 3 % (w/v) $FeCl_3$ (150 µl) were added successively to the mixture. After vortexing, samples were incubated at 37 °C for 60 min and the absorbance at 525 nm was recorded.

For the determination of total ascorbate, triethanolamine and potassium phosphate buffer were added to the metaphosphoric extract as above and then 75 µl DTT 10 mM were added to the sample

instead of water. The mixture was incubated for 15 min at 25 °C to reduce DAsA of the sample into AsA. Then excess of DTT was removed by adding 75 µl of 0.5 % (w/v) *N*-ethylmaleimide. After mixing, the samples were incubated for 2 min at 25 °C and determination of AsA proceeded as above. Standards of AsA and DAsA (50–600 µM) prepared in 5 % (w/v) metaphosphoric acid were used for calibration.

Statistics

In all experiments three replicates were performed for each treatments and all experiments were repeated three times. Data presented here are the means ± standard deviations of the three independent experiments. Significance of differences between samples was determined by the Student's *t*-test and p-values ≤ 0.05 were considered significant.

Results

Glutathione

In controls, total glutathione content was 117 ± 4.13 nmol g FW^{-1} and it was found mainly in the reduced form (GSH), accounting for more than 90% of the total number of GSH equivalents. Total glutathione content slightly decreased when copper concentration increased but the reduction became significant only from 500 µg l^{-1}, as shown in figure 1. The glutathione content reached 70 ± 3.28 nmol g FW^{-1} (59.82 % of the control) with 1 mg l^{-1} of the metal (figure 1). GSH content varied according to a similar trend while the GSSG content steadily increased from the lowest concentration (non-significant, $p < 0.05$) and reached a steady state of approximately 7.6 nmol g FW^{-1} (134% of a control level of 5.66 ± 0.23 nmol g FW^{-1}) with 200 µg l^{-1} of copper (figure 1). The GSH/GSSG ratio was 18.68 ± 0.60 in controls. When *L. minor* was contaminated by copper this ratio significantly decreased ($p < 0.05$) from the lowest concentration (25 µg l^{-1}) and reached 13 ± 3.14 and 12.05 ± 1.44 with 100 and 500 µg l^{-1} of copper respectively (table 1). This ratio was only 45 % of the control one with the highest copper concentration.

When *L. minor* was exposed for 48 h to diuron a significant concentration-dependent increase of total glutathione content occurred from 25 µg l^{-1} (figure 1). This content reached 152.76 nmol g FW^{-1} with 100 µg l^{-1} of the herbicide (130 % of the control). A similar trend was observed with reduced and oxidized forms, but in the latter case the increase became significant only with 50 µg l^{-1} of the herbicide. The GSH/GSSG ratio did not vary significantly with diuron concentration, as shown in table 1.

In the absence of folpet, GSH level was 111 nmol g FW^{-1}. Reduced glutathione content slightly increased with folpet concentration. A 10% increase was observed with 1 mg l^{-1}, however this increase was not significant at $p < 0.05$ level (figure 1). Higher concentration significantly increased the GSH content up to 142 nmol g FW^{-1} (+ 28%) with 10 mg l^{-1}. Oxidized glutathione content also increased with folpet concentration (figure 1). The control level was 5.7 nmol g FW^{-1} and it increased with increasing folpet concentrations, reaching 7.5 nmol g FW^{-1} (+ 32 %) with 10 mg l^{-1}. The redox state of the glutathione pool was not significantly modified in response to folpet treatments, as shown in table 1.

Ascorbate

Total ascorbate content was higher than that of glutathione. Like glutathione, ascorbate was predominantly found in its reduced state (AsA) which represented

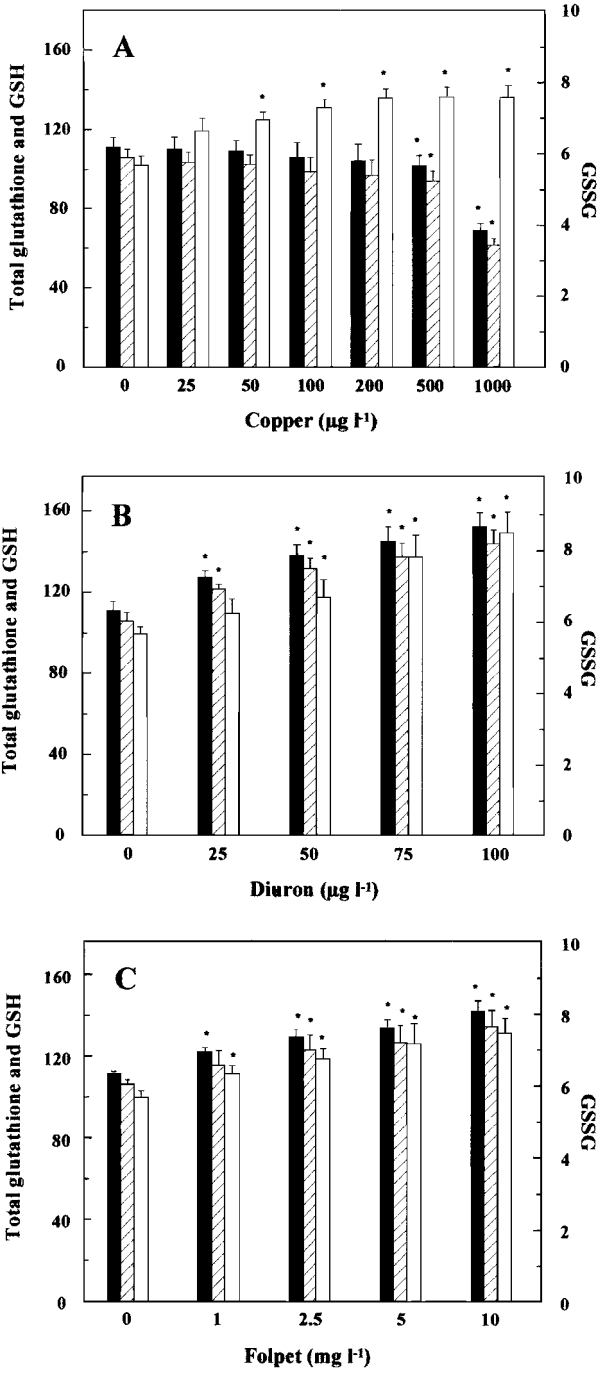

Figure 1. Influence of copper (A), diuron (B) and folpet (C) on total glutathione (■), GSH (▨) and GSSG (□) contents in *L. minor* after a 48-h treatment. Data (in nmol g FW^{-1}) are mean values ± SD of at least three independent experiments with triplicates. Asterisks indicate a significant difference from the control at $p \leq 0.05$ level.

Table 1. Effect of copper, diuron and folpet on GSH/GSSG and AsA/DAsA ratios in *L. minor* after a 48 h treatment. Data are mean values ± SD of at least three independent experiments with triplicates. Asterisks indicate a significant difference from the control at $p \leq 0.05$ level.

Compound	Concentration * (μg l^{-1} or mg l^{-1})	GSH/GSSG ± SD	AsA/DAsA ± SD
Cu (μg l^{-1})*	0	18.68 ± 0.60	3.39 ± 0.52
	25	16.02 ± 0.81*	3.23 ± 0.34
	50	14.76 ± 1.45*	2.87 ± 0.34*
	100	13.00 ± 3.14*	2.90 ± 0.17*
	200	12.32 ± 1.44*	2.87 ± 0.39*
	500	12.05 ± 2.47*	3.77 ± 0.39*
	1000	8.08 ± 0.80*	4.78 ± 0.81*
Diuron (μg l^{-1})*	0	18.62 ± 0.97	3.33 ± 0.25
	25	19.72 ± 1.33	3.24 ± 0.59
	50	18.95 ± 3.08	3.24 ± 0.45
	75	18.52 ± 2.60	2.90 ± 0.48
	100	17.74 ± 2.98	2.60 ± 0.36*
Folpet (mg l^{-1})*	0	18.47 ± 1.62	3.20 ± 0.96
	1	18.35 ± 1.83	3.23 ± 0.58
	2.5	17.11 ± 2.79	3.52 ± 0.44*
	5	16.92 ± 2.08	3.82 ± 0.55*
	10	17.22 ± 2.90	4.21 ± 0.37*

75% of the total ascorbate. As a response to copper contamination, an increase of total ascorbate content was observed up to 2.8 μmol g FW^{-1} (160% of a control level of 1.75 μmol g FW^{-1}) with 500 μg l^{-1} (figure 2).

Ascorbic acid content was 1.43 ± 0.12 μmol g FW^{-1} in the control. Figure 2 shows that a concentration-dependent increase of AsA content occurred after a 48 h exposure to copper. This increase became significant at 100 μg l^{-1} and reached a maximum value of 153% of the control, i.e. a content of 2.23 μmol g FW^{-1} with 500 μg l^{-1}. Beyond this latter concentration, ascorbic acid content reached a steady state.

The general aspect of the diagram showing variation of DAsA after copper contamination was nearly identical to the AsA one with only slight differences (figure 2). A concentration-dependent increase of DAsA content was observed from a control level of 0.43 ± 0.013 μmol g FW^{-1} up to a maximum of 0.67 ± 0.08 μmol g FW^{-1} (137% of the control) with 200 μg l^{-1}. Beyond that concentration, DAsA content progressively decreased and reached the control level at 1 mg l^{-1} of the metal. In the control, the AsA/DAsA ratio was 3.39 ± 0.52. When *L. minor* was exposed to copper this ratio first decreased up to 200 μg l^{-1}, at which concentration it reached 2.87 ± 0.39, and then it increased with higher concentrations, reaching 3.77 ± 0.39 and 4.78 ± 0.81 with respectively 500 and 1000 μg l^{-1} (table 1).

Diuron caused a significant decrease of both reduced and oxidized ascorbate, as shown in figure 2. In the absence of herbicide, AsA content was 1.42 ± 0.1 μmol g FW^{-1}. A 10% decrease was observed with 25 μg l^{-1}, however this decrease was not significant at $p < 0.05$. Higher concentrations significantly decreased the AsA content up to 0.9 ± 0.14 nmol g FW^{-1} (63% of the control) with 100 μg l^{-1}. Likewise, as a response to diuron exposure, DAsA content decreased, reaching 0.345 ± 0.034

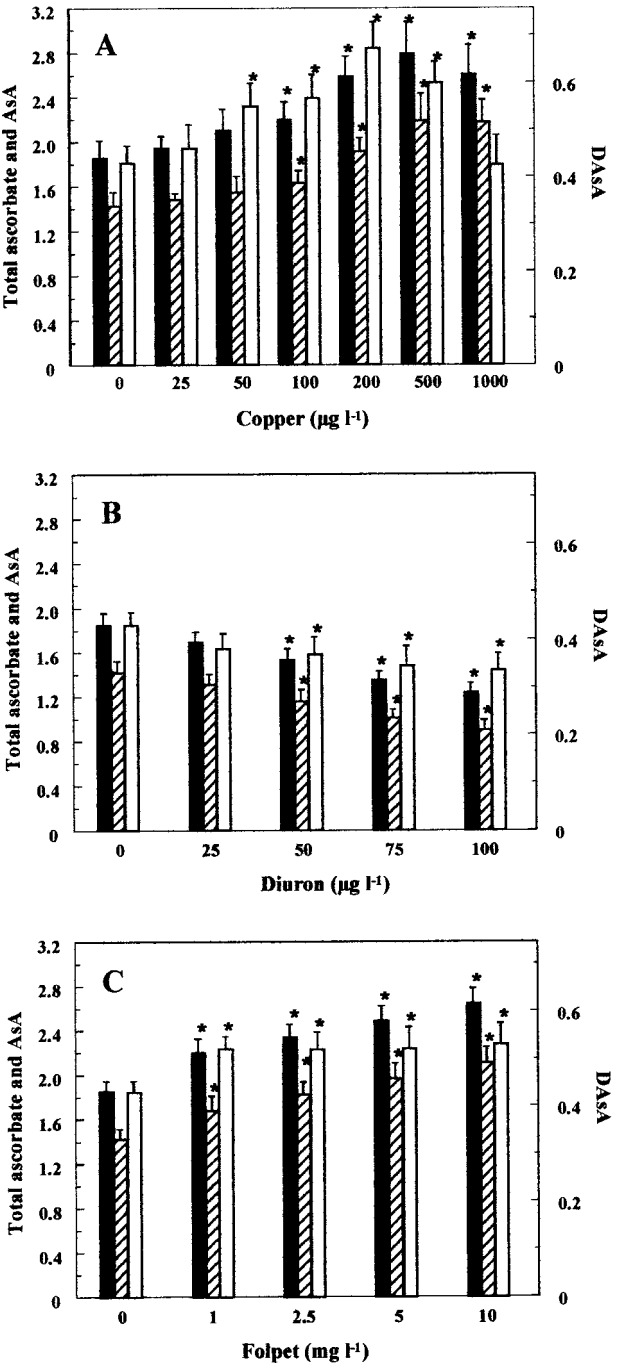

Figure 2. Influence of copper (A), diuron (B) and folpet (C) on total ascorbate (■), AsA (▨) and DAsA (□) contents in *L. minor* after a 48-h treatment. Data (in µmol g FW^{-1}) are mean values ± SD of at least three independent experiments with triplicates. Asterisks indicate a significant difference from the control at $p \leq 0.05$ level.

μmol g FW^{-1} with 100 μg l^{-1} from a control level of 0.44 ± 0.035 μmol g FW^{-1} (figure 2). The AsA/DAsA ratio decreased with increasing concentrations of diuron and reached values of 2.9 and 2.69 with respectively 50 and 100 μg l^{-1} of diuron (87 and 80 % of the control, table 1) indicating an oxidation of the ascorbate pool.

The total ascorbate pool increased as a response to folpet treatment (figure 2). The control level of 1.4 μmol g FW^{-1} of ascorbic acid increased with folpet concentration, reaching 2.1 μmol g FW^{-1} (+ 50 %) with the highest concentration. This increase was observed from 1 mg l^{-1} of the fungicide but it became significant at 2.5 mg l^{-1}. The lowest concentration of folpet tested caused a significant 20 % increase in DAsA content from a control level of 0.44 μmol g FW^{-1} (figure 2). Further increasing the concentration did not cause any additional change in the DAsA content which remained stable at 0.53 μmol g FW^{-1}. The redox state of the ascorbate pool was slightly altered by folpet treatment (table 1). The ratio AsA/DAsA slightly increased from 3.20 ± 0.96 without folpet to 4.21 ± 0.37 with 10 mg l^{-1} of the fungicide, indicating a reduction of the ascorbate pool.

Discussion

Effect of a 48 h contamination by the three toxicants on antioxidant content

Our results showed that GSH and GSSG contents in *L. minor* increased after diuron- and folpet-exposure. The increased of total glutathione pool and the stability of its redox balance after both treatments suggested that the rise in reduced and oxidized forms did not result from a displacement of redox equilibrium but rather from a *de novo* synthesis. Indeed it was reported that the increase in the glutathione pool size following stress was often linked to an enhancement of its biosynthesis through the induction of γ–glutamylcysteine synthetase (Noctor *et al*. 1998). Increases in the glutathione level have been reported under physical and chemical stress conditions such as chilling, injury, drought, heat shock, exposure to O_3, SO_2 or pesticides such as paraquat and oxyfluorfen (Alsher 1989, May and Leaver 1993, Knörzer *et al*. 1996, Kocsy *et al*. 1996). The increase in the glutathione content observed after diuron- and folpet-treatments was in agreement with these conditions and it constituted an indicator of the stress generated by the herbicide and the fungicide in *L. minor*. It should be noticed that increases in GSH and/or GSSG occurred from concentrations of both toxicants which caused significant inhibitions of growth and reduction of chlorophyll contents after 7 days of exposure. Thus, an increase in GSSG content was observed from 1 mg l^{-1} of folpet, a concentration responsible for a 10 % reduction of growth and chlorophyll content (Teisseire *et al*. 1998). Likewise, significant modifications of GSH and GSSG contents were observed with 25 μg l^{-1} of diuron, a concentration leading to a 50 % inhibition of growth (Teisseire *et al*. 1999). This suggested that the increase of GSH and/or GSSG content in *L. minor* after a 48 h exposure might be a potential early indicator of stress.

The lack of variation of the total glutathione content in *L. minor* during a treatment with 'low' copper concentrations (25–200 μg l^{-1}) suggested that the observed changes of GSH and GSSG contents resulted from a modification (oxidation) of the redox balance of the glutathione pool caused by cupric ions. This suggested that GSSG formation exceeded GR capacity, indicating a partial loss of the cellular ability to keep glutathione in its reduced form in presence of copper.

Total and reduced glutathione depletion, observed with high copper concentrations, has already been reported in mussels exposed to this metal (Doyotte *et al*. 1997) while Galli *et al*. (1996) reported an increase of GSH content in maize treated by copper. Other heavy metals, such as cadmium and mercury, were also reported to cause GSH depletion (Gullner *et al*. 1998).

Folpet and copper contamination caused an increase of both reduced and oxidized ascorbate of *L. minor*. Rise in the ascorbate pool size was a general response of stressed plants which resulted in the strengthening of their antioxidative defence mechanisms (Foyer *et al*. 1994). Increases in the ascorbate level have already been reported in plants exposed to oxidative stress-generating agents such as oxyfluorfen or ozone (Knörzer *et al*. 1996, Ranieri *et al*. 1996). The oxidation of the ascorbate pool observed with copper ($< 200\ \mu g\ l^{-1}$) might directly result from the oxidative properties of cupric ions or indirectly through an overproduction of ROS. This suggested that the copper-induced formation of DAsA and MDHA (oxidized forms of ascorbate) overloaded the regenerating capacity of the cell (GR, MDHAR, DHAR, GSH and photoreduction). When copper concentrations exceed $200\ \mu g\ l^{-1}$, the AsA/DAsA ratio goes up, passing beyond the control level, which could either indicate that the reducing capacity of the cell was enhanced or that the formation of DAsA and MDHA had decreased.

Unlike the fungicide and the metal, diuron exposure leaded to a significant depletion of the ascorbate pool. Decrease of ascorbate content has already been reported in drought- and oxyfluorfen-stressed plants (Knörzer *et al*. 1996, Bandurska *et al*. 1997). The ascorbate pool, like any cellular component, is continuously renewed and at a given time its content results from catabolism and synthesis. So the decrease of ascorbate content observed after a diuron treatment might result from the displacement of the catabolism/synthesis balance following the decrease of photosynthetic yields. Indeed, as a photosynthesis inhibitor, diuron reduced the formation of glucose (Kleczowski 1994), which is indirectly involved in the ascorbate biosynthesis through glucuronate (Guignard 1996).

Antioxidant content and their redox balance as potential biomarkers

As main antioxidants, the increases of glutathione and/or ascorbate pool size after contamination by copper, diuron and folpet was an attempt to strengthen the antioxidative defence mechanism which could permit duckweed to overload the stress imposed by the three compounds. This could characterize the resistance stage in which defence and adaptation metabolisms were stimulated leading to 'a hardening of plants by establishing a new physiological standard' (Lichtenthaler 1996). Since they were involved in the general defence strategy to cope with ROS and reactive compounds, induction of antioxidants reflected the exposure to an unsafe environment responsible for the increase of harmful compounds. So one could consider this increase of the antioxidant pools as a potential biomarker of exposure which could be suitable for monitoring water quality and pollution.

While increase in antioxidant content indicated a hardening of the plant's defence mechanism, both depletion of ascorbate and glutathione and the decrease of GSH/GSSG ratio observed with diuron and/or copper reflected a precarious state of the cell in which sensitivity to stress was enhanced. Indeed, as main antioxidants and because of their important functions in various metabolisms, the reduction of the ascorbate and glutathione pool size weakened the antioxidative

defence mechanism and made the cell more sensitive to ROS, leading to toxicity. Since they indicated a lower capacity of the cell to cope with these ROS, and then to overcome an oxidative stress, ascorbate and glutathione pool size and GSH/GSSG ratio decreases appeared to constitute useful biomarkers of stress. Insofar as a high GSH/GSSG ratio is necessary to sustain the role of glutathione as an antioxidant and a reductant (Foyer *et al*. 1997) its perturbation reflected cellular alteration and the partial loss of the regenerating capacity of the cell. So the decrease of this ratio after a 48 h exposure to copper could constitute an early indicator of a precarious state of the cell which might be a prelude to the decrease of plant vitality and inhibition processes. To confirm this view it should be observed that the modification of the redox balance of glutathione occurred from a copper concentration which caused significant inhibition (> 10 %) of growth and reduction of chlorophyll content when duckweed was exposed for 7 days to the metal (Teisseire *et al*. 1998). In addition, GSH/GSSG ratios ranging between 12 and 13 were observed with copper concentrations responsible for growth inhibition > 50 % and strong chlorosis after a 7-day treatment of duckweed (Teisseire *et al*. 1998). Likewise, 25 μg l^{-1} of diuron both caused a depletion of ascorbate after a 48 h exposure and a 50 % inhibition of growth after 7 days of contamination (Teisseire *et al*. 1999). This suggested that decreases in the redox ratio and depletion of antioxidants might constitute early indicators of toxicity insofar as the weakening of the antioxidative defence mechanism for which they were responsible, as a prelude to an overproduction of prooxidant. This conclusion agreed with the proposition of GSH depletion as an early marker of toxicity suggested by Doyotte *et al*. (1997).

The present results, obtained under laboratory conditions with three 'model' toxicants, suggested that antioxidant content in duckweed might serve as a suitable biomarker of exposure and an early indicator of toxicity. Further research was required to validate their use under field conditions. So as a first stage of validation, and since it represents a more complex pollution (mixture of toxicants, interactions, modified bioavailability, etc.), we are now investigating the effect of agricultural leachates coming from vineyards on antioxidant content in *L. minor*.

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