Aquatic Toxicology
September 2013, Volume 140-141, Pages 380-388
http://dx.doi.org/10.1016/j.aquatox.2013.06.016

Multigenerational exposure of the microalga *Tetraselmis suecica* to diuron leads to spontaneous long-term strain adaptation

Sabine Stachowski-Haberkorn[a, *], Marc Jérôme[b], Julien Rouxel[a], Célia Khelifi[a], Maéva Rincé[a], Thierry Burgeot[a]

[a] Ifremer, Laboratoire d'Écotoxicologie, rue de l'île d'Yeu, BP 21105, 44311 Nantes cedex 03, France
[b] Ifremer, Laboratoire de Science et Technologie de la Biomasse Marine, rue de l'île d'Yeu, BP 21105, 44311 Nantes cedex 03, France

*: Corresponding author : Sabine Stachowski-Haberkorn, tel.: +33 02 40 37 41 70 ; fax: +33 02 40 37 41 75 ; email address : Sabine.Stachowski.Haberkorn@ifremer.fr

Abstract:

To investigate the ability of microalgae to develop stable, long-term resistance to herbicides, the marine microalga *Tetraselmis suecica* was exposed to the herbicide diuron (5 µg/L) for a 43-generation exposure period followed by a 12-generation depuration phase. During the first 25 generations, diuron-exposed cultures showed doubling times ranging from 1.95 to 2.6 days, which was 2 to 2.5-fold longer than control cultures. Between generations 25 and 38, during diuron exposure, two out of the three exposed cultures exhibited a spontaneous drop in doubling time. These results provided evidence of culture adaptation to diuron. To assess persistence of the diuron adaptation observed on growth performance, one of the adapted cultures (D_3) was maintained for 12 months in unexposed conditions and then tested by a second, short-term exposure to diuron 5 µg/L, in parallel with a control culture (C_1) for six generations. Flow cytometry analyses were used to monitor cell density, viability, morphology, relative chlorophyll content and intracellular reactive oxygen species (ROS) level. Under these conditions, diuron induced a strong increase of doubling time in exposed-C_1 cultures (2.5-fold longer than unexposed-C_1 cultures), but no significant increase occurred in exposed D_3-cultures compared with unexposed D_3- and unexposed C_1-cultures, showing the persistence of adaptation in the previously-exposed strain D_3. Intracellular ROS level showed the same trend. Significant differences were observed between these strains, with weaker effects of diuron on strain D_3 compared with strain C_1: forward scatter (FSC), representing relative cell size, decreased in exposed cultures (67.8% and 95% of the controls for C_1 and D_3, respectively), whereas FL3 as relative chlorophyll content increased in exposed cultures (115.6% and 108.6% of the controls for C_1 and D_3, respectively).

Results of second exposure to diuron revealed that the adaptation of strain D_3 had persisted after 12 months of depuration, as no growth impairment was observed. This study demonstrates the possible appearance of stable diuron resistance in microalgae in cases of strong, multigenerational chronic exposure to this herbicide in polluted environments.

Highlights : Microalga *T. suecica* was exposed to diuron 5 µg/L on a multigenerational time scale ► After 25 to 32 generations, two culture replicates became resistant to diuron ► Resistance was shown to be stable over time after a one-year depuration period ► Growth of resistant strains, when exposed to diuron, was similar to initial strains ►This genetic adaptation did not induce any growth rate cost

Keywords : microalgae ; Diuron ; Multigenerational ; Adaptation ; flow cytometry

1. Introduction

Pesticides have been used extensively worldwide to improve agricultural productivity. Among the thousands of pesticide molecules on the market, herbicides are the most widely consumed, accounting for 48% of worldwide pesticide consumption in 2005 (Zhang et al., 2011). Diuron (phenylurea) is a broad-spectrum herbicide, persistent in seawater (Thomas et al., 2002). The documented toxicity of diuron for aquatic organisms led to its registration on the priority substance list of the Water Framework Directive (2000/60/CE). Diuron inhibits photosystem II (PSII) by competing with plastoquinone at the Q_B binding site of the D1 protein in the PSII reaction centre, which inhibits energy transfer (Oettmeier, 1992). Contamination of aquatic environments with anthropogenic substances such as herbicides may induce adverse effects on non-target organisms, including microalgae. As unicellular photosynthetic organisms, microalgae have physiological homologies with higher plants that make them potentially vulnerable to herbicides (DeLorenzo et al., 2001 and Dorigo et al., 2004). As primary producers, microalgae sustain the development of higher trophic levels. Damage caused by herbicides to microalgae in the natural environment could thus affect the quantity and quality of food available for primary consumers (Pennington and Scott, 2001).

In addition to their major ecological role, microalgae are also valuable laboratory model organisms. With many genera and species easy to cultivate, their short generation time makes microalgae perfect biological tools in ecotoxicity testing of pollutants. Numerous laboratory studies have been published using microalgae to test for and to compare pesticide toxicity (Bengtson Nash et al., 2005, Fernandez-Alba et al., 2002, Gatidou and Thomaidis, 2007, Geoffroy et al., 2002 and Magnusson et al., 2008, 2010; Stauber et al., 2008). However, in such

tests, microalgae are usually exposed to chemicals for 48, 72 or 96 h, which is only sufficient to ensure a few cell divisions. Their short doubling time makes it easy to work on dozens of generations, and thus to address the question of multigenerational effects of pollutants, acclimation and/or adaptation, on eukaryote phototrophic organisms. It is well known that organisms are able to deal with stress in their environment (including pollutants) by the modification of gene expression, which allows physiological acclimation (Bradshaw and Hardwick, 1989). However, if levels of stress conditions exceed physiological limits, then only the best adapted genotypes will be able to survive. This genetic variability, offering the potential for adaptation or resistance, is mainly due to mutations that occur randomly across the genome (Sniegowski and Lenski, 1995).

Long-term exposure experiments using genetic approaches can help us to investigate the mechanisms that lead to resistance to a molecule. In such studies, Luria–Delbrück fluctuation analysis can be applied, which uses growth monitoring qualitatively as a resistance indicator. Specifically, it assesses whether a microalga strain is able to grow under strong selection pressure induced by the tested molecule. Fluctuation analysis allows the calculation of the mutation rate that occurred in a resistant population. Studies on various pollutant-microalga pairs have shown the ability of freshwater and/or marine microalgae to resist petroleum (Carrera-Martínez et al., 2010; Romero-Lopez et al., 2012), the insecticide lindane (González et al., 2012), and the herbicides simazine, diquat (Marvá et al., 2010), atrazine (Reboud et al., 2007) and diuron (Costas et al., 2001; Lopez-Rodas et al., 2001).

Long-term exposure experiments may also be performed using an ecotoxicological approach to study the multigenerational effects of toxicants on quantitative growth (percentage of growth inhibition) (Pennington and Scott, 2001), which provided the first evidence of effects on population dynamics. When microalgae growth is inhibited to some extent under exposure to a toxicant, it is important to understand whether inhibition i) will remain at the same level or increase over generations or; ii) will fall, indicating resistance. Growth impairment may be associated with genetic changes (Labra et al., 2007) that can be detected in the whole genome using genetic fingerprinting methods. Among these methods, amplified fragment length polymorphism (AFLP) allows the whole DNA extract to be worked upon without prior knowledge of target sequences (Vos et al., 1995). The AFLP method has been successfully applied on various microalgae species for genetic diversity studies (Gaebler et al., 2007; John et al., 2004; Logares et al., 2007) but has only, to our knowledge, been used in one microalgae ecotoxicology study (Labra et al., 2007). In addition to growth and genetic fingerprinting, other cellular endpoints can be monitored to detect possible effects of toxicants on a cell

population. Using flow cytometry, numerous parameters can be measured on thousands of cells in a short time. Without staining, some of the morphological criteria possibly affected by toxicants can be examined, including relative cell size (forward scatter: FSC), complexity (side scatter: SSC) and cell red autofluorescence (relative to chlorophyll content) (Cid et al., 1995; Franqueira et al., 2000; Prado et al., 2011; Stauber et al., 2002). After staining, viability, reactive oxygen species (ROS) intracellular level and cytoplasmic membrane potential can also be measured (Jamers et al., 2009; Prado et al., 2012; Saison et al., 2010; Yu et al., 2007).

As has been described for higher plants and microorganisms, appearance of resistance to xenobiotic substances may modify balance of nature in an ecosystem. Using Luria–Delbrück fluctuation analysis, several studies showed that microalgae also have the ability to resist pollutants by the mean of rare spontaneous mutations. However, from an ecotoxicological point of view, information is lacking on the impact of such resistance, leaving a number of questions to be answered: 1) Can these mutants arise and replace other variants in a population subjected to chronic environmental exposure? 2) On what time scale does this process take place? 3) Are these mutants able to persist under unpolluted conditions? 4) Which physiological properties distinguish them from the initial population? 5) What could be the eventual consequences for the whole system?

In order to address questions 1, 2, 3 and in part question 4, the marine microalga *Tetraselmis suecica* was exposed to the herbicide diuron in both a long-term and a short-term experiment. The objectives were: i) to study the effects of diuron on growth and DNA in a long-term, multigenerational exposure and to test whether diuron exposure would induce noticeable changes in the strain; ii) to check for the persistence of adaptation to diuron after a long depuration period by a study of the strain's responses to short-term exposure.

2. Material and methods

2.1. Chemical / toxicant preparation

The herbicide diuron was purchased from Sigma Aldrich (diuron Pestanal®, analytical standard at 100 mg/L in acetonitrile). The stock solution was diluted to a working solution of 2 mg/L, using sterile ultra-pure water. Acetonitrile concentration in the working solution was 2%.

2.2. Microalgal cultures

The marine microalga *Tetraselmis suecica* (CCMP 904) was obtained from the Provasoli–Guillard National Center for Marine Algae and Microbiota, and maintained in sterile f/2 medium (Guillard and Ryther 1962, Guillard 1975). Cultures were grown in 500 mL round glass flasks filled with 300 mL sterile f/2 medium. Flasks were first heated to 450°C for 6 h, filled with medium and then autoclaved 20 min at 121°C. Microalgal cultures were cultivated at 17 ± 1°C, under daylight fluorescent tubes (Philips, Master TL–D 90 Graphica) at 120 $\mu mol.m^{-2}.s^{-1}$ (Quantometer Li-Cor Li-250 equipped with a spherical sensor), with a dark:light cycle of 8:16 h. For exposure experiments, a stock culture in exponential growth phase was used for inoculation. Initial cell densities of cultures were 10,000 cell/mL.

2.3. Growth rate

The strain was maintained under growth phase by the means of successive batch cultures and dilutions in fresh medium. For each culture, growth parameters were calculated as follows: the growth rate (μ, day^{-1}) was the slope of the regression line from the plot of Ln (cell/mL) on time (days). The doubling time (T_D, days) was calculated as: $T_D = Ln(2)/\mu$. The number of generations (N_G) was calculated as: $N_G = t/T_D$, t being the duration of the culture (days). In the long-term exposure experiment, for each culture, N_G obtained for successive cultures were added so that every culture had its own biological time scale or "age".

2.4. Exposure experiments

2.4.1. Long-term first exposure and depuration phases

Based on previous experiments, diuron was added to the culture medium to give a final concentration of 5 µg/L. The acetonitrile concentration was 0.005%. Therefore, the control cultures were inoculated in f/2 medium containing a final concentration of 0.005% acetonitrile. Control and exposed cultures were run in triplicate: solvent controls (C_1; C_2; C_3) and 5 µg/L diuron (D_1; D_2; D_3). Every day, 1 mL of each culture was sampled in order to assess cell density using flow cytometry: when a culture reached the end of the exponential growth phase (decrease of instantaneous growth rate), it was diluted to 10,000 cell/mL in a new flask containing fresh medium in order to maintain the experiment over many generations (on average a 2% dilution was made for controls and a 4% dilution for exposed cultures). Before each dilution, a part of the culture (60 to 100 mL, depending on the cell density) was taken for DNA analysis. Successive dilutions for each of the six cultures (8 for

controls and 10 to 11 for exposed cultures) were carried out over 55 generations. Toxic pressure was maintained for D cultures for about 43 generations, using diuron-contaminated medium. The multigenerational exposure ended with a short depuration period of 12 generations.

At the end of this 12-generation depuration step, one replicate from the control treatment (C_1) and one replicate from exposed treatment (D_3) were maintained under similar unexposed conditions as follows. Cultures were successively diluted (1.5% dilution for both) once a week over about 300 generations (exactly 307 and 294 generations for C_1 and D_3, respectively, corresponding to a 13-month period for C_1 and a 12-month period for D_3), resulting in an average doubling time of 1.19 days and 1.24 days for C_1 and D_3 cultures, respectively. After this long depuration phase, a short-term second exposure was carried out, starting on the same date for each strain (at 362 and 350 generations for C_1 and D_3, respectively).

2.4.2. Short-term second exposure

To test whether the adaptation of strain D_3 had persisted over the unexposed period, and to compare its response with strain C_1, a short-term exposure to diuron was carried out for six generations, using a single inoculation. The two strains, C_1 and adapted *T. suecica* (D_3), were compared in an experiment that exposed the cultures to 5 µg/L of diuron (C1-exposed and D3-exposed) and included control cultures containing acetonitrile 0.005% (C1-control and D3-control). All cultures were carried out in triplicate. Every day, 2 mL of each culture were sampled to check the cell density, morphology, relative chlorophyll content, viability, and intracellular ROS content, using flow cytometry. The experiment was run twice independently.

2.5. Microalgal analysis using flow cytometry

For the long-term exposure experiment, samples were run using a FACScalibur (BD Biosciences, San Jose, CA, USA) flow cytometer equipped with a 488 nm argon laser. For the short-term exposure experiment, samples were run using an Accuri C6 flow cytometer (Becton Dickinson Accuri) equipped with a blue (488 nm) and a red (640 nm) laser.

2.5.1. Growth rate measurement

Samples of 500 µL were fixed with glutaraldehyde (final concentration 0.25%). Tubes were vortexed and left for 15 minutes at room temperature in the dark before analysis. For the long-

term exposure experiment *T. suecica* cells were visualized on a Forward Scatter (FSC) and FL3 (red fluorescence, > 650 nm, chlorophyll-related) cytogram. Counts were estimated from the flow-rate measurement of the flow cytometer (Marie et al., 1999) as all samples were run for 1 min. For the short-term exposure experiment *T. suecica* cells were examined with a FL1 (green fluorescence, 533/30 nm) and FL3 (red fluorescence, > 670 nm, chlorophyll related) cytogram. Counts were available directly in the Cflow® software with the precise measurement of the analysed volume.

2.5.2. Morphology and relative chlorophyll content (short-term exposure experiment)

Fresh 500 µL samples were run immediately after sampling in order to analyse relative cell size (forward scatter, FSC), cell complexity (side scatter, SSC) and relative chlorophyll content (FL3). For each variable, data collected were mean values of the cell population gated, expressed in arbitrary units (a.u.).

2.5.3. Cell viability (short-term exposure experiment)

The effects of diuron exposure on cell viability were assessed using the fluorescent dye SYTOX Green (Molecular probes, Eugene, OR, USA), which penetrates cells with damaged membranes. Thus, cells stained by SYTOX Green are considered as dead, which allows the calculation of a viability percentage in the cell population gated (Veldhuis et al., 2001). SYTOX Green was used at 0.05 µM final concentration in 500 µL-fresh samples. Tubes were incubated in the dark at room temperature for 30 min before analysis. SYTOX Green fluorescence was measured by FL1 (green fluorescence, 533/30 nm).

2.5.4. Reactive oxygen species (ROS) (short-term exposure experiment)

To check for the diuron effect on the presence of intracellular ROS, the fluorescent dye 2',7'–dichlorodihydrofluorescein diacetate (H_2DCFDA) was used (Molecular probes, Eugene, OR, USA). This non-polar dye can enter the cells, where it is activated by esterases into a non-fluorescent form (2',7'–dichlorodihydrofluorescein: H_2DCF). It is then converted into the highly fluorescent 2',7'–dichlorofluorescein (DCF) if ROS are produced inside the cell. This fluorescent compound can be measured by green fluorescence (FL1, 533/30 nm). A stock solution of 10 mM H_2DCFDA in dimethyl sulfoxide (DMSO) was used to make a 2 mM working solution in phosphate buffered saline (137 mM NaCl; 2.7 mM KCl, pH 7.4) (DMSO concentration in the working solution: 20%). Preliminary experiments were carried out to determine the optimal H_2DCFDA concentration and incubation time to apply to *T. suecica*

cultures, using cultures exposed to 100 µM H_2O_2 (final concentration) as a positive control. In the present experiments, H_2DCFDA was used at 80 µM final concentration (0.8% DMSO) in fresh 500 µL samples. Samples were incubated in the dark at room temperature for 30 min before analysis. In order to prevent possible signal variations due to diuron influence on FL1 fluorescence, the mean FL1 values of H_2DCFDA-stained samples were divided by the mean FL1 values of the same fresh samples analysed for morphology. Results were thus expressed as FL1 ratios.

2.6. Genetic fingerprints

To obtain genetic fingerprints from the cultures of the long-term exposure experiment, Amplified Fragment Length Polymorphism (AFLP) analyses were carried out. After adequate extraction, the complete DNA was digested into fragments using restriction enzymes. Ligation of oligonucleotide adapters to the fragments then allowed their amplification by PCR using adapter-specific primers. Primer sequences end with 1 to 3 selective nucleotides corresponding to the first nucleotides of the unknown genome region to amplify. Using this technique, any restriction fragment originating from any DNA region can be amplified, supposing that the unknown sequence begins with the selective nucleotides added to the primers.

2.6.1. DNA extraction and purification

Culture volumes sampled before each successive dilution were filtered on 0.45 µm cellulose acetate membranes. Each membrane was washed in 15 mL TNE buffer (0.1 M Trizma base, 0.1 M NaCl, 0.05 M EDTA, pH 8). Cell suspension was centrifuged for 15 min at 9000 rpm at 4°C. The supernatant was discarded and cell pellet was suspended in 500 µL TNE buffer, 75 µL SDS 10%, 75 µL sarkosyl 10% and 12 µL proteinase K at 25 mg/mL. After gentle stirring, the tube was incubated at 40°C for 2 h. Lysate was purified by extraction with an equal volume of phenol–chloroform–isoamyl alcohol (PCI, 25:24:1), and the residual phenol was removed by extraction with an equal volume of chloroform–isoamyl alcohol (CI, 24:1). Then, 2 µL RNase (10 mg/mL) were added to the aqueous phase containing the DNA, and the tube was incubated at 60°C for 1 h. RNase was eliminated as previously described by an extraction with PCI followed by an extraction with CI. Two volumes of absolute ethanol and sodium acetate (10% of the total volume, 3 M, pH 5.2) were then added to the aqueous phase. The DNA pellet formed was recovered using a sterile glass pipette, and rinsed in ethanol 70%. After drying for a few seconds, the pellet was resuspended in sterile TE buffer (10 mM

Tris–HCl, 1 mM EDTA, pH 8) and left to dissolve overnight at 4°C. DNA quality and concentration were checked using the Nanodrop ND-1000 spectrophotometer.

2.6.2. Fragment analyses

AFLP analyses were performed using the AFLP® Analysis System I and AFLP® Starter Primer kits from Invitrogen™ (Paisley, UK). The restriction enzymes used were EcoRI and MseI. Invitrogen™ protocol was slightly modified to accommodate with the non-radioactive labelling of selective primers *Eco*RI–AXX and the capillary electrophoresis. Pre-selective PCR amplifications were performed with the *Eco*RI–A and *Mse*I–C primer combination. For selective PCR amplifications, eight D4-dye labelled *Eco*RI–AXX primers (WellRED™ fluorescent dye D4 primers, purchased from Sigma–Aldrich®, Paris, France) and the eight unlabelled selective primers *Mse*I–CXX of the Invitrogen™ kit (Table 1) were used. The entire 64 (8 × 8) combinations were tested, out of which five primer combinations (E1/M3, E1/M5, E5/M6, E6/M2, E7/M5, Table 1 in italics) were finally selected based on the quality of the peaks produced. Amplified DNA fragments were separated by capillary electrophoresis in the CEQ™ 8000 DNA analysis system (Beckmann Coulter): 0.5 µl volume of selective PCR product and 0.5 µl of DNA internal size standard (CEQ DNA Size standard 600 Beckmann Coulter) were added to 30 µl of sample loading solution (Beckmann Coulter). Samples were loaded into the CEQ™ 8000 system and run following Beckmann Coulter running conditions. A DNA fingerprint was obtained for each primer pair tested and each sample, where DNA fragments were identified according to their size (number of nucleotides). Fragment analysis was carried out using the "fragment analysis" module of the CEQ 8000 software (CEQ™ 8000 Genetic Analysis System V8). DNA fragments from 80 to 710 nucleotides were included in the study in order to generate a binary matrix (fragment presence/absence) containing all primer pairs tested for each sample. On the basis of a fragment presence/absence matrix at each number of generations (N_G) analysed, a dendrogram was constructed applying the Dice coefficient and the Unweighted Pair Group Method of Averages (UPGMA), using Matlab software R2007a (The Mathworks, Natik, MA).

2.7. Statistical analysis

2.7.1. Long-term exposure experiment

In order to test for significant differences between the DNA fingerprints obtained from control and exposed cultures, analyses of similarity (Clarke, 1993) were performed on binary

matrices from electrophoregrams using the one-way ANOSIM function in Past v2.17b software (Hammer et al. 2001).

2.7.2. Short-term exposure experiment

Analyses of variance (ANOVA) using general linear models (GLM) were performed to check for significant differences between the C_1 and D_3 strains. One-way ANOVAs were then performed on each strain to check for significant differences in growth, morphology and intracellular ROS level between control and diuron-exposed cultures. A p value < 0.05 was considered statistically significant. When significant differences were observed, the multiple range Newman–Keuls test was used to compare means. All statistical analyses were performed using Statgraphics® Centurion XVI software.

3. Results

3.1. Multigenerational exposure experiment

3.1.1. Evolution of doubling time

The experiment lasted about 55 generations (54 to 57, depending on the culture). While control cultures (C) were grown without exposure to diuron over 55 generations, exposed cultures (D) were exposed to 5 µg/L diuron over about 43 generations, followed by a depuration period of 12 generations. These 55 generations corresponded to 8 successive dilutions and cultures for control cultures, whereas diuron-exposed cultures needed 10 to 11 successive dilutions and cultures to reach the same number of generations. For each successive culture, the growth curve was used to calculate the corresponding doubling time (T_D) and number of generations (N_G). Evolution of T_D over N_G is shown in Fig. 1.

Throughout the experiment, control cultures exhibited T_D values that fluctuated by around 1 day, ranging from 0.78 days (C_1 at $N_G = 36$) to 1.19 days (C_3 at $N_G = 54$) (Fig. 1). The triplicate values were very similar to one other.

During the first five successive dilutions, the diuron-exposed (D) replicates exhibited relatively close T_D values and similar fluctuations between 1.95 and 2.6 days, which was twice as long as the C cultures (Fig. 1). The replicates then evolved differently (Fig. 1). Among the three cultures exposed to diuron 5 µg/L, two exhibited a spontaneous decrease in their T_D during exposure: D_3 and D_2 doubling times dropped after 25 and 32 generations, respectively, and then remained between 1.18 and 1.44 days until depuration (Fig. 1). During depuration generations in the D treatments ranged from 0.89 to 1.28 days, which was same range as the control cultures. For the D_1 replicate, T_D values varied between 1.9 and 2.53 days

during the whole exposure time and dropped when depuration began after 41 generations, remaining at 1.20–1.31 days during the depuration phase (Fig. 1).

At the end of this multigenerational exposure experiment, replicates C_1 and D_3 from control and exposed cultures, respectively, were maintained for a further 13 and 12 months (for C_1 and D_3, respectively) under unexposed conditions.

3.1.2. Genetic fingerprints

For AFLP analysis, samples of the multigenerational exposure experiment were considered according to the age of the cultures in terms of their generation number N_G. AFLP analyses were carried out and dendrograms were constructed from samples taken from cultures at different ages (data not shown): 5.7–6.3 generations and 5.1 generations for C-cultures and D-cultures, respectively (1st culture); 23.6–24.9 generations for the whole set of cultures (just before the T_D drop of D_3); 30.8 generations and 27.7–30.7 generations for C-cultures and D-cultures, respectively (as D_3 T_D dropped); 47.2–48.9 generations and 46.7–51.6 generations for C-cultures and D-cultures, respectively (just after the beginning of depuration).

Among the four dendrograms generated from DNA fingerprints (data not shown), no significant difference was shown between control and exposed cultures (ANOSIM, $p > 0.05$).

3.2. Short-term exposure experiment

The short-term exposure experiment took place 12 months after the end of the first depuration phase of the D_3 multigenerational exposure and was performed twice, independently, over six generations, with a single dilution: growth lasted seven days. As similar results were obtained for both short-term experiments, only one of these is presented.

3.2.2. Cell viability

During the experiment, cultures contained at least 95% of viable cells except on the first day where viability percentages were in the range 92–95% (data not shown). There was no difference in cell viability between the controls and the cultures exposed to diuron.

3.2.2. Growth and intracellular ROS level

Daily cell density monitoring allowed calculation of the average T_D of the whole growth phase for each culture condition (Fig. 2). Exposure of strain C_1 to 5 µg/L diuron significantly increased T_D and ROS intracellular level (Fig. 3) on the last day of the experiment ($p < 0.001$): T_D was 2.5-fold longer compared with C_1-control cultures (Fig. 2A) and mean FL1

ratio was 2.4-fold higher (Fig. 3A). For strain D_3, no significant difference was detected for growth (Fig. 2B) or intracellular ROS level (Fig. 3B) in D_3-exposed cultures ($p > 0.05$). No significant difference was detected between C_1 and D_3 strains ($p > 0.05$).

3.2.3. Morphology and relative chlorophyll content

On the last day of the experiment, relative cell size (FSC), complexity (SSC) and chlorophyll content (FL3) were compared between strains C_1 and D_3, and between control and exposed cultures (Fig. 4). No significant difference was detected between strains C_1 and D_3, whatever the parameter considered ($p > 0.05$).

Exposure to diuron significantly decreased relative cell size of C_1-exposed cultures (67.8% of the C_1-control, $p < 0.001$) and D_3-exposed cultures (95% of the D_3-control, $p < 0.05$). Under diuron exposure, relative chlorophyll content of the cells was significantly increased in both strains: mean FL3 value was 115.6% of the C_1-control value in C_1-exposed cultures ($p < 0.001$) and 108.6% of D_3-control value in D_3-exposed cultures ($p < 0.05$). Although significant, differences observed in strain D_3 were less marked than for strain C_1.

While relative cell complexity of C_1-exposed cultures was significantly lower than C_1-control cultures ($p < 0.01$), no significant difference was detected between D_3-exposed cultures and the D3-control.

4. Discussion

Long-term exposure of *T. suecica* to diuron 5 µg/L, a concentration representative of polluted environments, first led to a strong growth inhibition of the triplicate cultures. After 25 to 32 generations of exposure, two triplicates had become resistant to diuron, as shown by their sudden decrease in doubling time. This resistance was demonstrated to be persistent over time in a second, short-term exposure, conducted after about a year of culture under unexposed conditions. This short-term exposure experiment also revealed the persistence of slight but significant effects of diuron on the morphology and physiology of resistant cells.

4.1. Multigenerational exposure experiment

The purpose of this experiment was to assess the effects of diuron on *T. suecica* under conditions of chronic multigenerational contamination to see whether changes occurred in the strain. During the five first successive dilutions of the experiment (Fig. 1), doubling times (T_D) of exposed cultures were about twice those of the controls. The corresponding average growth inhibition was 56.5%, indicating that IC_{50} value would probably be close to 5 µg/L.

This result is consistent with other studies, as several marine microalgae species exposed to diuron exhibited very similar IC_{50} values (*Navicula* sp. 7–9 µg/L, *Nephroselmis pyriformis* 7–9 µg/L in Magnusson et al., 2008; *Dunaliella tertiolecta* 5.9 µg/L in Gatidou and Thomaidis, 2007). *T. suecica* may even be considered a sensitive species, as other growth inhibition tests performed on a variety of marine microalgae resulted in IC_{50} values in the range 27–63 µg/L (Gatidou and Thomaidis, 2007; Koutsaftis and Aoyama, 2006; Silkina et al., 2012).

Diuron concentrations currently encountered in freshwater environments range from ng/L (Dorigo et al., 2010; Pesce et al., 2010a) to peaks of several µg/L (8.5 µg/L in Mitchell et al., 2005; 7.9 µg/L in Pesce et al., 2010b). In coastal environments, peak concentrations of 1–3 µg/L diuron have been measured (Lamoree et al., 2002; Okamura et al., 2003; Thomas et al., 2002) though values usually remained below 1 µg/L (Lewis et al., 2009; Martínez Bueno et al., 2009; Munaron et al., 2012; Shaw et al., 2008). Thus, the concentration used in the present work is higher and would correspond to extremely polluted conditions in an enclosed coastal area.

Throughout the experiment, T_D fluctuations occurred from one culture to another, across a range of 20–25% in both treatments. Despite this variability, the similarity of T_D values of triplicates in each condition during the first five dilutions illustrate a high repeatability of this experiment.

For data analysis and interpretation, results of the multigenerational exposure experiment were considered according to the age of the cultures in terms of their number of generations N_G. This parameter is more biologically meaningful than absolute time, because absolute time does not take into account the differences of generation time between the control and exposed cultures.

Among the three replicates of exposed cultures, two showed a decrease in their T_D, at different ages: after 25 and 32 generations for D_3 and D_2, respectively. These decreases in doubling time suggest that the cultures have a kind of resistance to the herbicide, which was later confirmed by the results obtained from the two subsequent short-term exposures of the D_3 strain to diuron. The decreased T_D could result from genetic mutation(s). Two kinds of mutation could be involved: 1) an adaptive mutation induced by the herbicide during the chronic exposure, or 2) a mutation of the type that generates genetic diversity. In our case, the original cultures were polyclonal, having their own genetic diversity that resulted from mutations occurring randomly during each cell division. Among these, a mutation conferring advantage under herbicide exposure could have been present in the cultures independently from diuron exposure. In this case, this particular mutation, i.e., a particular resistant

genotype, could be selected and become more dominant under the selection pressure of diuron. Differences between exposed replicates could be explained by spontaneous mutations occurring at a different times/ages in each culture during exposure, or the presence of such mutations in the culture inoculums before exposure followed by dominance of resistant variants appearing at different times/ages in each culture. At this point in study, neither of these hypotheses could be demonstrated. Nevertheless, the hypothesis of a genetic mutation already existing in the cultures prior to exposure is supported by results from previous studies that used Luria and Delbrück's (1943) fluctuation analyses on microalgal clones exposed to the insecticide lindane (González et al., 2012), and the herbicides simazine and diquat (Marvá et al., 2010) and diuron (Costas et al., 2001; López-Rodas et al., 2001). The authors demonstrated that pesticide-resistant cells in microalgae cultures arose only by rare spontaneous mutations occurring randomly prior to exposure to the tested chemicals.

In our experiment, exposure to diuron was not carried out on clones but on polyclonal cultures, meaning that genetic diversity was present. One of our goals was to assess the possible multigenerational effects of diuron on DNA of the microalga. As this species was not completely sequenced, it was chosen for work on fragment polymorphism using the AFLP technique (Vos et al., 1995). This DNA fingerprinting technique was recommended for similarity assessment of microalgae strains within and between populations (de Bruin et al., 2003). In the present study, no significant effect could be seen on DNA from cultures during and after chronic exposure to diuron, as compared to control cultures. This technique, in our case, did not seem sufficiently discriminant. Using AFLP, Labra et al. (2007) demonstrated genotoxic effects induced by increasing concentrations of potassium dichromate in the freshwater microalga *Pseudokirchneriella subcapitata*. We can hypothesize that such effects need to be vast and to occur in a large part of the population sampled in order to be detected with AFLP, as this is performed on DNA extracted from millions of cells. For further investigations, it would be preferable to check for differential expression of genes involved in various cellular functions.

4.2. Short-term exposure experiment

The aim of this experiment was to check for the durability of adaptation to diuron in strain D_3 previously obtained and maintained during a year without any contact with the herbicide. Viability measurements carried out throughout the experiment showed that exposure to 5 µg/L diuron did not induce mortality under any conditions: about 95% of cells were viable during the experiment. The average T_D calculated at the end of experiment highlighted a

strong growth inhibition of C_1-exposed cultures (Fig. 2), whereas D_3-exposed cultures were not affected by the herbicide. Not only did the adaptation allow a growth of D_3-exposed cultures similar to D_3-control cultures, but the latter grew as much as the C_1-control cultures. We can thus suggest that during the long-term experiment, adapted cells probably out-competed sensitive cells due to their improved generation time. This result provided strong evidence that adaptation observed during the multigenerational exposure experiment was persistent and stable through time. Consequently, we can confirm strain D_3 to be genetically resistant to diuron. Other microalgae species have also been shown to develop resistance to diuron: Grizeau et al., (1985) isolated what appeared to be a spontaneous mutant resistant to diuron from a *Dunaliella bioculata* culture after diuron exposure at 350 µg/L. The resistance was maintained in this strain for more than three years even when grown in the absence of diuron.

Showing the same trend as growth, at the end of experiment intracellular ROS level was strongly increased in C_1-exposed cultures, when compared to C_1-control cultures, which had a level similar to the D_3-control and D_3-exposed cultures. It is well known that diuron can induce the formation of reactive oxygen species as a secondary effect of PSII inhibition (Fuerst and Norman, 1991; Oettmeier, 1992), although studies testing for the presence of ROS or antioxidative responses upon diuron exposure are scarce. The fact that intracellular ROS level was not increased in D_3-exposed cultures could be interpreted as follows: i) no ROS production occurred in the cells; ii) ROS production occurred in the cells and their antioxidative system dealt with it very efficiently. Additional measurements on C_1 and D_3 strains, such as enzyme activities and/or antioxidative system gene expression could provide better insight into the mechanisms underlying this result.

Whereas growth and intracellular ROS level exhibited significant effects of diuron in the C_1-strain only, morphology and relative chlorophyll content pointed to some similar significant differences between strains C_1 and D_3: relative cell size was smaller and relative chlorophyll content was higher than in control cultures. These effects were weaker in strain D_3, but they demonstrated that diuron effects could still be detected to a lesser extent on resistant cells. Such effects on pigment content were already noticed in studies where microalgae were exposed to various PSII inhibitors like atrazine (Adler et al., 2007), isoproturon and terbutryn (Rioboo et al., 2002), but also diuron; thus, Magnusson et al., (2008) suggested that it could be a strategy to compensate diuron action. As the herbicide inhibits photosynthesis, cells could produce more chlorophyll in order to maximize light harvesting. In our case, relative chlorophyll content increase observed in the resistant D_3-exposed cultures would suggest that

diuron still had a slight inhibitory effect on photosynthesis, but not one that would affect growth.

In the present study, further investigations could provide more evidence for an acquired genetic resistance to diuron. Information about possible mutation locations are provided by several studies: Thuillier-Bruston et al., (1996) induced mutations conferring resistance to diuron in *Euglena gracilis* strains, after exposure to very high concentrations (5.8 to 112 mg/L). They showed that resistance was acquired through one to two mutations in the *psbA* gene sequence, coding for the D1 protein of PSII: they could weaken the binding of diuron with D1 protein. In a review concerning herbicide resistance, Oettmeier (1999) provides a list of mutations generated by site-directed mutagenesis in various microalgal species: some of these that cause diuron resistance were also expected to play a role in herbicide binding to D1 protein. As *psbA* sequence for *Tetraselmis suecica* CCMP 904 is known, the *psbA* sequences of D_3 and C_1 strains should be sequenced and compared in order to check for possible mutations as reported in previous cited studies.

5. Conclusion

After a first severe growth inhibition when exposed to diuron 5 µg/L, the microalga *Tetraselmis suecica* showed resistance to the herbicide during long-term exposure, most probably as a result of selection of a resistant variant. This resistance, which did not impair growth rate, was shown to be persistent after a year without exposure to diuron. These results suggest that chronic pollution of coastal areas with diuron could strongly disturb primary production and microalgae diversity: after a first drop in primary production as a consequence of growth inhibition and elimination of sensitive variants in the microalgae community, genetic variants adapted to the herbicide could arise and dominate, occupying vacant ecological niches. Even though this ability of some microalgae to survive and maintain high growth rates in polluted environments would allow primary production to be maintained, this would take place at the expense of genetic variability and biodiversity.

Acknowledgements

We thank two anonymous reviewers for their insightful comments, which helped us to improve the manuscript. The authors wish to thank Philippe Soudant, Christophe Lambert and Nelly Le Goïc who offered us the possibility to do flow cytometry analyses and also for their cordial welcome and assistance during these analyses. The authors are grateful to Hansy Haberkorn and Denis de la Broise for their helpful discussions and suggestions during

preparation of the manuscript. We also want to thank Dominique Ménard and Larissa Haugarreau for their technical assistance. This work was funded by Ifremer.

References

Adler, N.E., Schmitt-Jansen, M., Altenburger, R., 2007. Flow cytometry as a tool to study phytotoxic modes of action. Environ. Toxicol. Chem. 26, 297–306.

Bengtson Nash, S.M., Quayle, P.A., Schreiber, U., Muller, J.F., 2005. The selection of a model microalgal species as biomaterial for a novel aquatic phytotoxicity assay. Aquat. Toxicol. 72, 315–326.

Bradshaw, A.D., Hardwick, K., 1989. Evolution and stress–genotypic and phenotypic components. Biol. J. Linn. Soc. 37, 137–155.

Carrera–Martínez, D., Mateos–Sanz, A., López-Rodas, V., Costas, E., 2010. Microalgae response to petroleum spill: An experimental model analysing physiological and genetic response of *Dunaliella tertiolecta* (Chlorophyceae) to oil samples from the tanker Prestige. Aquat. Toxicol. 97, 151–159.

Cid, A., Herrero, C., Torres, E., Abalde, J., 1995. Copper toxicity on the marine microalga *Phaeodactylum tricornutum*: effects on photosynthesis and related parameters. Aquat. Toxicol. 31, 165–174.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Austral. Ecol. 18, 117–143.

Costas, E., Carrillo, E., Ferrero, L.M., Agrelo, M., Garcia-Villada, L., Juste, J., Lopez-Rodas, V., 2001. Mutation of algae from sensitivity to resistance against environmental selective agents: the ecological genetics of *Dictyosphaerium chlorelloides* (Chlorophyceae) under lethal doses of 3–(3,4–dichlorophenyl)–1,1–dimethylurea herbicide. Phycologia 40, 391–398.

de Bruin, A., Ibelings, B.W., Van Donk, E., 2003. Molecular techniques in phytoplankton research: from allozyme electrophoresis to genomics. Hydrobiologia 491, 47–63.

DeLorenzo, M.E., Scott, G.I., Ross, P.E., 2001. Toxicity of pesticides to aquatic microorganisms: a review. Environ. Toxicol. Chem. 20, 84–98.

Dorigo, U., Berard, A., Rimet, F., Bouchez, A., Montuelle, B., 2010. In situ assessment of periphyton recovery in a river contaminated by pesticides. Aquat. Toxicol. 98, 396–406.

Dorigo, U., Bourrain, X., Berard, A., Leboulanger, C., 2004. Seasonal changes in the sensitivity of river microalgae to atrazine and isoproturon along a contamination

gradient. Sci. Total Environ. 318, 101–114.

Fernandez-Alba, A.R., Hernando, M.D., Piedra, L., Chisti, Y., 2002. Toxicity evaluation of single and mixed antifouling biocides measured with acute toxicity bioassays. Anal. Chim. Acta 456, 303–312.

Franqueira, D., Orosa, M., Torres, E., Herrero, C., Cid, A., 2000. Potential use of flow cytometry in toxicity studies with microalgae. Sci. Total Environ. 247, 119–126.

Fuerst, E.P., Norman, M.A., 1991. Interactions of herbicides with photosynthetic electron transport. Weed Sci. , 458–464.

Gaebler, S., Hayes, P., Medlin, L., 2007. Methods used to reveal genetic diversity in the colony-forming prymnesiophytes *Phaeocystis antarctica , P. globosa and P. pouchetii* – preliminary results. Biogeochemistry 83, 19–27.

Gatidou, G., Thomaidis, N.S., 2007. Evaluation of single and joint toxic effects of two antifouling biocides, their main metabolites and copper using phytoplankton bioassays. Aquat. Toxicol. 85, 184–191.

Geoffroy, L., Teisseire, H., Couderchet, M., Vernet, G., 2002. Effect of oxyfluorfen and diuron alone and in mixture on antioxidative enzymes of *Scenedesmus obliquus*. Pestic. Biochem. Physiol. 72, 178–185.

González, R., García-Balboa, C., Rouco, M., Lopez-Rodas, V., Costas, E., 2012. Adaptation of microalgae to lindane: A new approach for bioremediation. Aquat. Toxicol. 109, 25–32.

Grizeau, D., Jeanne, N., Calvayrac, R., 1985. Isolation and characterization of a DCMU (3–(3,4–dichlorophenyl)–1,1–dimethylurea) resistant strain of a marine alga, *Dunaliella bioculata*. Physiol. Plant. 65, 189–195.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. pp 26–60. *In* Smith W.L. and Chanley M.H (Eds.) *Culture of Marine Invertebrate Animals*. Plenum Press, New York, USA.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. Can. J. Microbiol. 8, 229–239.

Hammer, Ø., Harper, D.A.T., Ryan, P.D. 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4(1): 9pp. http://palaeo–electronica.org/2001_1/past/issue1_01.htm

Jamers, A., Lenjou, M., Deraedt, P., Van Bockstaele, D., Blust, R., de Coen, W., 2009. Flow cytometric analysis of the cadmium-exposed green alga *Chlamydomonas reinhardtii* (Chlorophyceae). Eur. J. Phycol. 44, 541–550.

John, U., Groben, R., Beszteri, B., Medlin, L., 2004. Utility of Amplified Fragment Length Polymorphisms (AFLP) to Analyse Genetic Structures within the *Alexandrium tamarense* Species Complex. Protist 155, 169–179.

Koutsaftis, A., Aoyama, I., 2006. The interactive effects of binary mixtures of three antifouling biocides and three heavy metals against the marine algae *Chaetoceros gracilis*. Environ. Toxicol. 21, 432–439.

Labra, M., Bernasconi, M., Grassi, F., De Mattia, F., Sgorbati, S., Airoldi, R., Citterio, S., 2007. Toxic and genotoxic effects of potassium dichromate in *Pseudokirchneriella subcapitata* detected by microscopy and AFLP marker analysis. Aquat. Bot. 86, 229–235.

Lamoree, M.H., Swart, C.P., van der Horst, A., van Hattum, B., 2002. Determination of diuron and the antifouling paint biocide Irgarol 1051 in Dutch marinas and coastal waters. J. Chromatogr. A 970, 183–190.

Lewis, S.E., Brodie, J.E., Bainbridge, Z.T., Rohde, K.W., Davis, A.M., Masters, B.L., Maughan, M., Devlin, M.J., Mueller, J.F., Schaffelke, B., 2009. Herbicides: A new threat to the Great Barrier Reef. Environ. Pollut. 157, 2470–2484.

Logares, R., Rengefors, K., Kremp, A., Shalchian-Tabrizi, K., Boltovskoy, A., Tengs, T., Shurtleff, A., Klaveness, D., 2007. Phenotypically different microalgal morphospecies with identical ribosomal DNA: a case of rapid adaptive evolution? Microb. Ecol. 53, 549–561.

Lopez-Rodas, V., Agrelo, M., Carrillo, E., Ferrero, L.M., Larrauri, A., Martin-Otero, L., Costas, E., 2001. Resistance of microalgae to modern water contaminants as the result of rare spontaneous mutations. Eur. J. Phycol. 36, 179–190.

Luria, S.E., Delbrück, M., 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28, 491–511.

Magnusson, M., Heimann, K., Negri, A.P., 2008. Comparative effects of herbicides on photosynthesis and growth of tropical estuarine microalgae. Mar. Pollut. Bull. 56, 1545–1552.

Marie, D., Brussaard, C., Partensky, F. , Vaulot, D., 1999. Flow cytometric analysis of phytoplankton, bacteria and viruses. In: Current Protocols in Cytometry. John Wiley & Sons, Inc. 11.11.1–11.11.15.

Martínez Bueno, M., Hernando, M., Agüera, A., Fernández-Alba, A., 2009. Application of passive sampling devices for screening of micro-pollutants in marine aquaculture using LC–MS/MS. Talanta 77, 1518–1527.

Marvá, F., López-Rodas, V., Rouco, M., Navarro, M., Toro, F.J., Costas, E., Flores-Moya, A., 2010. Adaptation of green microalgae to the herbicides simazine and diquat as result of pre-selective mutations. Aquat. Toxicol. 96, 130–134.

Mitchell, C., Brodie, J., White, I., 2005. Sediments, nutrients and pesticide residues in event flow conditions in streams of the Mackay Whitsunday Region, Australia. Mar. Pollut. Bull. 51, 23–36.

Munaron, D., Tapie, N., Budzinski, H., Andral, B., Gonzalez, J.-L., 2012. Pharmaceuticals, alkylphenols and pesticides in Mediterranean coastal waters: Results from a pilot survey using passive samplers. Estuarine, Coastal Shelf Sci. 114, 82–92.

Oettmeier, W., 1992. Herbicides of photosystem II. Topics in photosynthesis 11, 349–408.

Oettmeier, W., 1999. Herbicide resistance and supersensitivity in photosystem II. Cell. Mol. Life Sci. 55, 1255–1277.

Okamura, H., Aoyama, I., Ono, Y., Nishida, T., 2003. Antifouling herbicides in the coastal waters of western Japan. Mar. Pollut. Bull. 47, 59–67.

Pennington, P.L., Scott, G.I., 2001. Toxicity of atrazine to the estuarine phytoplankter *Pavlova sp.* (prymnesiophyceae): increased sensitivity after long-term, low-level population exposure. Environ. Toxicol. Chem. 20, 2237–2242.

Pesce, S., Margoum, C., Montuelle, B., 2010a. In situ relationships between spatio-temporal variations in diuron concentrations and phototrophic biofilm tolerance in a contaminated river. Water Research 44, 1941–1949.

Pesce, S., Lissalde, S., Lavieille, D., Margoum, C., Mazzella, N., Roubeix, V., Montuelle, B., 2010b. Evaluation of single and joint toxic effects of diuron and its main metabolites on natural phototrophic biofilms using a pollution-induced community tolerance (PICT) approach. Aquat. Toxicol. 99, 492–499.

Prado, R., Rioboo, C., Herrero, C., Cid, A., 2011. Characterization of cell response in *Chlamydomonas moewusii* cultures exposed to the herbicide paraquat: Induction of chlorosis. Aquat. Toxicol. 102, 10–17.

Prado, R., Rioboo, C., Herrero, C., Suarez-Bregua, P., Cid, A., 2012. Flow cytometric analysis to evaluate physiological alterations in herbicide-exposed *Chlamydomonas moewusii* cells. Ecotoxicology 21, 1–12.

Reboud, X., Majerus, N., Gasquez, J., Powles, S., 2007. *Chlamydomonas reinhardtii* as a model system for pro-active herbicide resistance evolution research. Biol. J. Linn. Soc. 91, 257–266.

Rioboo, C., Gonzalez, O., Herrero, C., Cid, A., 2002. Physiological response of freshwater

microalga (*Chlorella vulgaris*) to triazine and phenylurea herbicides. Aquat. Toxicol. 59, 225–235.

Romero-Lopez, J., Lopez-Rodas, V., Costas, E., 2012. Estimating the capability of microalgae to physiological acclimatization and genetic adaptation to petroleum and diesel oil contamination. Aquat. Toxicol. 124–125, 227–237.

Saison, C., Perreault, F., Daigle, J.-C., Fortin, C., Claverie, J., Morin, M., Popovic, R., 2010. Effect of core-shell copper oxide nanoparticles on cell culture morphology and photosynthesis (photosystem II energy distribution) in the green alga, *Chlamydomonas reinhardtii*. Aquat. Toxicol. 96, 109–114.

Shaw, C.M., Lam, P.K.S., Mueller, J.F., 2008. Photosystem II herbicide pollution in Hong Kong and its potential photosynthetic effects on corals. Mar. Pollut. Bull. 57, 473–478.

Silkina, A., Bazes, A., Mouget, J.-L., Bourgougnon, N., 2012. Comparative efficiency of macroalgal extracts and booster biocides as antifouling agents to control growth of three diatom species. Mar. Pollut. Bull. 64, 2039–2046.

Sniegowski, P.D., and Lenski, R.E., 1995. Mutation and adaptation: the directed mutation controversy in evolutionary perspective. Annu. Rev. Ecol. Syst. 26, 553–578.

Stauber, J.L., Franklin, N.M., Adams, M.S., 2002. Applications of flow cytometry to ecotoxicity testing using microalgae. Trends Biotechnol. 20, 141–143.

Stauber, J.L., Binet, M.T., Bao, V.W.W., Boge, J., Zhang, A.Q., Leung, K.M.Y., Adams, M.S., 2008. Comparison of the Qwiklite™ algal bioluminescence test with marine algal growth rate inhibition bioassays. Environ. Toxicol. 23, 617–625.

Thomas, K.V., McHugh, M., Waldock, M., 2002. Antifouling paint booster biocides in UK coastal waters: inputs, occurrence and environmental fate. Sci. Total Environ. 293, 117–127.

Thuillier-Bruston, F., Calvayrac, R., Duval, E., 1996. Partial molecular analysis of the psbA gene in *Euglena gracilis* mutants exhibiting resistance to DCMU and atrazine. Zeitschrift für Naturforschung. C, J. Biosci. 51, 711–720.

Veldhuis, M.J.W., Kraay, G.W., Timmermans, K.R., 2001. Cell death in phytoplankton: correlation between changes in membrane permeability, photosynthetic activity, pigmentation and growth. Eur. J. Phycol.36, 167–177.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T.v.d., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. 23, 4407–4414.

Yu, Y., Kong, F., Wang, M., Qian, L., Shi, X., 2007. Determination of short-term copper toxicity in a multispecies microalgal population using flow cytometry. Ecotoxicol. Environ. Saf. 66, 49–56.

Zhang, W.J., Jiang, F.B., Ou, J.F., 2011. Global pesticide consumption and pollution: with China as a focus. Proceedings of the International Academy of Ecology and Environmental Sciences 1, 125–144.

Table 1

Primers	Sequence (5'- 3')
Primers for pre-selective amplification	
Eco-A	GAC TGC GTA CCA ATT C-A
Mse-C	GAT GAG TCC TGA GTA A-C
Primers tested for selective amplification	
(E1) Eco-AAC	*GAC TGC GTA CCA ATT C-AAC*
(E2) Eco-AGC	GAC TGC GTA CCA ATT C-AGC
(E3) Eco-AAG	GAC TGC GTA CCA ATT C-AAG
(E4) Eco-ACA	GAC TGC GTA CCA ATT C-ACA
(E5) Eco-ACT	*GAC TGC GTA CCA ATT C-ACT*
(E6) Eco-ACC	*GAC TGC GTA CCA ATT C-ACC*
(E7) Eco-ACG	*GAC TGC GTA CCA ATT C-ACG*
(E8) Eco-AGG	GAC TGC GTA CCA ATT C-AGG
(M1) Mse-CAA	GAT GAG TCC TGA GTA A-CAA
(M2) Mse-CAC	*GAT GAG TCC TGA GTA A-CAC*
(M3) Mse-CAG	*GAT GAG TCC TGA GTA A-CAG*
(M4) Mse-CAT	GAT GAG TCC TGA GTA A-CAT
(M5) Mse-CTA	*GAT GAG TCC TGA GTA A-CTA*
(M6) Mse-CTC	*GAT GAG TCC TGA GTA A-CTC*
(M7) Mse-CTG	GAT GAG TCC TGA GTA A-CTG
(M8) Mse-CTT	GAT GAG TCC TGA GTA A-CTT

Captions

Table 1 – Primers tested and used for pre-selective and selective amplifications in the AFLP analysis. Primers shown in italics are those selected for analysis in the long-term experiment.

Figure 1 – Evolution of doubling time (T_D) of *Tetraselmis suecica* over culture generations (N_G) during the long-term first exposure and depuration (first and second phases): C_1, C_2, and C_3 are triplicates of control cultures and D_1, D_2, and D_3 are triplicates of cultures exposed to 5 µg/L diuron. Each point represents the T_D and N_G calculated from one dilution (for each culture, N_G were cumulated throughout the experiment).

Figure 2 – Doubling time (T_D) of *Tetraselmis suecica* C_1 (A) and diuron-acclimated D_3 (B) strains under control and diuron-exposed conditions. Data are given as means ± standard errors (SE) from triplicates. Significant differences between an exposed strain and its own control are indicated with asterisks (***: $p < 0.001$).

Figure 3 – Reactive oxygen species at the intracellular level (FL1 ratios) in *Tetraselmis suecica* C_1 (A) and diuron-acclimated D_3 (B) strains under control and diuron-exposed conditions. Data are given as means ± standard errors (SE) from triplicates. Significant differences between an exposed strain and its own control are indicated with asterisks (*** $p < 0.001$).

Figure 4 – Morphology (relative cell size, complexity) and chlorophyll content of *Tetraselmis suecica* C_1 and diuron-acclimated (D_3) strains under control and diuron-exposed conditions. Data are presented using arbitrary units (a.u.) obtained from an Accuri C6 flow cytometer. Data are given as means ± standard errors (SE) from triplicates. Significant differences between an exposed strain and its own control are indicated with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 1

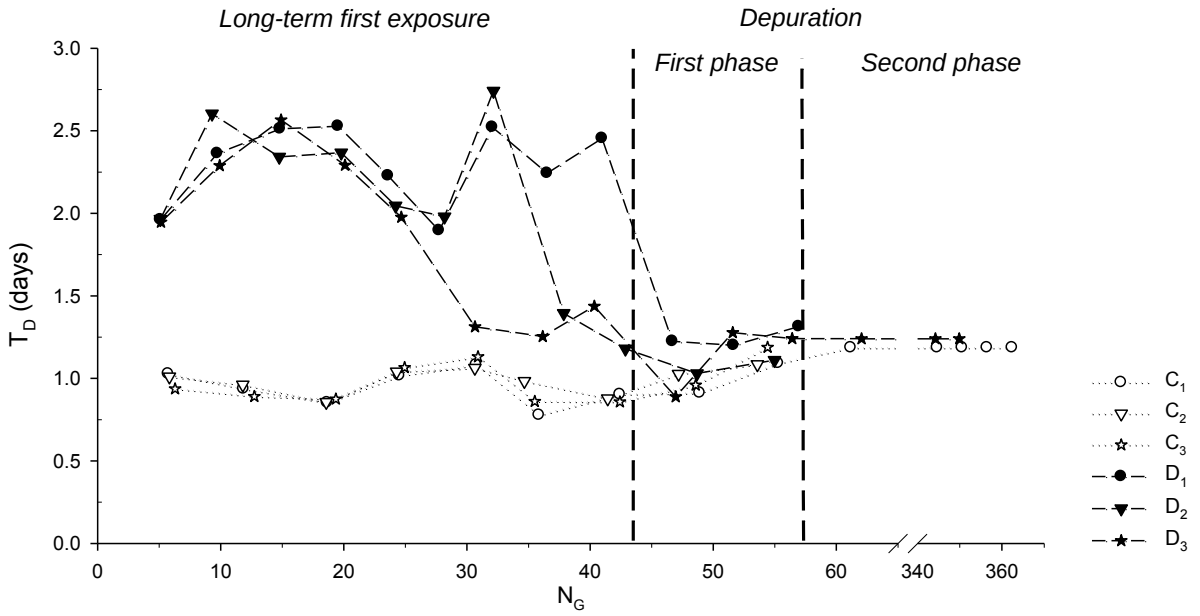

Figure 2

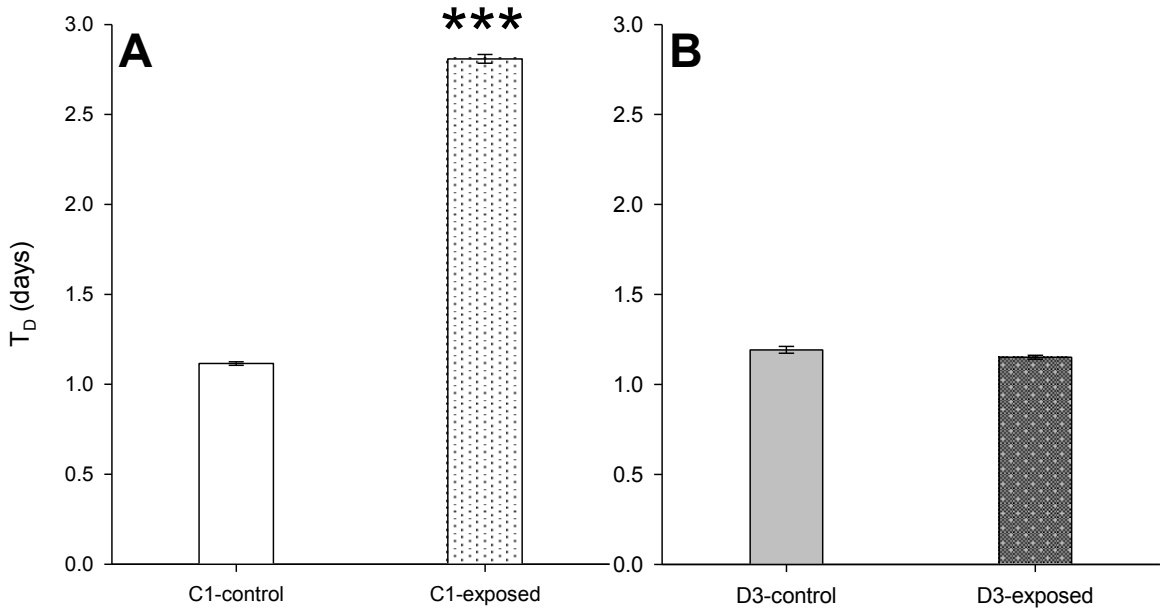

Figure 3

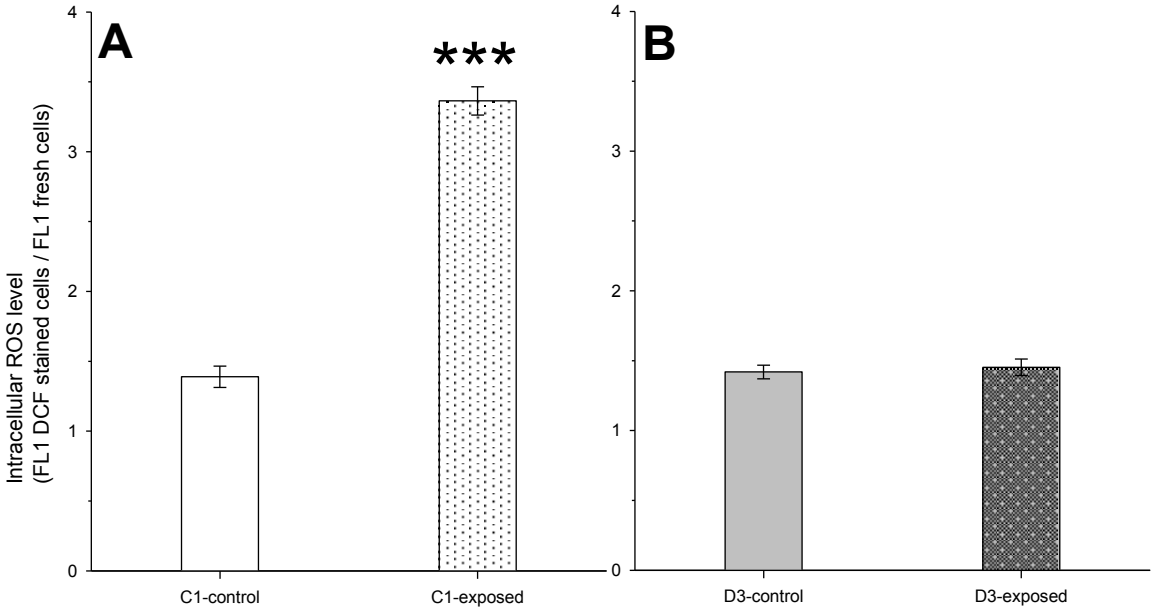

Figure 4