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Impact of Diuron on Aneuploidy and Hemocyte Parameters in Pacific Oyster, *Crassostrea gigas*

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Abstract. Diuron is a substituted urea herbicide used for agricultural and nonagricultural weed control. Its widespread use and relatively slow breakdown led us to analyze its influence on aneuploidy level (lowered chromosome number in a percentage of somatic cells) and hemocyte parameters in Pacific oysters, *Crassostrea gigas*. Adult oysters were subjected to two diuron concentrations (300 ng L^{-1} and 3 μg L^{-1}) for 11 weeks. Significantly higher aneuploidy level was observed in diuron-treated oysters compared with the control. Furthermore, the observed impact on aneuploidy persisted to the next generation as offspring exhibited significantly higher aneuploidy levels when their parents had been exposed to diuron. Significant increases in hemocyte parameters (cell mortality, phagocytosis, granulocyte percentage, reactive oxygen species, and lysosome presence) of the adults were also observed after 4 weeks of diuron exposure. The effects observed on oyster aneuploidy level and hemocyte parameters could have serious environmental and practical consequences.

Diuron [3−(3′,4′-dichlorophenyl)-1,1-dimethylurea] is a phenylurea herbicide (Brian 1964) used for pre- and post-emergence weed control in agriculture. This herbicide may enter aquatic ecosystems by spraydrift, leaching, runoff, or accidental spills (Thurman *et al.* 1991; Squillace and Thurman 1992). Diuron is little affected by natural degradation processes (Radosevich *et al.* 1975), resulting in almost permanent contamination of surface and ground waters. This situation continues in France, even though the use of diuron has been deliberately reduced since 1999. Diuron is considered as a "Priority Hazardous Substance" by the European Commission (Malato *et al.* 2002). Because of its slow breakdown (a month to a year) (Wauchope *et al.* 1992; Okamura 2002), diuron can be found in many environments such as the soil and water (Field *et al.* 2003; Okamura *et al.* 2003).

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Crassostrea gigas has a diploid chromosome number of 2n = 20 (Thiriot-Quiévreux 1986), but hypodiploid aneuploid cells are regularly found (*e.g.*, Leitão *et al.* 2001a). Aneuploidy mainly originates from the nondisjunction of chromosomes during mitosis or meiosis (Bond and Chandley 1983; Martin and Rademaker 1990). Somatic aneuploidy in *C. gigas* is negatively correlated with growth rate (Leitão *et al.* 2001a), and some evidence has been shown for genetics influencing the level of this phenomenon (Leitão *et al.* 2001b). Moreover, differential chromosomal susceptibility was observed in aneuploid cells (Leitão *et al.* 2001c; Bouilly *et al.* 2005). Recently, the herbicide atrazine was shown to significantly increase levels of aneuploidy in *C. gigas* (Bouilly *et al.* 2003, 2004).

Mollusks have an open circulatory system that is continually exposed to the fluctuations of environmental factors including contaminants (Cheng 1981). The cellular defense system of bivalves is mediated by hemocytes that perform various actions including phagocytosis, intracellular degradation of pathogens by enzymatic hydrolysis, and production of reactive oxygen metabolites (Cheng and Rodrick 1975; Pipe 1992). In invertebrates, these cellular activities may be affected by the presence of pollutants (Cheng 1988a, 1988b, 1990). Diuron has already been shown to disturb the metaphase spindle microtubules by splitting microtubule organizing centers that eventually divide into multiple poles along with the associated chromosomes (multipolar anaphase) (Chauhan *et al.* 1998). Thus, diuron disrupts mitosis by inhibiting polymerization of tubulin subunits of microtubules and one might expect effects such as aneuploidy.

Although numerous studies have been made concerning the toxic effects of diuron in organisms such as phytoplankton (Devilla *et al.* 2005) and fish (Okamura *et al.* 2002; Saglio and Trijasse 1998), no investigation has been carried out to study the genotoxicity and the immunotoxicity of this herbicide on bivalves.

The aim of the present work was to study the genotoxicity and immunotoxicity of diuron in the Pacific oyster at concentrations equivalent to those found in the environment. We investigated whether diuron had any effect on (1) chromosome number (aneuploidy) in adults and their offspring in a controlled environment, and on (2) hemocyte parameters (cell mortality, phagocytosis, granulocyte percentage, reactive oxygen species (ROS) production and lysosome presence) for adults monitored using flow cytometry.

Materials and Methods

Diuron Exposure

Pacific oysters, *Crassostrea gigas*, were collected at Bonne Anse, Charente-Maritime, on the French Atlantic coast. These oysters were used in an experiment performed under controlled conditions in the nearby IFREMER hatchery. As 3-year-old adults, the oysters were exposed to diuron added to seawater pumped directly from Marennes-Oléron Bay. Diuron was given by the Institut National de la Recherche Agronomique (INRA, Saint-Laurent-de-la-Prée, Charente-Maritime, France). Pure seawater from Marennes-Oléron Bay was used as a control (treatment 1) and two concentrations of diuron were tested: 300 ng L^{-1} and 3 μg L^{-1} (treatments 2 and 3, respectively). For each treatment, two replicate tanks (A and B) were used, each containing 74 oysters for the aneuploidy study and 60 oysters for monitoring hemocyte parameters. Oysters were first acclimated to the hatchery environment for 4 weeks in raceways. The experiment was then conducted over 11 weeks in tanks without automatic water renewal. Each tank contained 130 L of seawater that was changed every day and maintained at 20°C ± 1°C. Diuron, in the form of diluted solutions, was added to the tanks for treatments 2 and 3. Oysters were fed daily with a mixture of algae (*Skeletonema costatum*, *Isochrysis galbana*, *Tetraselmis suecica*, and *Chaetoceros gracialis*).

Offspring of Diuron-Exposed Oysters

At the end of the diuron exposure, six males and six females from each treatment replicate were used to make crosses. The crosses were performed between parents from the same replicate (1A, 1B, 2A, 2B, 3A, or 3B) using all 12 animals for mass fertilization and leading to 6 crosses in all. Gametes were obtained by strip-spawning sexually mature animals. For each fertilization, 400 million spermatozoids were added to approximately 4 million oocytes suspended in 500 mL of seawater. For each cross, the fertilized gametes were divided into two replicates and these were placed in 50-L fiberglass larval tanks of seawater. Larvae from these crosses were reared for 23 days under standard conditions at 23–24°C and fed with a mixture of algae (*Isochrysis galbana*, *Tetraselmis suecica*, and *Chaetoceros calcitrans forma pumillum*). Larval density was evaluated directly by microscope counts for each batch. Larval growth was studied every 2 or 3 days by measuring the size of 50 larvae using an image analysis system. Larvae were transferred to a micronursery when they were ready to settle. These offspring were examined for aneuploidy after 6 months.

Chromosome Preparation for Aneuploidy Analysis

Oysters (adults or juvenile offspring) were incubated for 7–8 h in seawater containing 0.005% colchicine. The gills were then dissected in seawater, treated for 40 min in 0.9 % sodium citrate, and fixed in a freshly prepared mixture of absolute ethanol–acetic acid (3:1) with two 10-min changes and then two 20-min changes. Slides were prepared from a piece of gill tissue, following the air drying technique of Thiriot-Quiévreux and Ayraud (1982). The preparations were stained for 10 min with Giemsa (4 %, pH 6.8).

Aneuploidy Scoring

Chromosome counts were made directly by microscope observation (Olympus BH2 microscope) choosing apparently intact and well-spread metaphase cells. Cells with 2n = 19, 18, or 17 chromosomes were considered as aneuploid. The mean percentage aneuploidy per replicate was estimated by counting 30 metaphase cells per animal in 10 animals per replicate (300 well-spread metaphase cells per replicate in total).

Sampling of Circulating Hemocytes

Oyster hemocytes were collected at 0, 1, 2, 4, and 11 weeks. On each occasion, 10 oysters per raceway were analyzed. Hemolymph was withdrawn from the pericardial cavity using a 1-mL syringe equipped with a needle (0.9 × 25 mm). For each oyster, 0.5–1 mL of hemolymph was collected and conserved on ice to prevent hemocyte aggregation. Hemolymph samples were pooled from five oysters giving two pools per raceway and four pools per treatment.

Cell Analysis by Flow Cytometry

Flow cytometry protocols were as described in Gagnaire *et al.* (2003, 2004). For each sample, 3000 events were counted using an EPICS XL 4 flow cytometer (Beckman Coulter). Results were depicted as cell cytograms indicating cell size (FSC value) and cell complexity (SSC value) with the fluorescence channel(s) corresponding to the marker used. A gate was defined on the basis of FSC value in order to eliminate cell debris. The type of fluorescence recorded depended on the parameter to be monitored: ROS production, lysosomes, and phagocytosis were measured using green fluorescence and cell mortality using red fluorescence. The EPICS XL 4 software allowed differentiation between supposed populations of granulocytes and hyalinocytes based on their FSC and SSC values. Mortality was quantified using 200 μL of hemolymph. Hemocytes were incubated in the dark for 30 min at 4°C with 5 μL propidium iodide (PI, 1.0 mg mL^{-1}, Interchim). Lysosome presence was measured using a commercial kit (LysoTracker® Green DND-26, 1 mM in dimethyl sulfoxide, Molecular Probes). A 1-μL aliquot of LysoTracker was added to 200-μL hemocyte suspension. Cells were incubated for 2 h in the dark at room temperature, and the reaction was then stopped by placing the tubes on ice for 5 min. ROS production was measured using dihydrorhodamine 123 (DHR123, Molecular Probes), specific to the superoxide anion O_2^-. A 1-μL aliquot of a DHR123 solution (145 μM) was added to 200-μL hemocyte suspension. Cells were incubated for 30 min in the dark at room temperature and the reaction was then stopped by placing the tubes on ice for 5 min. Phagocytosis was measured by ingestion of fluorescent beads. Two hundred microliters of hemolymph was incubated for 1 h in the dark at room temperature with 10 μL of a 1/10 dilution of Fluorospheres® carboxylate-modified microspheres (diameter 1 μm, Interchim).

Statistical Analysis

Because the number of metaphase cells evaluated per individual was the same in all of the studied material (30 per individual), it was possible to test the replicate effect using a two-way analysis of variance (ANOVA) (diuron concentration and replicate effect). Results were compared between parents and offspring using a three-way ANOVA in which the effects were generation (parents or offspring) diuron concentration and replicate effect. Effects of parental treatment on the offspring hatching rate and larval growth were analyzed with nonparametric Kruskal-Wallis tests.

For hemocyte parameters, results were expressed as the percentage of positive cells. Values were normalized using an r angular arcsinus (percentage of positive cells) transformation before analysis. ANOVA of the hemocyte parameters was carried out in order to detect any effect of the diuron. When H_0 was rejected, an *a posteriori* test was used. Significance was set at $p \leq 0.05$. For ROS and lysosomes,

gates were defined from the cytograms in order to distinguish different populations according to fluorescence intensity. Three cell populations were defined as follows: a population of negative cells, a population of moderately stained cells, and a population of strongly stained cells (Gagnaire *et al.* 2006). For both ROS and lysosomes, statistical analysis was only made on strongly stained cells.

Statistical analyses were performed using the computer programs Statgraphics® Plus version 5.1 and SYSTAT 9.0 by SPSS (Wilkinson 1990).

Results

Mortality and Developmental Parameters

Mortality rates of adult oysters ranged from 0 to 5.4% except for the replicate 3A, where this was higher (31.3%). The hatching rates in offspring batches bred from parents exposed to 0 (control), 300 ng L^{-1}, and 3 µg L^{-1} of diuron were 59.2%, 68%, and 45.7%, respectively (Figure 1A). There was no significant difference in hatching rate between treatments ($F = 5.34$; $p = 0.06$). The offspring of parents exposed to the two different diuron concentrations did not show any significant difference in larval growth ($p > 0.05$) (Fig. 1B).

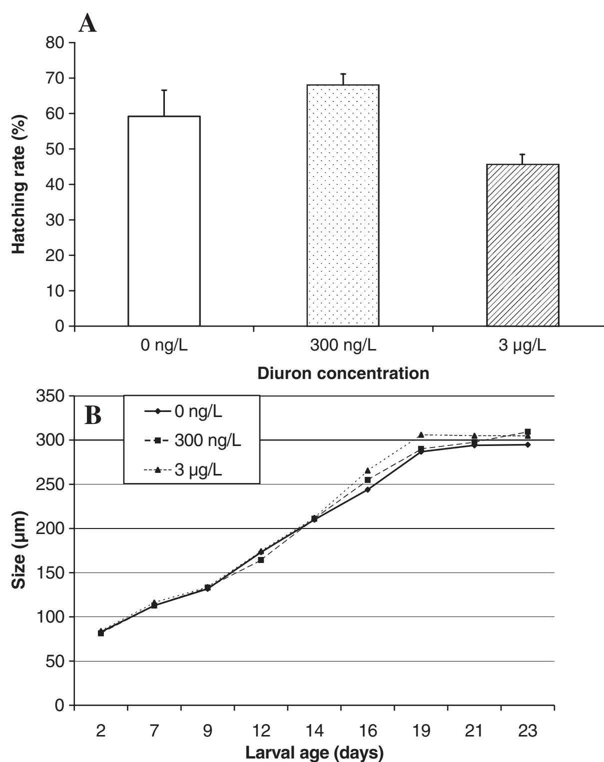

Fig. 1. **A** Hatching rate (%) of offspring of Pacific oysters, *Crassostrea gigas*, exposed to different diuron concentrations (0, 300 ng L^{-1} and 3 µg L^{-1}). Bars indicate standard deviation of the mean. **B** Size (µm) of larvae bred from parental Pacific oysters, *Crassostrea gigas*, exposed to different diuron concentrations (0, 300 ng L^{-1} and 3 µg L^{-1})

Aneuploidy

Aneuploidy levels in adult oysters from treatments 1 (control), 2 (300 ng L^{-1}), and 3 (3 µg L^{-1}) ranged from 7.3% to 10%, 16.3% to 16.7%, and 14.7% to 15.7%, respectively (Figure 2). One chromosome per cell was more often lost than two or three (Table 1). Treatment effect was significant ($F = 13.658$; $p < 0.001$), and there was no significant difference between replicates ($F = 0.115$; $p = 0.736$). Significant differences were observed between treatments 1 and 2 ($F = 25.598$; $p < 0.001$), as well as between treatments 1 and 3 ($F = 17.09$; $p < 0.001$) (Figure 2). No significant difference was observed between the two diuron treatments (2 and 3) ($F = 0.617$; $p = 0.437$).

Offspring aneuploidy levels ranged from 10% to 11.8%, 13.3% to 14.7%, and 15.9% to 17.3% for treatments 1, 2, and 3, respectively (Figure 2). Treatment effect was significant ($F = 3.547$, $p = 0.036$), and there was no significant difference between replicates ($F = 0. 137$, $p = 0. 713$). A significant difference was observed only between treatment 1 and treatment 3 ($F = 7.289$, $p = 0.011$). There was no significant difference when aneuploidy was compared between parents and offspring ($F = 0.142$, $p = 0.707$).

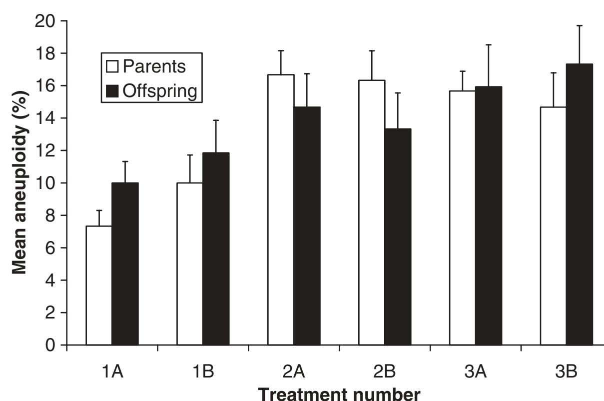

Fig. 2. Mean aneuploidy percentage of adult Pacific oysters, *Crassostrea gigas*, in controls (1A and 1B) and diuron treatments at two concentrations (2A and 2B, 300 ng L^{-1}; 3A and 3B, 3 µg L^{-1}), and of their offspring. Bars indicate standard deviation of the mean

Hemocyte Parameters

The first significant differences in hemocyte parameters were only found after 4 weeks of exposure to diuron. All hemocyte parameters tested were higher in the two diuron treatments (2 and 3) than the control (1) (Figure 3). Cell mortality was 13% in the control and 17.3% and 16.4% for 300 ng L^{-1} (treatment 2) and 3 µg L^{-1} (treatment 3), respectively ($p < 0.05$). Granulocyte percentage was 11.2% in the control and 17.2% for both diuron concentrations ($p < 0.05$). The largest differences appeared for phagocytosis: values were 37% for the control and 59.9% and 58.8% for diuron treatments 2 and 3, respectively ($p < 0.01$ between the control and the two diuron treatments). For ROS production, a similar result was observed (25.9% for the control and 32.6% for both diuron concentrations) ($p < 0.05$). For lysosome detection, values were 27.7% for the control and 40.4% and 41.9% for diuron treatments 2 and 3, respectively. Although there was a

Table 1. Observed aneuploidy of Pacific oysters *Crassostrea gigas* (parents) exposed to different diuron treatments: treatment 1 (control 0 ng L^{-1}), treatment 2 (300 ng L^{-1}) and treatment 3 (3 μg L^{-1}) and observed aneuploidy of their offspring

Population	Treatment number	Percentage of aneuploid cells with			Mean aneuploidy (%)
		2n = 19	2n = 18	2n = 17	
Parents	1	3.5	2.8	2.3	8.6
Parents	2	6.0	4.8	5.7	16.5
Parents	3	8.0	4.5	2.7	15.2
Offspring	1	6.7	2.6	1.6	10.9
Offspring	2	8.7	2.8	2.5	14.0
Offspring	3	10.0	4.0	2.6	16.6

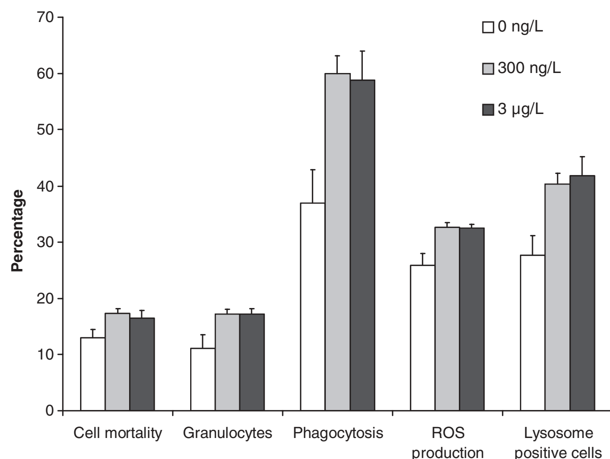

Fig. 3. Hemocyte parameters of adult Pacific oysters, *Crassostrea gigas*, for control (0 ng L^{-1}) and both diuron concentrations (300 ng L^{-1} and 3 μg L^{-1}) after 4 weeks of contamination. Values are means of four replicate pools of five animals each. Bars indicate standard deviations of the mean. ROS, reactive oxygen species

significant difference between diuron-treated samples and the control ($p < 0.05$), the difference between the diuron treatments (2 and 3) was not significant.

Despite these clear effects after 4 weeks exposure, by 11 weeks diuron had no effects on hemocyte parameters (data not shown). Moreover, hemocyte parameters from control and contaminated oysters showed variations during the 11 weeks of experiments (data not shown).

Discussion

An effect of an environmental contaminant on aneuploidy level in *Crassostrea gigas* was previously demonstrated with atrazine. A positive relationship was shown between atrazine concentration and aneuploidy level in *C. gigas* adults and juveniles, and effects persisted to the next generation (Bouilly *et al.* 2003, 2004).

In the present study, a significant increase in aneuploidy level of *C. gigas* adults was observed at both diuron concentrations tested, compared with the control. Aneuploidy is a quite common response in *C. gigas*, but observed aneuploidy levels can be more or less high. Aneuploidy levels less than 5% could be classified as very weak; between 5% and 10%: weak; between 10 and 14%: normal weak; between 14 and 18%: normal high; between 18 and 30%: high; and more than 30%: very high. Observed aneuploidy levels after diuron exposure are not so high when comparing with some other studies where aneuploidy levels could exceed 30%, although this was observed in slow-growing juveniles (Thiriot-Quiévreux *et al.* 1992). The oysters used in this study had about the same size and grew very well. With an exposure to 10 μg L^{-1} of atrazine (Bouilly *et al.* 2003), observed aneuploidy was at the same level as in that study. The number of aneuploid cells with one missing chromosome was higher than the ones with two or three, which could be related to a lower viability of cells with only 17 or 18 chromosomes. This study is the first reported evidence of an effect of diuron, or its derived products, on aneuploidy in a bivalve. In mammalian cells, a diuron degradation product (3,4-dichloroaniline), may induce aneuploidy by interacting with the mitotic apparatus (Bauchinger *et al.* 1989). The concentration used in treatment 2 (300 ng L^{-1}) is equivalent to that found in polluted environments. It is close to the peak value found in water from the river Vilaine (Férel, Morbihan, France), which was 440 ng L^{-1} in August 2003. The highest environmental values that have been reported were found in estuary and harbor waters, 700 ng L^{-1} and 6740 ng L^{-1}, respectively (Readman *et al.* 1993; Thomas *et al.* 2001). Open estuaries usually have lower environmental concentrations than harbors, because of higher water exchange (Thomas *et al.* 2001).

The exact mechanisms of how pesticides induce aneuploidy are not well characterized. Diuron may potentially interact with a variety of cellular targets whose modification may lead to aberrant chromosome segregation. Targets for aneugenic interactions potentially include all the components of the cell cycle (Parry and Sors 1993), and thus metabolite interactions may occur throughout it. The synthesis and formation of the microtubules and the formation of the division-spindle are of central importance in the segregation of chromosomes (Bond 1987), and hence they are potential targets for aneugenic effects of chemicals (Parry *et al.* 1996). Modifications in the synthesis, division, and functioning of the centrioles and polar bodies or in the assembly and functioning of the kinetochore proteins and the centromeric DNA may also induce aneuploidy (Parry *et al.* 1996).

In our study, diuron did not have a significant effect on the mortality of *C. gigas*. Mortality rates were low for all three treatments. Although the mortality rate in one batch of treatment 3 was higher, this was probably due to zootechnical problems. Tanguy *et al.* (2005) observed no mortality when they treated oysters for 4 weeks with a mixture of three herbicides (2 g L^{-1} atrazine, 0.5 g L^{-1} diuron, and 1 g L^{-1}

isoproturon), even though the pollutant concentrations were much higher than those used in the present study. This agrees with results from other experiments (see review in Giacomazzi and Cochet 2004) that showed that diuron only had lethal effects on fish and aquatic invertebrates in acute exposure episodes with higher concentrations (1–42 mg L^{-1}).

Although numerous studies have examined the effect of pesticides on hemocyte parameters, little work has been done on the effects of diuron in bivalves. Diuron induced no modification of the hsp70 promoter, a stress indicator, in human cells (Aït-Aïssa *et al.* 2000). However, hemocyte aggregation decreased in *C. gigas* when diuron (1 μg L^{-1}) was tested in a mixture with other herbicides (atrazine and isoproturon) (Auffret and Oubella 1997).

Most herbicides act on photosynthesis, and both atrazine and diuron have the same mode of action on plant metabolism. They inhibit photosynthesis by reversibly binding to the Q_B binding site on the D_1 protein (often called the ''herbicide-binding'' protein), thus inhibiting electron flow (see review by Oettmeier 1992). However, this does not suggest that herbicides would act directly on hemocyte parameters, and a previous study reported that atrazine had no effect on them (Gagnaire *et al.* 2003). In the present study, diuron increased all the hemocyte parameters studied (cell mortality, granulocyte percentage, ROS production, lysosome-positive cells, and phagocytosis), although effects were only detectable after 4 weeks of contact. Oysters may be able to respond to diuron exposure by increasing values of hemocyte parameters. We may hypothesize, therefore, that if hemocyte parameters are modulated, defense capacities may also be altered. After 11 weeks, diuron seemed to have no effect on hemocyte activity, suggesting that the oysters had acclimated to diuron. This kind of response has already been reported for other pollutants (Larson *et al.* 1989). Hemocyte parameters may recover in the long term, suggesting that mechanisms of cell restoration may possibly be at work (Russo and Lagadic 2004). Laboratory conditions could also induce a stress on hemocyte parameters, as observed after a cadmium contamination in laboratory conditions (Bouilly *et al*. 2006). Hence, variations were observed in control and contaminated oysters during the whole experiment (data not shown).

The investigation of the persistence of diuron impact on aneuploidy in the Pacific oyster is of particular importance because diuron may cause irreversible damage to genetic material. Considering the persistence of diuron in habitats adjacent to application areas, and the fact that diuron is applied preferentially in the spring, pollution could negatively impact aquatic organisms during their breeding periods. It was therefore important to study the aneuploidy level of oyster offspring. We examined the offspring of the adult oysters exposed (or not) to diuron for 11 weeks, during which gametogenesis was taking place, and observed a significantly higher aneuploidy level in those bred from parents exposed to diuron compared with those bred from the control. Statistical analysis revealed no significant difference in aneuploidy level between parents and offspring in our study. The impact of diuron on Pacific oyster aneuploidy and the level of this damage therefore persist to the next generation. The same result has been observed with atrazine exposure (Bouilly *et al.* 2004). These herbicides may have a similar mode of action on the genome.

In our study, the presence of diuron in the environment of the parents did not have an effect on the hatching rate of offspring or on larval growth. In previous other studies, however, diuron did affect survival and growth in amphibian embryos and tadpoles (Schuytema and Nebeker 1998), cladocerans, amphipods, midges, minnows, worms, and snails (Nebeker and Schuytema 1998), although these effects were observed at concentrations higher than those used in field applications. Reproductive success of oysters exposed to diuron and condition of the offspring did not appear to be significantly affected. Thus, no physiological impairments were observed during the maturation of oysters and gamete growth. No physiological study was performed during the experiment, but Photosystem II herbicides (including diuron) appear to have little direct effect on invertebrate physiology (Jones 2005); thus, this effect can be expected to be the same in oysters. Diuron has a low but measurable toxicity to aquatic invertebrates (see reviews in Jones 2005; Giacomazzi and Cochet 2004).

Our study showed that diuron worsened aneuploidy in *C. gigas* and that effects persisted to the next generation, indicating that this herbicide may cause irreversible cytogenetic damage. Diuron also had an impact on hemocyte parameters after 4 weeks of exposure, but this appeared to be reversible, because after 11 weeks of exposure these effects were no longer observed. However, at the tested concentrations, the diuron exposure response does not seem to be biologically relevant because parameters such as mortality, hatchering rate and larval growth were not affected.

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