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Study of genetic damage in the japanese oyster induced by an environmentally-relevant exposure to diuron: evidence of vertical transmission of dna damage

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Abstract:

Pesticides represent a major proportion of the chemical pollutants detected in French coastal waters and hence a significant environmental risk with regards to marine organisms. Commercially-raised bivalves are particularly exposed to pollutants, among them pesticides, as shellfish farming zones are subject to considerable pressure from agricultural activities on the mainland.
The aims of this study were to determine (1) the genotoxic effects of diuron exposure on oyster genitors and (2) the possible transmission of damaged DNA to offspring and its repercussions on oyster fitness. To investigate these points, oysters were exposed to concentrations of diuron close to those detected in the Marennes-Oleron Basin (two 7-day exposure pulses at 0.4 and 0.6 µg L^{-1}) during the gametogenesis period. Genomic abnormalities were characterized using two complementary approaches. The Comet assay was applied for the measurement of early and reversible primary DNA damage, whereas flow cytometry was used to assess the clastogenic and aneugenic effect of diuron exposure. Polar Organic Chemical Integrative Samplers (POCIS) were used in exposed and assay tanks to confirm the waterborne concentration of diuron reached during the experiment.
The results obtained by the Comet assay clearly showed a higher level of DNA strand breaks in both the hemocytes and spermatozoa of diuron-exposed genitors. The transmission of damaged genetic material to gamete cells could be responsible for the genetic damage measured in offspring. Indeed, flow cytometry analyses showed the presence of DNA breakage and a significant decrease in DNA content in spat from diuron-exposed genitors. The transmission of DNA damage to the offspring could be involved in the negative effects observed on offspring development (decrease in hatching rate, higher level of larval abnormalities, delay in metamorphosis) and growth.
In this study, the vertical transmission of DNA damage was so highlighted by subjecting oyster genitors to short exposures to diuron at medium environmental concentrations. The analysis of POCIS showed that oysters were exposed to integrated concentrations as low as 0.2 and 0.3 µg L^{-1}, emphasing the relevance of the results obtained and the risk associated to chemical contamination for oyster recruitment and fitness.

Highlights

► Oyster genitors were exposed to environmental concentrations of diuron during gametogenesis. ► A genotoxic effect was observed in hemocytes and spermatozoa of diuron-exposed genitors. ► DNA damage in spermatozoa could be involved in the negative effects observed on oyster recruitment. ► Vertical transmission of DNA damage was evidenced by DNA aneuploidy in spat from exposed genitors.

Keywords : Herbicide ; Diuron ; Oyster ; Genotoxicity ; Comet assay ; Cytogenetics

1. Introduction

Coastal ecosystems are exposed to various forms of pollution from human activities. Over 80% of marine pollution comes from the mainland and originates from industrial, agricultural and urban activities (UNEP, 2004; SOES, 2011). France is the leading user of agrochemicals in Europe and the third in the World (Jacquet et al., 2011; UIPP, 2012). As a result of this high consumption of phytosanitary products, pesticides can contaminate coastal waters through various processes, including run-offs, leaching and spray drift. Some pesticides are notoriously toxic; indeed, out of the 41 priority substances identified by the EU Water Framework Directive (Directive 2008/105/EC), 14 are pesticides. Their presence in coastal waters is therefore a potential major environmental hazard for marine organisms.

The ecological characteristics of mollusks, and in particular bivalves and gastropods (filter feeders, sessile mode of life and ability to bioaccumulate pollutants), makes them highly sensitive to chemical stresses. They are therefore considered as reliable species for the study of pollutant effects in the marine environment (His et al., 1999). Moreover, the Pacific oyster *Crassostrea gigas* is one of the foremost aquaculture resources on a worldwide level (FAO, 2011). *C.gigas* has been farmed in France since the late 1970s. France is currently Europe's leading producer, with 82,800 metric tons in 2010/2011, 20,000 of which (24%) were farmed in the Poitou-Charentes region alone (CNC, 2012). High mortality rates for this species have been observed in summer for several years; this phenomenon does not appear to be due to a single pathogenic cause, but a combination of several abiotic (physico-chemical environmental parameters, pollution and aquaculture practices) and biotic factors (e.g. pathogens and physiological status of the oyster) (Samain et al., 2007).

In the main French farming basins where Pacific oysters reproduce naturally, flow cytometry analysis performed in wild spat within a biomonitoring network highlighted a DNA aneuploidy rate as high as 20% (Benabdelmouna et al., 2011). Inputs of pollutants such as pesticides have already been detected in the Marennes Oleron basin in Spring due to rainfall events during the highly sensitive period of oyster gametogenesis (Munaron et al., 2003; Munaron, 2004; Burgeot et al., 2008). This latter observation suggest that the DNA hypodiploidy detected in production areas may be linked to genotoxic pollutant discharges in water. As oysters have been shown to be particularly vulnerable to physiological and environmental stressors during the reproduction period, pollutants could have a direct genotoxic effect on the genitors themselves, together with an indirect genotoxic effect on offspring due to the exposure of parental germ cells.

Genotoxicity is common to several families of major environmental pollutants. Pollutant genotoxicity has been well-investigated in marine invertebrates and in particular in oysters and mussels (Bouilly et al., 2003; Wessel et al., 2007; Marcheselli et al., 2011; Akcha et al., 2012; Dallas et al., 2013). Exposing organisms to genotoxicants can result in reversible and irreversible DNA lesions. Primary DNA lesions, such as single or double-strand breaks, DNA base modifications and DNA adducts can be rapidly overcome by the cellular DNA repair machinery (Mateuca et al., 2006). Conversely, irreversible chromosomal damage may result in perturbations of cell division leading to chromosomal aberrations and variations of nuclear DNA content. In addition to somatic cells, gametes have been shown to be particularly sensitive to genotoxicant exposure (Speit et al., 2009; Favret and Lynn, 2010; Akcha et al., 2012; Lewis and Ford 2012). Despite nuclear DNA damage, male gametes can nevertheless contribute to the following generation (Lewis et Galloway, 2009; Devaux et al., 2011). The parental transmission of pollutant-induced DNA damage further to exposure to herbicides and cadmium has already been demonstrated in oysters in terms of aneuploidy (Bouilly et al., 2004, 2007). Moreover, various phenotypic consequences have been associated with the transmission of DNA damage in terms of developmental abnormalities (Lewis and Galloway, 2009; Devaux et al., 2011; Lacaze et al., 2011; Santos et al., 2013). In oysters, aneuploidy has been shown to be negatively correlated with growth rate and survival rates during summer mortality in adults by karyotyping (Leitao et al., 2001; Wilson et al., 2002), and spat by flow cytometry (Benabdelmouna et al., 2011), respectively.

In this study, oyster genitors were exposed to diuron during gametogenesis with the aim of studying the parental transmission of herbicide-induced DNA damage and its potential consequences on oyster physiological fitness. Diuron is the most widely-found biocide in antifouling paints and one of the WFD's priority pollutants. Although it has been banned in France since 2008 (JO n°204 September 4, 2007), diuron is still found in high concentrations in coastal waters (Munaron et al., 2012; Caquet et al., 2013). Moreover, diuron has already been reported as having diverse toxic effects on oysters, including somatic and gamete cell genotoxicity (Bouilly et al., 2007; Wessel et al., 2007; Akcha et al., 2012; Mai et al., 2013), embryotoxicity (Akcha et al., 2012; Mai et al., 2013), and immunotoxicity (Gagnaire et al., 2006, 2007; Luna-Acosta et al., 2012).

For this purpose, the extent of genomic abnormalities was analyzed in genitors, their gametes, larvae and spat for the detection of somatic and/or heritable genetic effects using two complementary approaches. The Comet assay was applied to detect early reversible primary DNA damage, while flow cytometry (FCM) was used to measure the prevalence of abnormal

genomic profiles (variability in CV and genome size). In marine invertebrates, flow cytometry has been widely used to highlight genomic abnormalities (DNA breakage and DNA ploidy) (Barsiene et al., 2006; Goanvec et al., 2008; Jung et al., 2011; Theodorakis et al., 2012) and is recognised as a high precision technique (Bihari et al., 2003). Moreover, it has an advantage over other cytological assays because a larger number of cells can be analysed rather than in chromosome or micronucleus assay (Deaven, 1982; Bickham, 1990), allowing a rapid analysis of a large number of samples.

The presence of DNA damage in the spermatozoa of diuron-exposed genitors was also assessed in order to investigate the potential links between damaged DNA transmission and embryo-larvae development and growth, up to spat stage.

2. Materials and methods

2.1 Chemical reagents

Diuron (Pestanal, analytical standard), acetonitrile (anhydrous, 99.8%), NaCl, Trizma base, DAPI, Dimethyl sulfoxide (DMSO), normal and low melting point agarose, Triton X-100, fetal calf serum, and GelRead were purchased from Sigma Aldrich Chemicals (France). $MgCl_2$-$6H_2O$ was purchased from Euromedex. DNA reference calibrator (Trout Red Blood Cells, TRBC) was purchased from Beckman Coulter (France).

Pesticides analytical standards including deuterared pesticides used as internal standards were obtained from Cluzeau Info Labo (Ste-Foy-la-Grande, France; purity > 96%). Dichloromethane and methanol (HPLC reagent grade, Scharlau) were obtained from ICS (Belin-Beliet, France). Ultrapure deionised water was prepared using a Milli-Q system (Millipore, Molsheim, France).

All POCIS (Polar organic chemical integrative samplers) used were pharmaceutical POCIS with PRC (Performance Reference Compounds). Each POCIS contained 200 mg of Oasis® HLB bulk sorbent (60 µm) (Waters, Guyancourt, France) enclosed between two hydrophilic polyether sulfone (PES) membrane disc Filters (0.1 µm, 90 mm membrane diameter) (VWR, Fontenay-sous-Bois, France) and held together with stainless steel rings (hole diameters = 54 mm). Selected performance reference compounds (d5-DIA) were added into the sorbent of POCIS prior to exposure at a concentration of 10 µg g^{-1} (Mazzella et al., 2010).

2.2 Genitor origin and oyster husbandry

The adult Pacific oysters (*Crassostrea gigas*) used for this experiment originated from wild genitors collected in the Marennes-Oléron Bay (France). They were used in the framework of a selection program aimed at producing oyster families with an improved resistance to summer mortality. These oysters showed an average survival rate during mortality periods. For our experiment, they were transferred from the field to the hatchery for a one-month acclimatization period. Seawater was pumped directly from the Seudre river Estuary, filtered through a sand filter (40 µm) and passed through UV rays before draining the tanks in a continuous flow system (output : 55L h^{-1}). Good seawater oxygenation was hence provided by circulation, coupled with an additional bubbling system. Oyster feeding was controlled: oysters were fed daily with a mixture of four marine microalgae (*Skeletonema costatum:* $0.6x10^6$ cellules mL^{-1}, *Isochrysis galbana:* $4x10^6$ cellules mL^{-1}, *Tetraselmis suecica:* $0.8x10^6$ cellules mL^{-1}, *Chaetoceros gracialis:* $4x10^6$ cellules mL^{-1}). Water temperature was maintained at 8°C (± 1°C) throughout the acclimatization period. In order to initiate the oyster gonad maturation process, the temperature was then raised by two degrees per day for 1 week, to reach 19.8°C (± 0.3°C) at the start of the experiment.

2.3 Diuron exposure during oyster gametogenesis

Once gonad development had begun, the oysters were divided into three experimental groups: a seawater control, a solvent control and a diuron-exposed group. As diuron was prepared in acetonitrile, the solvent control group was exposed to acetonitrile at a concentration of 0.005%.

Three 250-L tanks were used for each experimental group, each receiving 240 oysters. Two 7-day exposure periods took place at the start and mid-course of gametogenesis by exposing oysters to 0.4 and 0.6 µg L^{-1} of diuron respectively. These short exposure pulses were chosen to mimic rain events with concentrations of diuron close to those detected in coastal waters (Table 1).

The oysters were maintained in a closed circulation system throughout the exposure period. The seawater was changed every morning and diuron and acetonitrile were added every day after seawater renewal to reach target concentrations in the tanks. The temperature was kept stable at 20°C and feeding was controlled as described previously. Mortality was assessed for the all duration of the experiment in each experimental tank.

It is noteworthy that mortality was very high in some tanks (up to 40% in control tanks) by the end of the first pulse of herbicide exposure. All moribund oysters displayed high bacterial

loads with, in particular, the presence of *Vibrio aestuarianus*: a pathogenic bacteria with high virulence for *Crassostrea gigas.* Some strains can induce a mortality rate of about 90% in oysters at seven days post-injection (Labreuche, 2006). In order to cope with this bacterial infection and avoid the total loss of the biological material, all oyster batches were subjected to a 5-day antibiotic treatment (10 mg L^{-1} of Flumequine). At the end of the treatment, the oysters were then allowed to recover for 11 days prior to the second herbicide pulse.

At the end of the second herbicide pulse, oysters from each group were then grown in normal conditions for an additional period of three weeks in order to reach maturity and allow production of the next generation (Fig. 1).

2.4 Gamete handling, fertilization, and larval and spat culture

At the end of oyster gametogenesis, crosses were performed using genitors from the same experimental group (n=70 oysters per group). Spawning was induced in males and females by thermal shock (from 18°C to 28°C for 30 min.). The number of spawning males and females was 24 and 45, 11 and 36, 20 and 32 for the seawater control, solvent control and diuron-exposed groups respectively. Gametes from each individual were filtered on a 45 µm sieve for males and a 75 µm sieve for females, then suspended in 1 µm filtered sea water (FSW) at 25°C. Oocytes and spermatozoa from each individual male and female were then pooled. For each experimental group, 9 000 000 oocytes were used and fertilization was achieved with a ratio of 200 spermatozoa per oocyte.

One hour post-fertilization, the embryos were divided into three replicates (n= 3 000 000 embryos per tanks) and transferred to 30-L glass fiber tanks filled with 1 µm FSW at 22-24°C. They were grown for 24 h up to D-larvae at a density of 100 larvae mL^{-1}. The tank water was changed every two days from this development stage onwards; the larvae were reared until metamorphosis was achieved at a temperature of 24-25°C and fed with a mixture of algae (*Isochrysis galbana, Chaetoceros gracilis, Skeletonema costatum*). Larvae density was assessed by means of microscope counts for each batch to further limit competition: larval concentration was progressively reduced from 10 to 5 and 3 larvae mL^{-1} on day 1, 5 and 7 post-fertilization respectively. At the eyed larvae (pediveliger) stage, the larvae were ready to settle; they were then transferred to the micronursery into 150 µm sieve-bottomed trays in a FSW downweller system at 20°C, using oyster shell cultch as a settlement medium. The sea water was enriched every day with a mixture of four algal species routinely produced at the hatchery (*Isochrisis galbana, Chaetoceros gracilis, Tetraselmis suecica,* and *Skeletonema costatum*). The sieves were washed daily and changed regularly depending on spat growth.

2.5 Sampling program

Sampling was performed at various stages of the oyster life cycle during the course of the experiment. The genitors exposed during gametogenesis were sampled at the beginning and end of the two herbicide pulses and at spawning time, when gametes from each genitor groups (controls and diuron-exposed) were also analysed. In order to study putative vertical effects on the next generation, larvae and spat originating from each genitor group were also sampled as described in Figure 1. The number of individuals and type of tissue/fluid sampled for each type of chemical and biological analysis are shown in Table 2.

2.6 Chemical analysis of seawater using Polar Organic Chemical Integrative Samplers

Polar organic chemical integrative samplers (POCIS) are used for the detection of hydrophilic pollutants ($1 < \log K_{ow} < 4$) such as pesticides, pharmaceutical substances, detergents and hormones. POCIS allow sampling of the dissolved water fraction that is thought to be bioavailable for living organisms over period of several weeks, allowing the determination of the Time Weighted Average (TWA) concentration of a substance. In our study, they were used in two aims, (1) to assess the chemical quality of seawater used at the hatchery and, (2) to obtain an integrated measurement of the concentration of diuron reached during the pulses. For this purpose, one out of the three tanks used for the seawater control and diuron-exposed tanks were equipped with a deployment cage. Two sampling strategies were adopted. POCIS were used in duplicate during the all experiment duration, with some replacements to avoid fouling onto the membranes. They were also used during the 7 day-exposure pulses to measure the TWA of diuron reached in the exposed tanks during the pulse.

POCIS were used for the detection of diuron and its main metabolites (1-(3,4 dichlorophenyl)-urea known as dcpu; 1-(3,4dichlorophenyl)-3 methyl-urea known as dcpmu) and of 54 other pesticides: 1-(2,4dichlorophenyl)-urea, acetochlor, acetochlor ESA (ethane sulfonic acid), acetochlor OA (oxanilic acid), alachlor, amethryne, atrazine, atrazine 2 hydroxy, azoxystrobine, bentazone, carbendazime, carbetamide, carbofuran, carbosulfan, chlorotoluron, chlorsulfuron, cyanazine, cyromazine, desethyl atrazine (DEA), desisopropyl atrazine (DIA), diflufenican, dimetachlor, dimethylaminosulfanilide (DMSA), N,N-dimethyl-N'-p-tolylsulfamide (DMST), flazasulfuron, fluazifop-p-butyl, flusilazole, hexazinone, hydroxysimazine, imidaclopride, irgarol, isoproturon, linuron, metazachlore, methiocarb, metolachlore, metolachlore ESA (ethane sulfonic acid), metolachlor OA (oxanilic acid),

metoxuron, metsulfuron-methyl, nicosulfuron, promethrine, propachlore, propazine, propiconazole, prosulfuron, pymethrozine, quizalofop-ethyl, quizalofop-p-tefuryl, simazine, terbutrine, terbutylazine, terbutylazine desethyl, thiamethoxan.

POCIS extraction and analysis were performed as described by Tapie et al. (2011). Briefly, the sorbent from each POCIS was transferred into an empty glass solid phase extraction (SPE) tube by rinsing it with ultra-pure water, then vacuum-dried for 1 h. Internal standards (d13-alachlor, d5-atrazine, d3-carbofuran, d6-diuron, d6-hexazinone, d9-irgarol, d10-simazine, d5-terbutylazine) were gravimetrically added and the sorbent was eluted successively using 10 mL of methanol, 10 mL of a methanol/dichloromethane mixture (v/v: 50/50), and finally 10 mL of dichloromethane. The extract was concentrated first by using a vaccum evaporation system, dried under a stream of nitrogen and then dissolved in 150 mL of methanol for pesticides analysis by LC/MS/MS. The quantification of the gross mass of each chemical sequestered is expressed below as the concentration per POCIS in ng g^{-1} of sorbent. From these data, TWA concentrations (Cw, ng L^{-1}) can be derived, corresponding to the average concentration of a contaminant present in the water sampled during the exposure period of the sampler. These TWA concentrations are derived from the concentration of contaminant sequestered in POCIS (Cp, ng g^{-1}), from the exposure time (t, days) and from the sampling rate (Rs, L day^{-1} g^{-1}) of each contaminant assessed during a laboratory calibration and are bound by the following equation (Alvarez et al., 2004; Vrana et al., 2005):

$$Cw = Cp.\ Rs^{-1}.\ t^{-1}$$

2.7 Time-course of genitor growth

Genitor growth was monitored in each assay group throughout the experiment by measuring shell length and wet weight at each of the sampling points.

2.8 Measurement of primary structural DNA damage by the Comet assay

Analysis of genitor and spat hemocytes. The hemolymph of each individual was collected by punction from the posterior adductor muscle sinus using a sterile hypodermic needle (23 G) in a 2 mL syringe pre-rinsed with the anticoagulant Alsever's solution (113.7 mM glucose, 27.2 mM sodium citrate, 58.44 mM sodium chloride, pH 6.1). A volume of Alsever's solution (0.1 mL) remained in the syringe to achieve a 1:5 dilution of the withdrawn hemolymph. The hemocytes were recovered by centrifugation (1500 rpm, 5 min) and the hemocyte pellet was resuspended in 1 mL of freezing medium (55 % RPMI 1640 medium, 25 % fetal calf serum,

20% DMSO) and stored in liquid nitrogen prior to analysis. Suspension in freezing medium and storage in liquid nitrogen was already performed and validated in hemolymph and gill cells of the blue mussel (Akcha et al., 2004). The samples were slowly defrosted at room temperature prior to the Comet analysis. Two slides were prepared for each sample and the Comet assay was conducted as previously described (Akcha et al., 2003; Wessel et al., 2007). DNA migration was performed for 15 min at 23V (390 mA, E=0.66Vcm^{-1}). At the end of electrophoresis, the slides were washed by incubation for 3×5 min in Tris base 0.4 M, pH 7.5. In order to obtain permanent preparations, the slides were immersed in absolute ethanol for 10 min to dehydrate, then dried at room temperature. Just prior to analysis, 75 µL of GelRed at 8 mg L^{-1} were spread over each slide using a cover glass. The slides were placed for at least 1 h in the dark at 4°C for coloration, then analysed using an optical fluorescence microscope (Olympus BX60, ×40) equipped with a CDD camera (Luca-S, Andor Technology) and image analysis system (Komet 6, Kinetic Imaging Ltd.). At least 50 nuclei were analysed per slide and the percentage of DNA present in the Comet tail (% Tail DNA) was measured for each observed nucleus.

Analysis of genitor spermatozoa. The semence from 18 males (6 per tank) was analysed individually in each assay group. At the outset of gamete emission, each identified male was isolated in a beaker containing 250 mL of FSW. Dilution in CMFS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 5.5 mM glucose) was performed (1:2 to 1:10) depending on the concentration of the sperm sample. The Comet assay was performed on fresh samples, as described previously in this paper for genitor hemocytes. Following agarose embedding, a prior digestion step by proteinase K was not added to the protocol to decondense the DNA as already performed in human sperm by other authors (Morris et al., 2001). As a matter of fact, the Comet assay was previously validated without a decondensation step in oyster sperm following exposure to the model direct genotoxicant, hydrogen peroxide (Akcha et al., 2012). In fish such the three-spined stickleback, the Comet assay was also applied with success to assess sperm DNA damage without proteinase K digestion (Santos et al., 2013).

2.9 FCM analyses of nuclear DNA damage

FCM analyses were performed individually on genitors and spat. Regarding genitors, the same individuals (identified by marking) were monitored throughout the experiment by anesthesia, whereas spat sampling was destructive. In both cases, nuclei were extracted from small pieces (1mm^2) of gills, mixed with 2 µl trout red blood cells (TRBC, Coulter DNA

Reference Calibrator, 629972) as internal standard solution, then stained with DAPI at a concentration of 2 µg mL^{-1} in 2 mL final volume. At least 2,000 nuclei were analyzed for each sample. FCM was performed on a PARTEC CyFlow ML with the following features: excitation-365 nm UV LED, UG 1 (290–410 nm, 3 mm), chromatic beam splitter (TK 420), emission-beam splitter (TK 420, TK 560) and barrier filter (CG 455) for DAPI signals. Peak positions and coefficients of variation (CVs) were calculated automatically (PARTEC PAS II software package). In this study, FCM analyses of genome size variation meant that changes in the number or composition of individual chromosomes were not measured directly by karyological analyses. Therefore, as suggested by Suda et al. (2006), classical cytogenetic terminology was preceded by the prefix "DNA" (DNA-ploidy level, DNA-hypo/hyperdiploidy, DNA-polyploidy, etc.). The DNA index is used to estimate DNA ploidy level variations (DNA aneuploidy and polyploidy) by measuring mean nuclear DNA content (genome size). CV is used to measure intra-individual genome size variability, in order to assess clastogenic effects (presence of different cell populations with different amounts of DNA breaks within an individual). High CVs have previously been shown to be associated with cell populations with varying DNA contents, most commonly resulting from chromosomal aberrations caused by experimental or environmental exposure to clastogenic agents (Lamb et al. 1995; Lowcock et al. 1997; Bickham et al. 1998).

For the purpose of our study, only samples with internal standard cells showing a CV below 3% were considered for subsequent analyses. The ratio between the respective positions of sample and TRBC G0/G1 peaks is characteristic of sample DNA ploidy level. In our laboratory conditions, this DNA index was equal to 0.42 (+/-5%) for diploids. Values below this ratio indicate a decrease in genome size (DNA hypodiploidy), whereas values above this ratio indicate an increase in genome size (DNA hyperdiploidy).

2.10 The effects of parental exposure on offspring development and growth

Hatching rate refers to the percentage of embryos reaching the D-larvae stage twenty-four hours after fertilization, according to the initial number of oocytes used for fertilization. All larvae were collected from each tank by sieving on a 45 µm nylon mesh, then placed in a graduated beaker. After homogenization, the larvae were counted using a binocular microscope (Olympus BX41, x100). At this stage, all larvae that were not D-shaped were considered as abnormal. Larvae samples were placed at a concentration of 50,000 individuals per liter of FSW and fixed using 50 µl of 8% neutralized formalin solution. The percentage of abnormal D-shell larvae was scored out of 3x100 individuals in each tank. Abnormalities (D-

larvae presenting mantle and/or shell abnormalities) were determined according to the criteria given by His et al. (1999) and Quiniou et al. (2005). To further characterize developmental abnormalities, fertilized oocytes from each assay group were saved in beakers at a concentration of 50,000 per liter prior to transfer to 30-L glass fiber tanks, in order to perform a standardized embryo-larval bioassay in accordance with French standards (AFNOR XP-T90-382, September 2009).

Larvae growth was then monitored in relation to different size classes (45-60-70-85-100-125-150-180-220µm) up to the spat stage.

2.11 Statistical analysis

Statistical tests were conducted using STATISTICA (StatSoft, Inc. (2011), version 10). Normality was checked using Lilliefor's test and variance homogeneity was evaluated using Bartlett's test. In order to achieve normality, raw data from the Comet assay were mathematically transformed (Ln x) before proceeding with an ANOVA, taking into account both the experimental group and sampling time as factors. ANOVA was also used to handle developmental abnormality data. When significant, a *posteriori* Tukey test was performed. Data on growth, hatching rate and flow cytometry could not be normalized, statistical differences between treatments were tested using the non-parametric Kruskal-wallis test.

3. Results

3.1 Seawater contamination

Background pesticide contamination of the seawater used at the hatchery . The analysis of the POCIS from the control tank provided information on the background level of pesticide contamination of the seawater used at the experimental hatchery. The results obtained are summarized in Table 3. They showed that out of the 57 pesticides analyzed, 36% were detected. Among the latter substances, metolachlor derivatives (metolachlor ESA and metolachlor OA) and DEA (desethyl atrazine) are those presenting the highest concentrations (close to 30 and 60 ng L^{-1} respectively). Of note, variations in the measured concentrations were observed between the two POCIS immersion periods for some of the detected substances. That was the case for DEA, which was detected at a concentration of 57 ng L^{-1} during the first 29 days of the experiment, but was present at 4 ng L^{-1} only during the second consecutive immersion period. During the experiment, the total pesticide concentration in the water from the hatchery was 0.15 and 0.08 µg L^{-1} for the two POCIS immersion periods.

Diuron was detected at a mean concentration of 77 and 62 ng L^{-1} that is nearly one order of magnitude lower than the concentrations tested in our study. None of its metabolites were detected.

Assessment of reached diuron concentration in assay tanks. Using the passive samplers, the diuron concentrations targeted during the experiment were shown to be around 20% and 49% lower than expected, respectively without and with PRC correction, e.g. oysters were exposed to 0.30 µg L^{-1} of diuron rather than 0.6 µg L^{-1} for the second herbicide pulse taking into account the data obtained after PRC correction (Table 4).

3.2 Effects of diuron exposure on genitors

3.2.1 Growth

At the end of the first herbicide pulse, the genitors exposed to diuron showed a significantly lower wet weight than those from the control groups, with values of 3.08 ± 1.01 g for diuron-exposed genitors versus 3.87 ± 1.27 g and 3.89 ± 1.10 g for genitors from the seawater and solvent control groups ($p<0.009$) (7 days at 0.4 µg L^{-1}). This lower wet weight was also observed at the end of the second herbicide pulse, with values of 3.35 ± 1.03 g for diuron-exposed genitors versus 3.98 ± 1.21 g (- 16 %) and 4.16 ± 1.28 g (- 19 %) for those from the seawater and solvent controls ($p<0.02$) (Fig. 2). No differences were observed in genitor growth in terms of shell length and width across the experimental groups ($p>0.05$).

3.2.2 DNA damage in hemocytes and gill cells

The results of the Comet assay revealed a significant increase in DNA strand breaks in the hemocytes of genitors exposed to diuron at the end of the first herbicide pulse ($p<0.001$). This increase was even higher at the end of the second pulse, reaching a mean percentage of 33% DNA in Comet tails, versus less than 20% in the controls ($p<0.001$). The amount of DNA strand breaks remained stable in the control groups, except at the end of the second herbicide pulse in the solvent control groups, when a slight increase was recorded ($p=0.01$) (Fig. 3).
Although primary DNA lesions were induced in diuron-exposed genitors, analysis of the data obtained by FCM showed no significant variations in the extent of chromosome breakage (CVs) and genome size in the gills of the same individuals. (Table 5).

3.2.3 DNA damage in male gametes

Male gamete analysis using the Comet assay showed a significantly higher level of DNA strand breakage in the spermatozoa of genitors exposed to diuron versus the controls ($p=0.001$) (Fig. 4). Despite a marked increase in DNA damage, the percentage of breaks in the Comet tail of spermatozoa nucleus remained below 10%.

3.3 Effect of parental diuron exposure on offspring

3.3.1 Hatching rate, embryo-larvae development and growth

Although no differences were found in fertilization success across the three groups, a significantly lower hatching rate was observed for offspring from genitors exposed to diuron: 42.4 ± 5.9% vs 79.3 ± 10.8% and 76.7 ± 12.1% for the seawater control and solvent control groups respectively ($p<0.005$) (Fig. 5). Moreover, a higher level of D-larvae abnormalities was observed in offspring from diuron-exposed genitors ($p<0.05$). Of note, the offspring of genitors exposed to acetonitrile also showed significant developmental abnormalities ($p<0.01$). The mean percentage of larval abnormalities was 20.8 ± 9.5 %, 38.9 ± 5.6% and 30.2 ± 5.2% respectively in offspring from seawater control, solvent control and diuron-exposed genitors (Fig. 5).

On the other hand, embryo-larval bioassay results revealed a significantly higher level of embryo-larvae abnormalities only in larvae originating from diuron-exposed genitors (18 ± 3 % versus 7 ± 3 and 6 ± 2 for larvae from the seawater and solvent controls).

A slower growth rate was only observed in larvae from genitors exposed to diuron. In the control groups, ready-to-settle pediveliger larvae were obtained after 20 days of rearing, whereas 24 days were required to reach the same stage for larvae from diuron-exposed genitors (Fig. 5).

3.3.2 DNA integrity of offspring

The DNA integrity of spat from the various experimental groups was analysed using the Comet assay to check for the parental transmission of diuron-induced DNA damage. No difference in the level of DNA strand breaks in spat hemocytes was observed across the various batches originating from the two control groups and diuron-exposed genitors ($p=0.23$) (Fig.6).

Conversely, FCM analysis showed both clastogenic and aneugenic effects in the gills of spat from diuron-exposed genitors: the nuclei isolated from gill tissue had significantly higher CVs

versus the controls, indicating more extensive clastogenic effects ($p< 10^{-6}$). The mean coefficient of variation was 5.72 ± 1.11 in spat from diuron-exposed genitors vs 4.58 ± 0.49 and 4.54 ± 0.49 in spat from the seawater and solvent controls respectively. Additionally, 30% of individual spat originating from diuron-exposed genitors showed high CVs (at least twice the value obtained for the internal standard TRBC), with values ranging from 6 to 11.5 %. In contrast, only 2% of spat from the control groups showed such high CVs, with a maximum value of 7.08% (Fig.7).

Moreover, a significant decrease ($p< 10^{-6}$) in nuclear DNA content was also measured in spat from diuron-exposed genitors (0.413 ± 0.020), with over 15% of individuals showing DNA hypodiploidy with a DNA index lower than the euploid DNA index of 0.400. Hypodiploid individuals accounted for just 2% in the control groups (0.427 ± 0.009 and 0.423 ± 0.008 respectively for the seawater and solvent control groups). The lowest DNA index values were 0.39, 0.38 and 0.34 for offspring from seawater control, solvent control and diuron-exposed genitors respectively (Fig. 7)

4. Discussion

4.1 Seawater contamination

Background pesticide contamination of the seawater used at the hatchery. POCIS are generally used to assess the contamination of water systems by hydrophilic substances. In this paper, their results initially provided information on the background level of contamination of the seawater used at the hatchery. In fact, UV treatment and filtration are not expected to eliminate the pollutants drained by Seudre River. The background contamination of the water used at the hatchery appeared low considering the few data available on the detection of pesticides in coastal water by the use of POCIS (Munaron et al., 2012, Alvarez et al., 2013). In the study from Munaron et al. (2012), POCIS were used to assess pollution by pharmaceuticals, alkylphenols, herbicides and biocides in the French Mediterranean coast. Among the herbicides analysed, diuron was the only one detected in all the samples and at concentrations as high as 30 ng L^{-1}, the other detected herbicides (terbuthylazine, atrazine, irgarol, simazine) being at concentrations lower than 2.5 ng L^{-1}.

In the present study, the use of POCIS also allowed us to show variations in water concentrations of some of the detected herbicides that are thought to result from irregular discharges of these molecules into the water, due to both agricultural practices and weather conditions. Metolachlor metabolites and DEA were the herbicides found at the highest

concentrations. Metolachlor is a member of the chloroacetanilide herbicide family, widely used for maize and sorgho treatment. It was banned in France in 2003, but its active isomere S-metolachlore is still authorized. ESA and OA metolachlor metabolites could originate from metolachlor itself or from S-metolachlore degradation. DEA is a residue of atrazine, which was banned in France in 2001. However, atrazine has a high remanence and is still regularly detected in its parent and residue forms in surface and coastal waters.

Assessment of reached diuron concentration in assay tanks. Diuron concentrations in the assay tanks at the end of the herbicide pulses were determined using POCIS and a diuron loss of up to 49% was observed. As previously demonstrated by Luna-Acosta et al. (2012), a 19% diuron loss due to volatilization or adsorption on tank walls and oyster shells can be achieved by exposing oysters to 1 µg L^{-1} of diuron for 24 hours. Diuron is fairly persistent in seawater and is not expected to degrade, as highlighted by the absence of diuron metabolites in water. Thomas et al. (2002) reported no degradation of diuron in seawater at 15°C over a 42 day-period.

In our study, oysters were exposed to even lower concentrations than expected. Our initial target concentrations were medium environmental concentrations (Table 1), hence highlighting the relevance of the effects observed on oyster DNA. Due to the general pollution of the water systems, the presence of pollutants at trace levels in the water used at the hatchery was shown by the use of POCIS. As a consequence, it can't be excluded the possibility of background pollutants contributing to the toxic effects reported in the present study. However, differences with seawater control groups were sufficiently obvious to allocate most of the toxic effects observed in oysters to diuron.

4.2 Genotoxicity assessment in genitors

Oysters used for our experiment were wild genitors and so naturally exposed to microorganisms present in the environment. There is nowadays no possibility to use germ free oysters. Some of these microorganisms are pathogenic for the oysters such as the bacteria *Vibrio aestuarianus*, those pathogenicity was revealed when increasing the temperature to initiate gonad maturation. It is noteworthy that the flumequine treatment performed between the two herbicide pulses to eradicate the bacterial infection could have led to the production of ROS-induced DNA damage as previously observed in other organisms (Kashida et al., 2002). However, flumequine is known to be rapidly eliminated as demonstrated in the sea bass (Rigos et al., 2002). Moreover, the oxidative damage induced by this molecule in terms of

DNA strand breaks have been shown to be rapidly formed and repaired in rodent (Kashida et al., 2002). The 11 day between the end of the flumequine treatment and the beginning of the second herbicide pulse is so expected to be sufficient to turn back to basal DNA damage level. The absence of variation in the level of DNA strand breaks in the control groups following flumequine treatment seemed to confirm this point.

In our study, the Comet assay revealed significant increases in DNA strand breaks in genitor hemocytes further to diuron exposure. Similar genotoxic effects of diuron have already been demonstrated using the Comet assay in adult European topminnow (*Phoxinus phoxinus*) exposed to chronic environmental concentrations of diuron (1–2 µg L^{-1}) and axoxystrobin (0.5–1 µg L^{-1}) (Bony et al., 2008). DNA strand breaks in diuron-exposed organisms could be associated with oxidative stress induction. Indeed, Bouilly et al. (2007) highlighted an increase in reactive oxygen species (ROS) production in oyster hemocytes following a 4-week exposure to 0.3 and 3.0 µg L^{-1} of diuron. Moreover, diuron appears to decrease also the activities of antioxidant defenses: a significant decrease in the activity of the antioxidant enzyme superoxide dismutase was already observed in the oysters further to 6 and 24h exposure to 1 µg L^{-1} of diuron (Luna-Acosta et al., 2012). The oxyradicals produced by diuron-exposure in oysters could hence act as chemical nucleases for the DNA, resulting in DNA strand breakage detectable by the Comet assay. The mechanisms involved in ROS production haven't been identified yet for diuron but it may be linked with its biotransformation. Oysters are seemigly capable of biotransforming diuron, as suggested by the low bioconcentration factor (BCF) of diuron in oyster. The latter is reported to be as low as 17 and 7 in the paper from Luna-Acosta et al. (2012) and Buisson et al. (2008) respectively, highlighting the low ability of oyster to bioaccumulate diuron probably due to metabolic activities.

Although diuron-induced primary DNA lesions were observed in genitor hemocytes, no evidence of clastogenic or aneugenic effects was observed in gill cells using flow cytometry. For a direct comparison, it would have been better to measure both genotoxicity biomarkers in the same tissue cell types. As a matter a fact, DNA strand breaks were measured in hemocytes by the Comet assay whereas clastogenic and aneugenic effects were analysed by flux cytometry in gill cells. Cytometry analyses were also performed in hemocytes but the results obtained were difficult to interprete due to the different cell populations present in oyster hemolymph. On the other hand, the application of the Comet assay on gill cells requiered a dissociation step that can be fastidious to operate when handling a lot of samples.

In the present study, despite primary DNA damage was observed in hemocytes, no damage was measured by flow cytometry in gill cells that are known to be particularly sensitive to genotoxicant exposure (Akcha et al., 2000) and so target cells to assess DNA damage. This difference in results can be explained by the fact that flow cytometry is dedicated to the measurement of more severe DNA damage than the Comet assay. In fact, Bouilly et al. (2007) showed diuron to significantly increase somatic aneuploidy (lowered chromosome number in a percentage of somatic gill cells) when oysters were exposed to concentrations of 0.3 µg L^{-1} and 3.0 µg L^{-1} but for a much longer exposure period of 11 weeks. Aneuploidy occurs when replicated chromosomes (or chromatids) fail to segregate properly during cell division, resulting in the production of cells with an abnormal number of chromosomes. Several molecular mechanisms are involved in the induction of aneuploidy by chemicals during the cell cycle, such as damage to the spindle apparatus (centrioles and kinetochores), effects on chromosome (fragmentation, condensation or stickiness), induction of chromosome interchanges, persistence of the nucleolus during mitosis or meiosis, alterations in ion concentrations during mitosis (e.g. Ca+) and damage to the nuclear membrane (Aardema et al., 1998). Pesticide aneugenic activity has already been investigated, with the induction of chromosome loss and non-disjunction observed in human lymphoblastoid cells further to trichlorfon exposure (Doherty et al., 1996). Trichlorfon has been shown to interfere with the mitotic spindle assembly by blocking cells in mitosis in vertebrates (Sun et al., 2000; Tian et al., 2000; Cukurcam et al., 2004). Data from Mattiuzo et al. (2006) also showed that dichlorvos can block cells in mitosis, possibly through interference with the mitotic spindle assembly.

In parallel to primary DNA alterations, a significantly lower growth (wet weight) was observed in genitors exposed to diuron versus the controls. Little data is available on the effect of diuron on growth, and the diuron concentrations tested were very high and not representative of those found in the natural environment. Effects on weight and growth have already been observed in worms (*Lumbriculus variegatus*) and snails (*Physa gyrina*) (Nebeker and Schuytema, 1998) when exposed to concentrations of 29.1 and 15.3 mg L^{-1} respectively. Embryos from *P. regilla* and *X. laevis* had a higher rate of developmental abnormalities and a reduced growth when exposed to diuron concentrations exceeding 20 mg L^{-1} (Schuytema and Nebeker, 1998). Regarding oysters, only one paper has previously reported a negative impact of diuron on growth: a synergistic negative effect on growth (size) was reported following 13 days of juvenile exposure to a herbicide (Basamaïs, 10µg L^{-1}) and

fungicide (Opus, 10µg L^{-1}) (Stachowski-Haberkorn et al., 2008). As reported by Buisson et al. (2008), diuron can have adverse effects on oysters at low concentrations in the case of short exposure. In fact, after exposing oysters to a concentration of 1 µg L^{-1} for 1 week, a negative effect was observed on both reproduction (partial spawning) and nutrition (atrophy of the digestive epithelium).

4.3 DNA damage in semence and effects on offspring

Gamete cells (spermatozoa) are known to be sensitive to pollutant exposure (Speit et al., 2009; Favret and Lynn, 2010; Lewis and Ford, 2012) and specifically to diuron in *C. gigas* (Akcha et al., 2012; Mai et al., 2013). By *in vitro* exposure, Akcha et al. (2012) showed diuron to have significant genotoxic effects on oyster spermatozoa from concentrations of 0.05 µg L^{-1} upwards. In our study, indirect genotoxic effects on oyster spermatozoa were observed after exposing genitors during gametogenesis; it is hence probable that spermatogonia DNA was damaged by diuron biotransformation. Despite the low level of DNA strand breaks measured in spermatozoa, the induction of damage by diuron was significant compared to control groups. It is probable that the measured level of DNA breakage could have been higher if a DNA decondensation step was added to the Comet protocol prior to electrophoresis.

The presence of DNA damage in gamete cells may dramatically reduce the success of fertilization and consequently impair reproduction. Mai et al. (2013) demonstrated that sperm cells *in vitro*-exposed to 0.04 µg L^{-1} of diuron had a reduced fertilization ability. A similar observation was made in the sea urchin *Paracentrotus lividus* following exposure to 1 mg L^{-1} of diuron (Manzo et al., 2006). Our study showed the transmission of DNA damage to be potentially responsible for a significant decrease in the hatching rate of offspring originating from diuron-exposed genitors. Moreover, it may also be involved in the developmental abnormalities observed in D-stage larvae. Of note, variations were observed in the levels of developmental abnormalities determined in the present study by the embryo-larval bioassay versus direct sampling from the nursery tanks, probably due to differences in test conditions. In the nursery tanks, the embryotoxic effect observed in the solvent control group was not expected and not confirmed by the embryo-larval bioassay. Moreover, acetonitrile never impacted growth and DNA integrity in both genitors and their offspring (spat) during the all experiment.

Some phenotypic consequences have already been associated with the transmission of damaged DNA during fertilization. Regarding invertebrate species, Lewis and Galloway

(2009) demonstrated that in the polychaete *Arenicola marina* and mussel *Mytilus edulis*, paternal exposure to genotoxins (methyl methanesulfonate and benzo(a)pyrene) resulted in significant DNA damage in sperm, leading to teratogenic impacts on larval development. In fish, the exposure of the three-spined stickleback spermatozoa to an alkylating agent (methyl methanesulfonate) revealed a significant relationship between abnormal development and sperm DNA damage (Santos et al., 2013).

The transmission of damaged DNA during fertilization is likely to be responsible for the detection of DNA damage in the following generation. Indeed, clastogenic and aneugenic effects were observed via FCM in spat from diuron-exposed genitors. In this group, 15% of analysed individuals showed DNA hypodiploidy; interestingly, this percentage is in a similar range (15-22%) to that determined in French oyster farming areas most highly-impacted by summer mortality (Benabdelmouna et al., 2011). The absence of primary DNA lesions in spat, as measured by the Comet assay, could be explained by the capacity of DNA repair machinery to eliminate this type of lesion, whereas chromosomal damage is irreversible. These results underline the good complementarity of the genotoxic approaches adopted in the present study.

The cross-generation transmission of chromosomal abnormalities has already been demonstrated in a variety of species. Hypodiploid spat have been observed in the Pacific oyster following parental diuron exposure (Bouilly et al., 2007). In rainbow trout, males exposed to estrogen 17-ethynylestradiol (EE2) showed high levels of aneuploid sperm, strongly correlated with levels of embryonic aneuploidy (Brown et al., 2008).

5. Conclusion

In this study, the vertical transmission of DNA damage was highlighted by subjecting oyster genitors to short exposures (two 7-day pulses) to diuron at environmental concentrations (0.3 µg L^{-1}) during gametogenesis. The presence of structural DNA lesions was demonstrated in male gamete DNA using the alkaline Comet assay. Despite a loss of DNA integrity, male gametes were still efficient at fertilizing oyster oocytes. However, the transmission of damaged DNA is thought to be involved in the negative impact observed on oyster recruitment (decreased hatching rate, higher levels of larvae abnormalities and reduced larvae growth). The parental transmission of DNA damage to the next generation was demonstrated by the detection of a high rate of DNA aneuploidy (up to 15%) in spat from diuron-exposed genitors. The analysis of POCIS showed that oysters were exposed to integrated concentrations as low as 0.2 and 0.3 µg L^{-1}. Compared to the range of concentrations

668 measured in coastal waters, such observed effects could represent a threat for the reproduction
669 and survival of wild or cultivated Pacific oyster, with high consequences on economic
670 activities.
671 Investigations will be continued in order to determine whether the decrease in the DNA
672 content observed by FCM can be explained by chromosome breaks or chromosome loss. The
673 existence of a statistical link between the presence of genomic abnormalities induced by
674 diuron in spat and their physiological fitness will subsequently be studied in the field, in terms
675 of growth and performances against oyster mortality.
676

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681 **References**

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Fig. 1. General diagram of the experiment (black arrows represent different sampling points)

Fig. 2. Effect of diuron exposure on genitor weight at the end of the first (Tep1) and second (Tep2) exposure pulses. Data that do not share the same letter are significantly different.

Fig. 3. Effect of diuron exposure on levels of DNA strand breaks in genitor hemocytes at the end of the first (Tep1) and second (Tep2) exposure pulses. Data that do not share the same letter are significantly different.

Fig. 4. Effect of parental diuron-exposure on levels of DNA strand breaks in spermatozoa. Asterisks indicate the statistical differences observed between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

Fig. 5. Effects of parental diuron-exposure on offspring. Percentages of hatching rate (A), abnormal larvae 24h post fertilization (B) and size of larvae until metamorphosis (C). Asterisks indicate the statistical differences observed between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

Fig. 6. Effect of parental diuron-exposure on the level of DNA strand breaks in spat hemocytes. Asterisks indicate the statistical differences observed between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

Fig. 7. Levels of chromosomal damage in spat from controls and diuron-exposed genitors, indicated by coefficient of variation (A) and DNA index (B) measured by flow cytometry. Asterisks indicate the statistical differences observed between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

998 Table 1

999 Examples of diuron concentrations detected in coastal waters/estuaries in different parts of the
1000 World and France

1001

Location	Concentration (µg L^{-1})	Reference
World		
Spain	0.02-0.20	Sánchez-Rodríguez et al., 2011
Italy	0.01-1.38	Landa et al., 2009
Japan	0.03-3.05	Okamura et al., 2003
The Netherlands	0.09-1.13	Lamoree et al., 2002
United Kingdom	0.01-6.74	Thomas et al., 2001
France		
Bay of Vilaine	0.44-0.78	Caquet et al., 2013
Mediterranean coastal waters	0.33	Munaron et al., 2012
Bay of Veys	0.02-0.254	Buisson et al., 2008
Charente estuary	0.51	Munaron, 2004

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1003

1004

Table 2

Number of individuals and type of tissue/fluid sampled for each type of chemical and biological analysis

Development stage	Sampling point	Tissue/Fluid	Number of individuals/experimental group	Type of analysis
Genitors	T0	Gills	60	Flow cytometry
		Hemolymph	30	Comet assay
		all	30	Growth
	Tep1	Gills	60	Flow cytometry
		Hemolymph	30	Comet assay
		all	30	Growth
	Tep2	Gills	60	Flow cytometry
		Hemolymph	30	Comet assay
		all	30	Growth
	Spawning	Spermatozoa	Pool	Comet assay
D-larvae	24h post-fertilization		100	Larval abnormalities
Spat	7 months	Gills	150	Flow cytometry
		Hemolymph	30	Comet assay

1010 Table 3

1011 Chemicals detected in the seawater used at the experimental hatchery and their concentrations in
1012 POCIS in ng g^{-1} and ng L^{-1} with and without PRC. Compounds never detected were not reported here
1013 (nd : not detected).

1014

	Data in ng g^{-1}		Data in ng L^{-1} Rs low condition without PRC		Data ng L^{-1} Rs with PRC	
Immersion period (day/month)	28/02-28/03	28/03-10/05	28/02-28/03	28/03-10/05	28/02-28/03	28/03-10/05
Immersion time (day)	29	43	29	43	29	43
Phase weight (g)	0.17	0.19	0.17	0.19	0.17	0.19
Pesticide list						
Acetochlor	nd	9.06	nd	0.42	0	0.95
Acetochlor ESA	46.07	47.06	6.35	4.38	11.65	8.03
Atrazine	10.01	11.19	0.43	0.33	2.53	1.91
Atrazine 2 hydroxy	8.78	12.68	0.38	0.37	4.44	4.32
Carbendazime	1.84	3.93	0.14	0.2	0.46	0.67
Carbetamide	0.52	0.78	0.04	0.04	0.39	0.4
Carbofuran	0.56	0.8	0.03	0.03	0.14	0.14
Chlorotoluron	1.81	8.12	-	-	0.65	1.98
DEA	142.99	14.86	12.33	0.86	57.09	4
Diuron	4.25	5.11	0.27	0.22	0.77	0.62
DMSA	2.62	2.78	0.23	0.16	0.69	0.49
DMST	1.92	5.6	0.11	0.22	0.48	0.96
Flazasulfuron	0.67	1.17	0.08	0.09	0.47	0.54
Hexazinon	1.13	0.97	0.08	0.05	0.36	0.21
Isoproturon	5.33	5.58	0.25	0.17	2.89	2.04
Metazachlor	0.26	2.13	0.01	0.07	0.05	0.25

Metolachlor	9.5	63.08	0.39	1.73	1.8	8.07
Metolachlor ESA	95.24	109.78	10.95	8.51	34.4	26.75
Metolachlor OA	45.85	50.87	4.52	3.38	26.76	20.02
Simazine	1.49	2.57	0.05	0.06	0.66	0.77
Terbutylazine desethyl	nd	3.8	nd	0.11	0	1.77

1015

1016

Table 4

Example of derive TWA concentrations of diuron in the experimental tanks during the second herbicide pulse using k_u value of 0.188 for diuron.

		Seawater control tank	Diuron-exposed tank
Data in ng g^{-1}	replicate 1	0	1410
	replicate 2	0	1904
	mean	0	1657
Data in ng L^{-1}	replicate 1	0	258
with PRC correction	replicate 2	0	349
	mean	0	304
Target diuron concentration in ng L^{-1}		0	600

1022 Table 5

1023 Coefficient of variation (CV%), and DNA index (i.e genome size) (Mean ± S.D.) of genitor gill tissue
1024 throughout the experiment.

1025

Experimental group	Sampling point	CV%	DNA index
Seawater control	T0	5.162 ± 0.452	0.417 ± 0.007
	Tep1	5.092 ± 0.572	0.416 ± 0.005
	Tep2	5.617 ± 0.678	0.420 ± 0.009
Solvent control	T0	5.162 ± 0.452	0.417 ± 0.007
	Tep1	4.926 ± 0.284	0.417 ± 0.004
	Tep2	5.311 ± 0.530	0.419 ± 0.009
Diuron-exposed	T0	5.162 ± 0.452	0.417 ± 0.007
	Tep1	5.212 ± 0.435	0.415 ± 0.006
	Tep2	5.139 ± 0.844	0.423 ± 0.012

1026

1027

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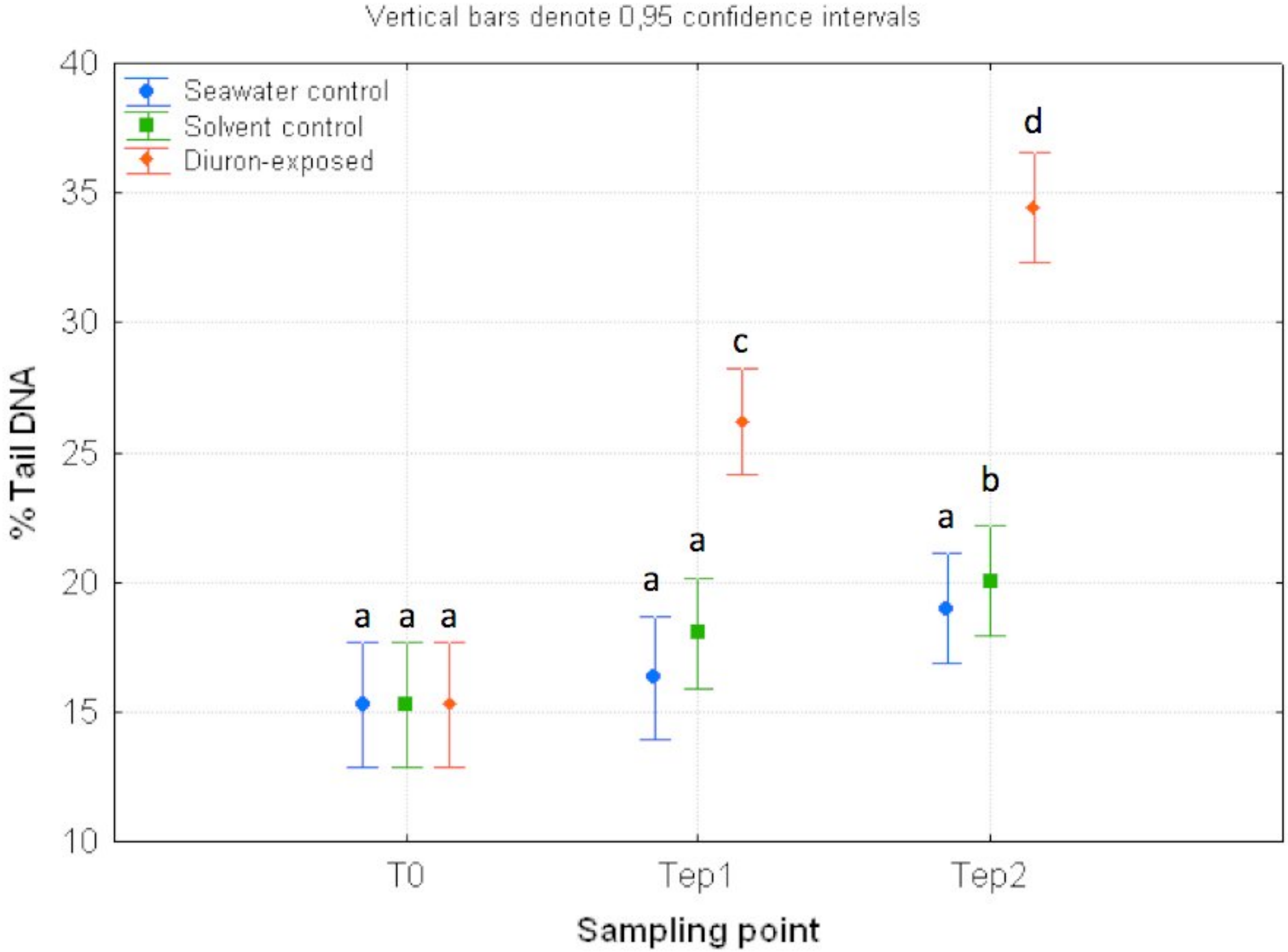
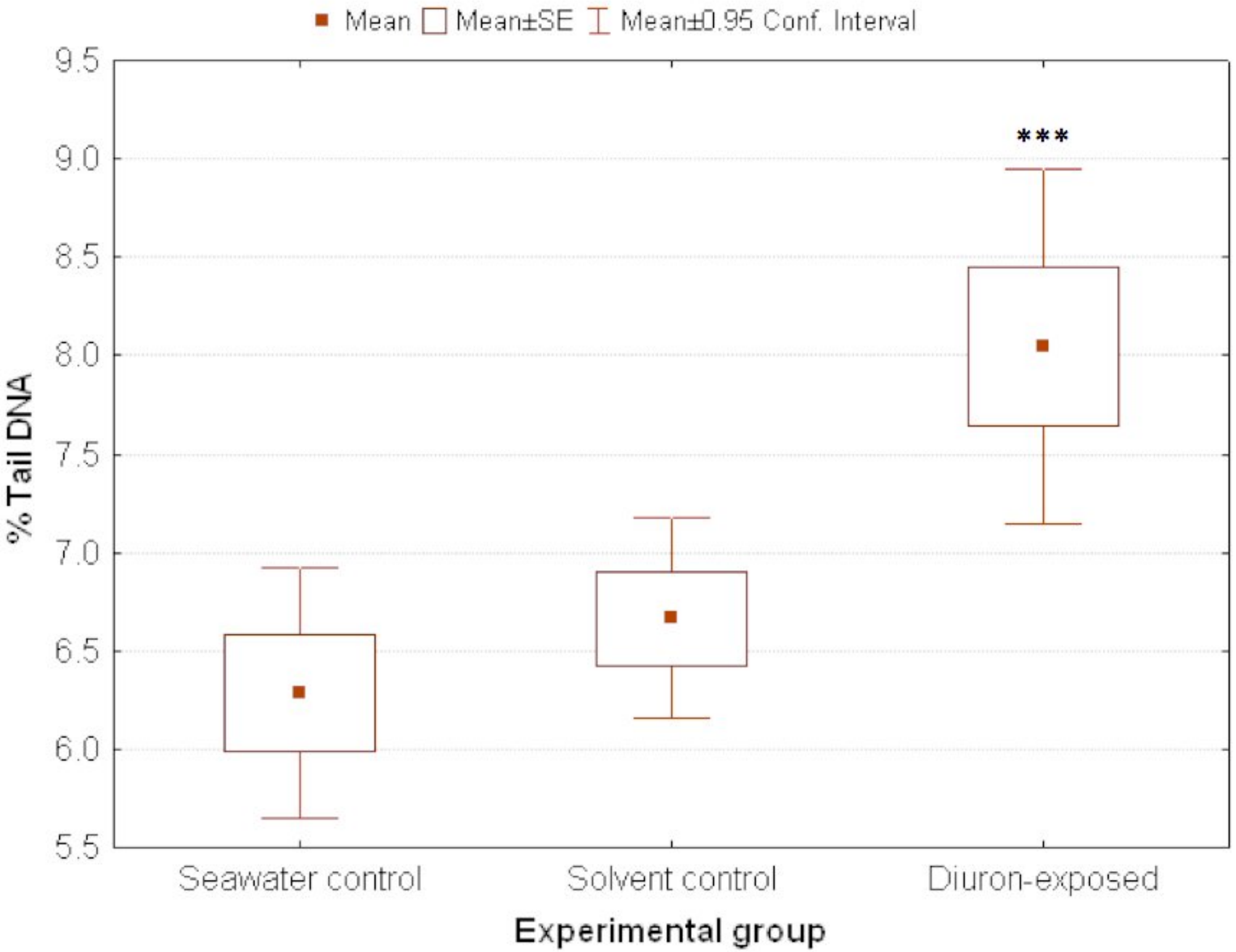
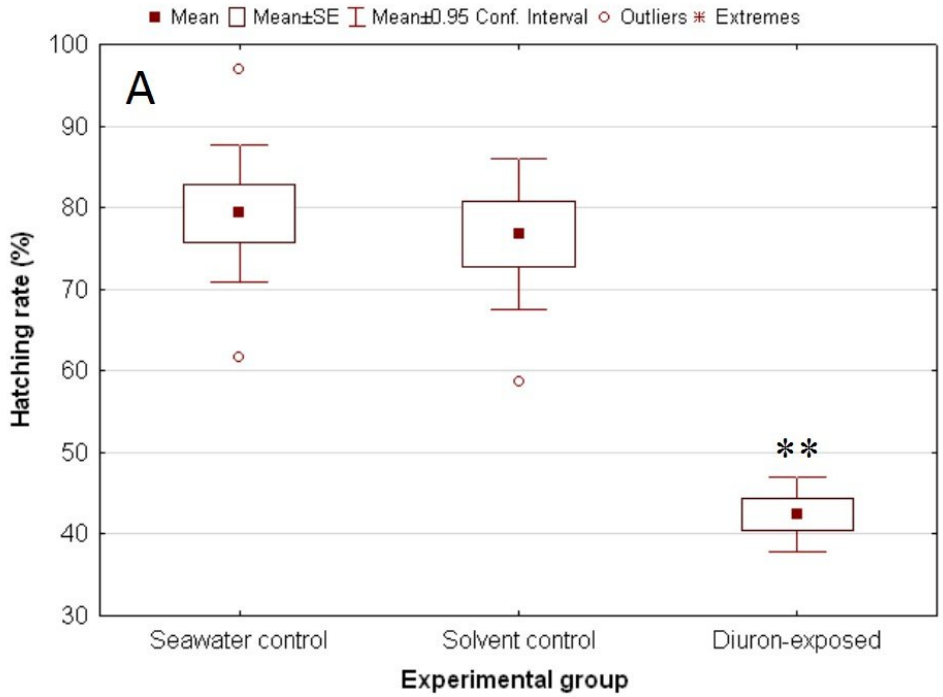
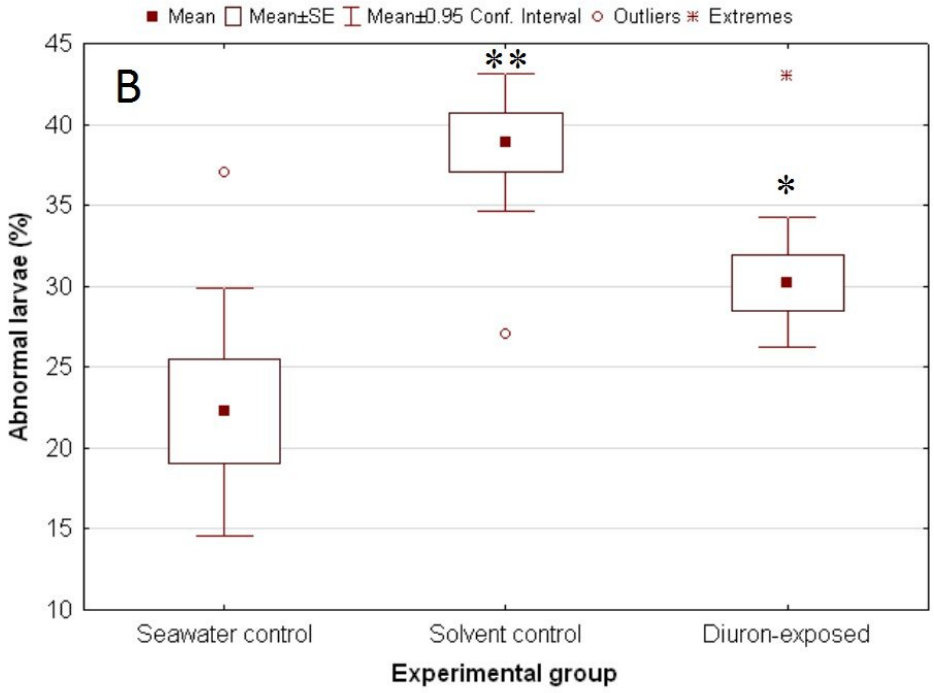
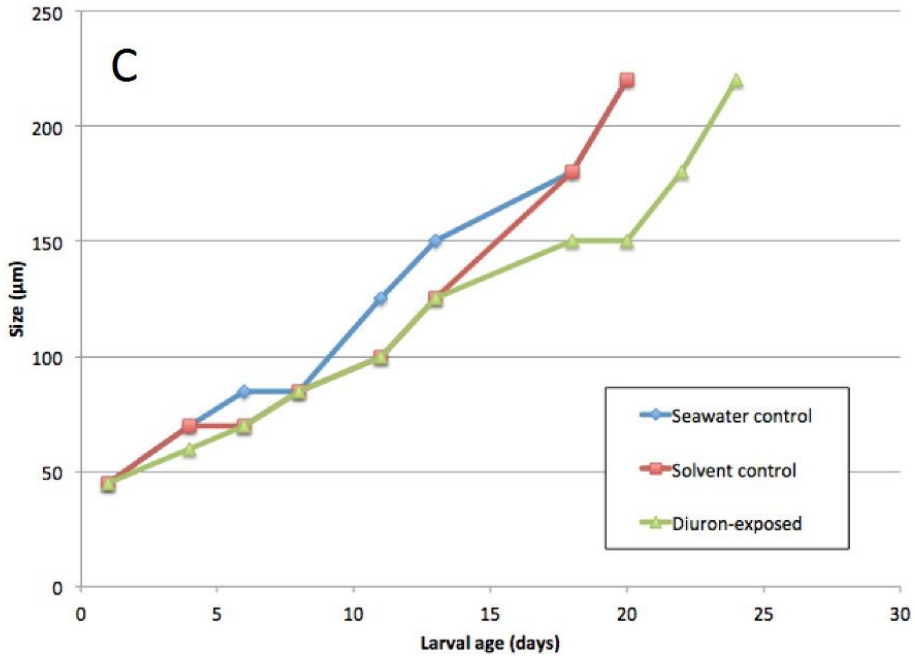
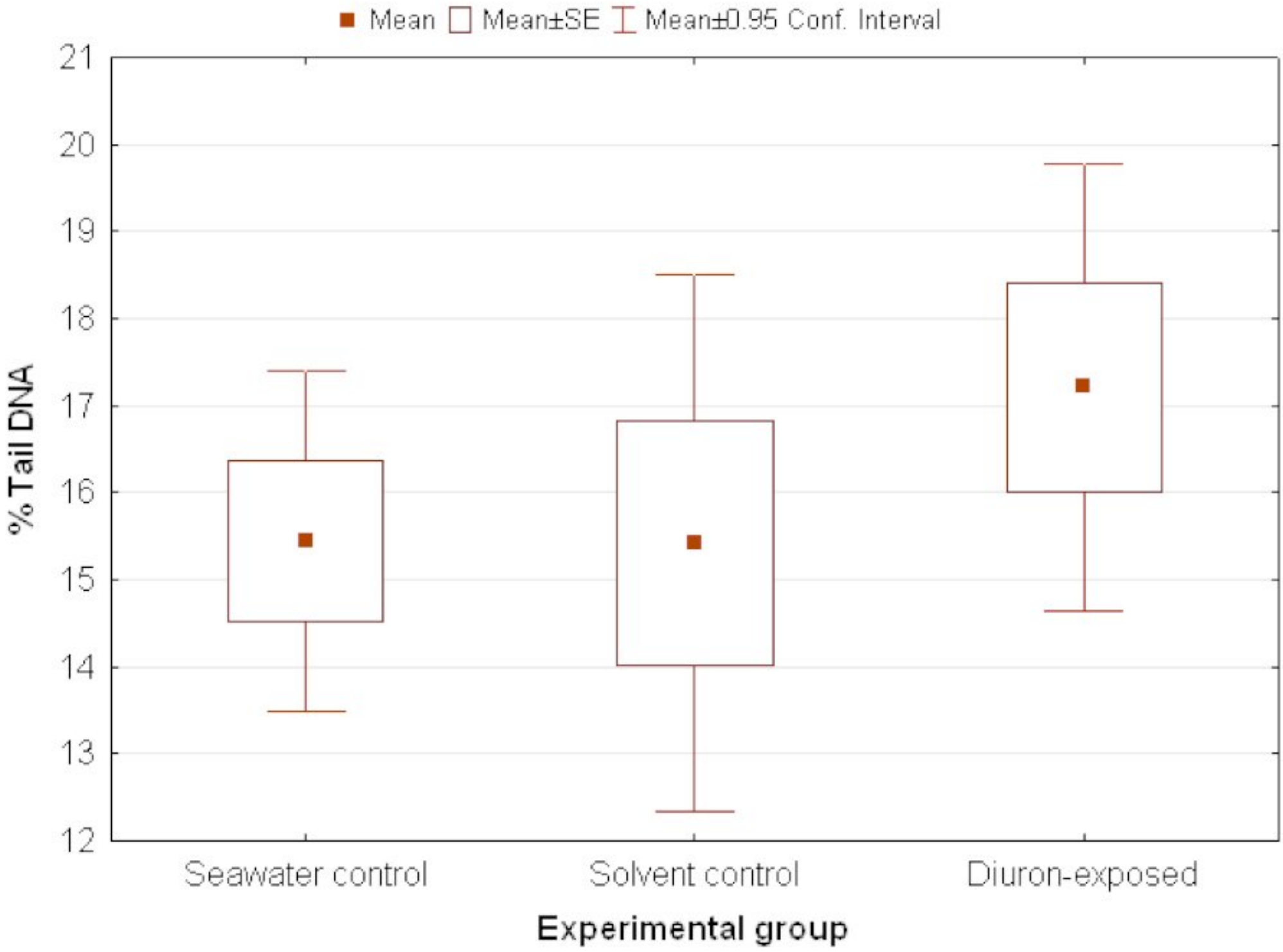

Vertical bars denote 0,95 confidence intervals
Seawater control
Solvent control
Diuron-exposed
% Tail DNA
40
35
30
25
20
15
10
a
a
a
a
a
c
a
b
d
T0
Tep1
Tep2
Sampling point

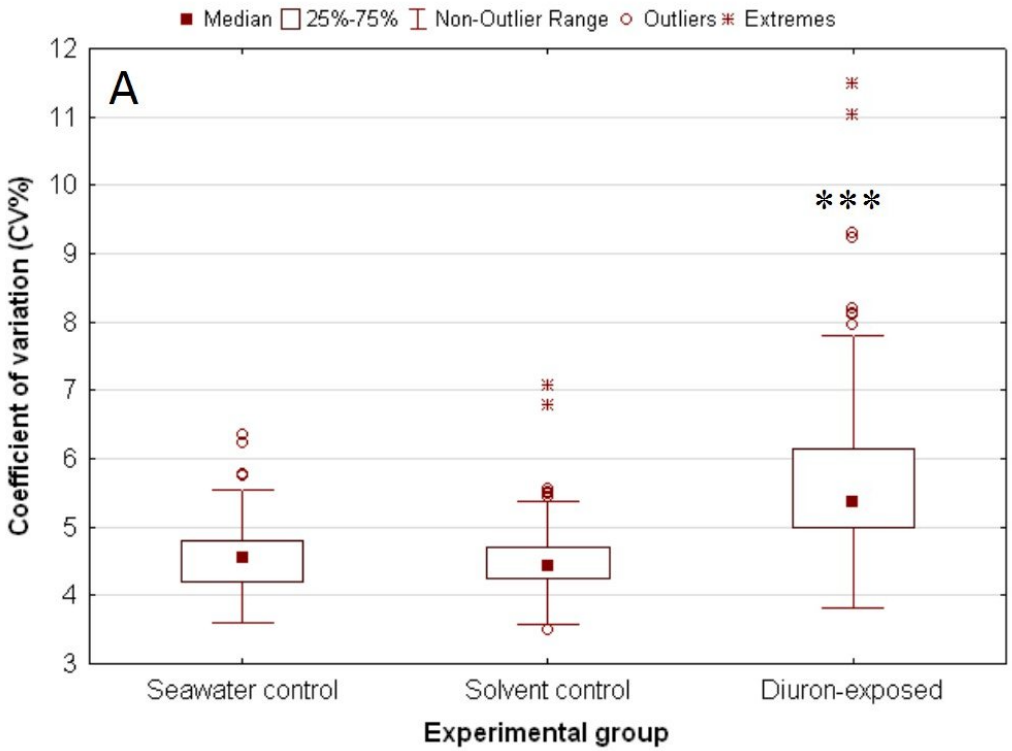

Mean
Mean±SE
Mean±0.95 Conf. Interval
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
% Tail DNA
***
Seawater control
Solvent control
Diuron-exposed
Experimental group


Mean Mean±SE Mean±0.95 Conf. Interval Outliers Extremes
A
Hatching rate (%)
100
90
80
70
60
50
40
30
**
Seawater control
Solvent control
Diuron-exposed
Experimental group


Mean Mean±SE Mean±0.95 Conf. Interval Outliers Extremes
B
Abnormal larvae (%)
45
40
35
30
25
20
15
10
**
*
Seawater control
Solvent control
Diuron-exposed
Experimental group


C
Size (µm)
250
200
150
100
50
0
0
5
10
15
20
25
30
Larval age (days)
Seawater control
Solvent control
Diuron-exposed


Mean
Mean±SE
Mean±0.95 Conf. Interval
21
20
19
18
17
16
15
14
13
12
% Tail DNA
Seawater control
Solvent control
Diuron-exposed
Experimental group


Median
25%-75%
Non-Outlier Range
Outliers
Extremes
A
Coefficient of variation (CV%)
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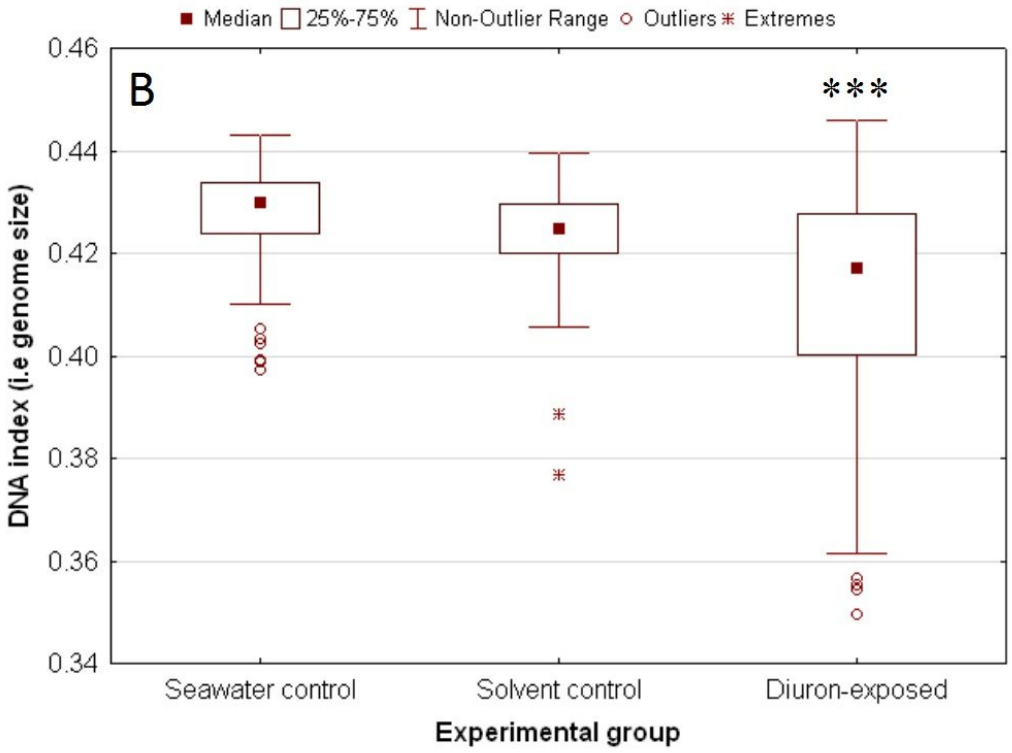
11
10
9
8
7
6
5
4
3
***
Seawater control
Solvent control
Diuron-exposed
Experimental group


Median
25%-75%
Non-Outlier Range
Outliers
Extremes
B
DNA index (i.e genome size)
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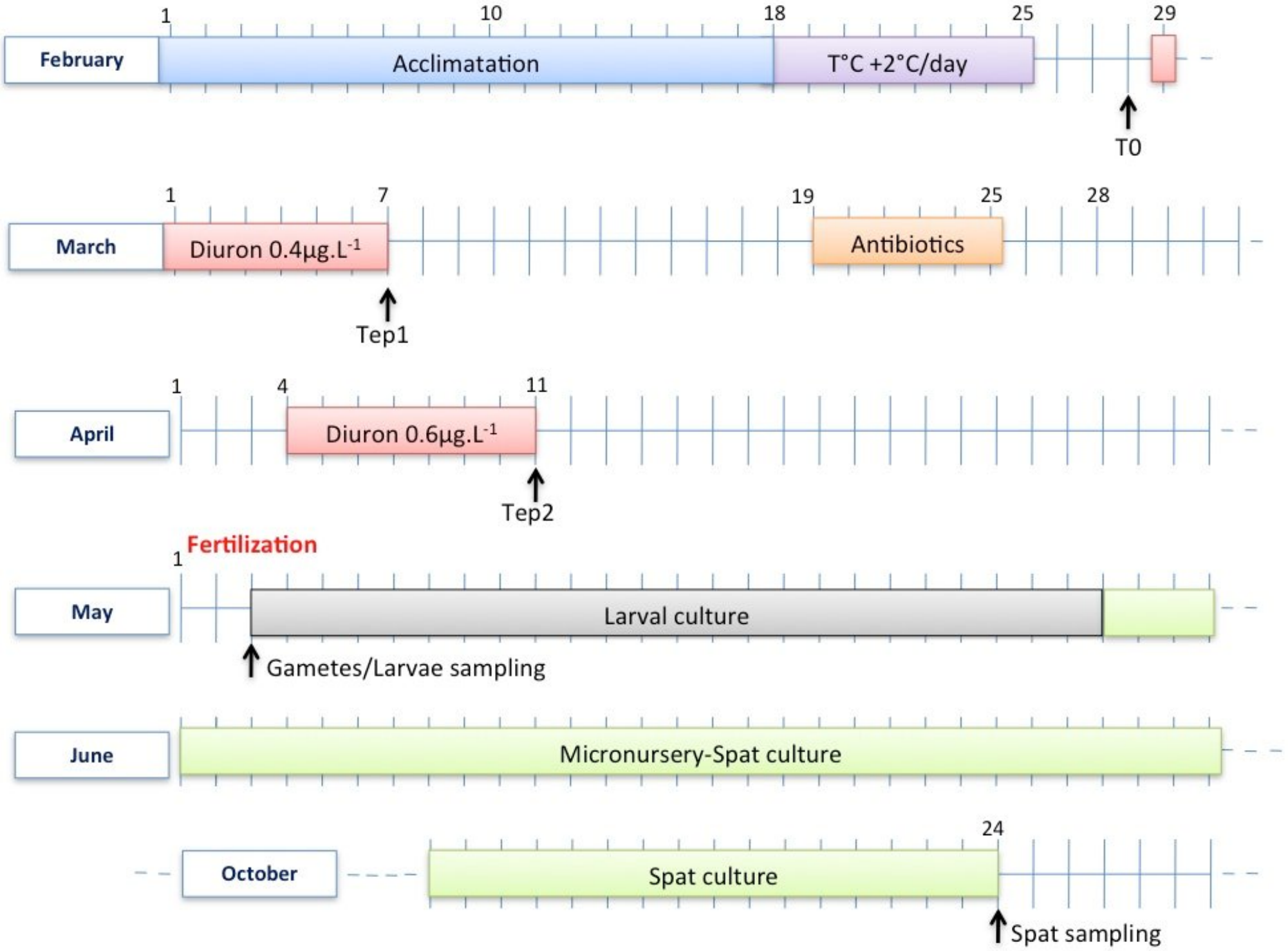
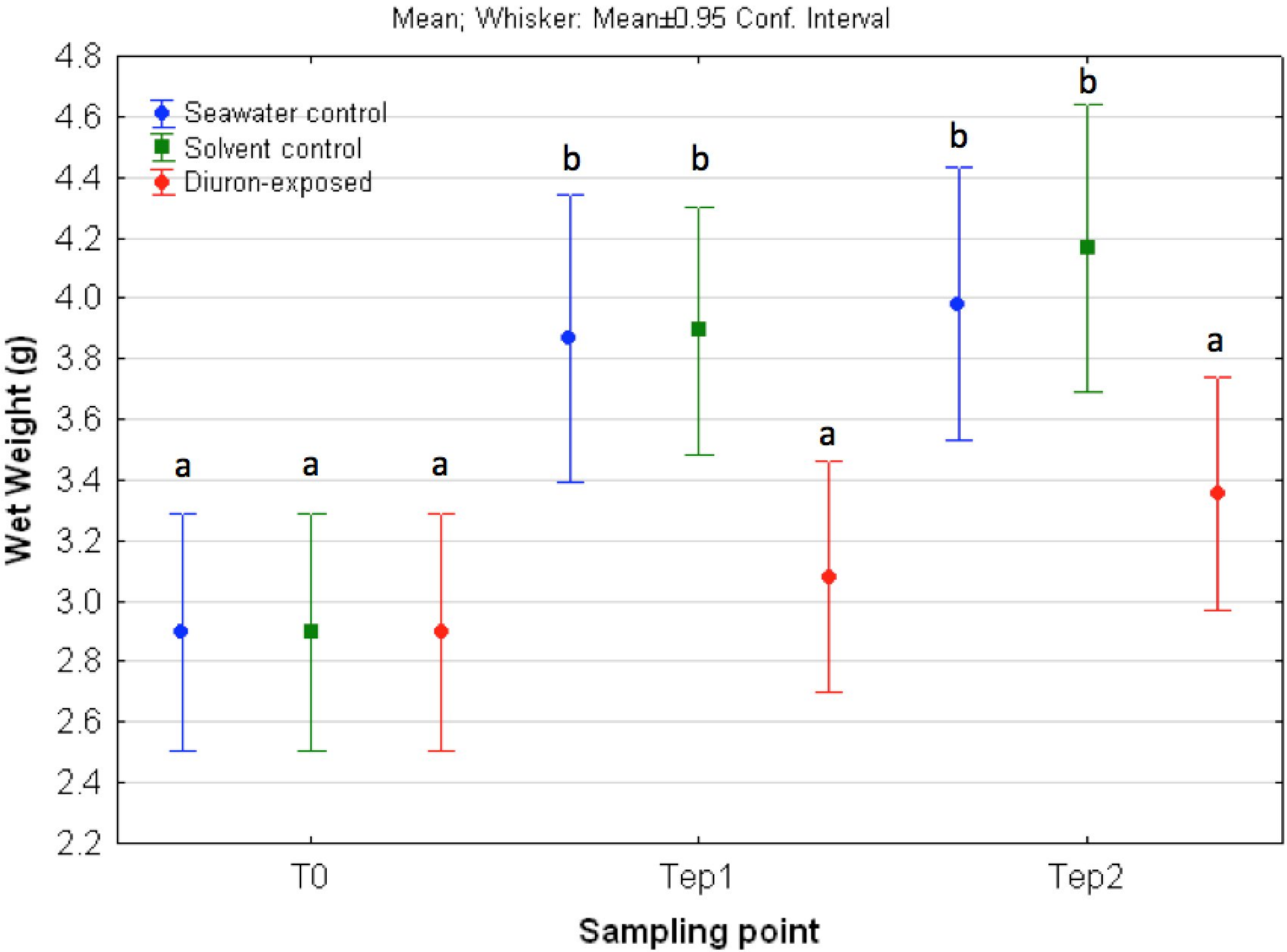
0.44
0.42
0.40
0.38
0.36
0.34
***
Seawater control
Solvent control
Diuron-exposed
Experimental group


1
10
18
25
29
February
Acclimatation
T°C +2°C/day
T0
1
7
19
25
28
March
Diuron 0.4µg.L-1
Antibiotics
Tep1
1
4
11
April
Diuron 0.6µg.L-1
Tep2
Fertilization
1
May
Larval culture
Gametes/Larvae sampling
June
Micronursery-Spat culture
24
October
Spat culture
Spat sampling


Mean; Whisker: Mean±0.95 Conf. Interval
Seawater control
Solvent control
Diuron-exposed
Wet Weight (g)
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4.6
4.4
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4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
a
a
a
b
b
a
b
b
a
T0
Tep1
Tep2
Sampling point