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Genotoxic and epigenetic effects of diuron in the Pacific oyster: in vitro evidence of interaction between DNA damage and DNA methylation

Akcha Farida [1, *], Barranger Audrey [1], Bachère Evelyne [2]

[1] Ifremer, Ecotoxicology Laboratory, Rue de l'Ile d'Yeu, BP21105, 44311, Nantes Cedex 03, France
[2] Ifremer, UMR 5244, IHPE Interactions Hosts Pathogens Environment, UPVD, CNRS, University of Montpellier, CC 80, 34095, Montpellier, France

* Corresponding author : Farida Akcha, email address : fakcha@ifremer.fr

Abstract :

Recently, research has contributed to better knowledge on the occurrence of pesticides in coastal water by identifying frequently detected substances, their concentration range and their acute and chronic toxicity for organisms. Pesticide pollution is of particular concern in France due to important agricultural activities and presence of several exoreic catchment areas that vehicle pesticides up to coastal waters, impacting non-target marine species. Several ecotoxicology questions remain to be addressed concerning the long-term effects of chronic pesticide exposure and the mechanisms involved in adaptation to chemical stress. In the present study, we brought new insights on the genetic and epigenetic effects of the herbicide diuron in oyster genitors. During gametogenesis, we exposed Crassostrea gigas to environmentally realistic herbicide concentrations (0.2–0.3 µg L−1 during two 7-day periods at half-course and end of gametogenesis). Diuron exposure was shown to decrease global DNA methylation and total methyltransferase activity in whole oyster tissue; this is consistent with the previous observation of a significant decrease in DNMT1 gene expression. Diuron effect seemed to be tissue-specific; hypermethylation was detected in the digestive gland, whereas diuron exposure had no effect on gill and gonad tissue. The genotoxicity of diuron was confirmed by the detection of one adduct in gonad DNA. By using in vitro approaches and human DNMT1 (DNMT1 has not been purified yet in bivalves), the presence of DNA lesions (adduct, 8-oxodGuo) was shown to interfere with DNMT1 activity, indicating a complex interaction between DNA damage and DNA methylation. Based on our results, we propose mechanisms to explain the effect of diuron exposure on DNA methylation, a widespread epigenetic mark.

Keywords : Genotoxicity, 8-oxodGuo, DNA adducts, DNA damage, DNA methylation, Oyster, Pesticide, Diuron

1. Introduction

Recently, the possibility for environmental chemical pollutants such as persistent organic pollutants has been proposed to impact epigenetic marks in human and all living organisms (Baccarelli and Bolatti 2009; Vandegehuchte and Janssen 2014; Jimenez-Chillaron et al. 2015). Pollutant induced-epigenetic effects are of great concern due to their expected involvement in adaptation to stress upon long-term chemical exposure (Vandegehuchte and Janssen 2014). By using vertebrate models in both *in vitro* and *in vivo* experiments, numerous chemicals were found to significantly affect epigenetic marks such as DNA methylation (Colotta et al. 2013). As a consequence, several laboratories started investigating the effects of pollutants on the epigenome of model or sentinel species in ecotoxicology with the aim to bring information on the mechanisms responsible for phenotypic variations within individuals and across generations (Kamstra et al. 2014). Proposals for epigenetic biomarkers of chemical pollutant exposure and effects are a targeted objective. However, much work remains to be done; the genome-wide characterisation of epigenetic marks requires integrative analyses of various omic data (e.g., methylome, transcriptome and proteome) and development of methods and tools for meta-analysis (Suarez-Ulloa et al. 2015).

Oyster epigenetic studies have recently been published on DNA methylation, but data remains limited for this ecotoxicologically relevant organism for which the potential role of DNA methylation on both gene expression and adaptation are almost unknown. Patterns of DNA methylation are bimodal in the Pacific Oyster *Crassostrea gigas*, similar to the honeybee *Apis mellifera* (Gavery and Roberts 2010). The two DNA methylation distributions are made of functionally distinct classes of genes as shown by *in silico* analysis of *Crassostrea gigas* transcriptome ('GigasDatabase' version 6). Genes involved in development, tissue-specific functions or response to environmental stimuli are poorly methylated and could be associated to higher phenotypic plasticity (Gavery and Roberts 2010; Roberts and Gavery 2012). DNA methylation is carried out by DNA methyltransferases that are expected to be present in oyster. Sequences with high homology to DNMT3 (*de novo* methylation), DNMT1 (methylation maintenance) and methyl-CpG-binding domain protein 2 are present in the pGigascontig Database version 6 (Wang et al. 2014a). DNA methylation seems to play a complex regulating role in oysters that could facilitate transcription and regulate expression of a portion of the oyster genome (Gavery and Roberts 2013). Male gametes from *C. gigas* have a positive association between gene expression and methylation status of gene bodies and putative CpG promoters (Olson and Roberts 2014). In oyster development, DNA methylation in Hox gene promotors has been shown to modify *in vitro* the transcription level of these genes encoding

proteins known to be major regulators of embryonic development (Saint-Carlier and Rivière 2015). This is in accordance with a previous demonstration of a functional role for DNA methylation in oyster development with stage–specific change in expressions of homeobox gene orthologues and DNMT (Rivière et al. 2013). Developmental abnormalities observed in oyster embryo following copper exposure could be related to an effect on the methylation pattern of homeotic genes, resulting in significant change in their expression level (Sussarellu et al. 2018).

Pesticide pollution is of great environmental concern in France and several herbicides have been detected in coastal seawater (Caquet et al. 2013; Munaron et al. 2012). For this reason, we studied the genetic and epigenetic effects of the herbicide diuron in *C. gigas*, a species that is also of valuable economical interest. In oyster genitors exposed during gametogenesis to realistic environmental concentrations of diuron, RT-qPCR analysis showed a decrease in the expression level of DNMT1; these results do not show a decrease in DNMT3A (Akcha et al. 2016). A significant hypermethylation of the DNA was observed in oyster gametes and offspring by HPLC-UV analysis (Bachère et al. 2017). The effect of parental diuron exposure on spat methylome was further confirmed by analysis of whole genome bisulfite sequencing and target gene DNA methylation (Rondon et al. 2017). Most of the differentially methylated regions occurred within coding sequences; changes in methylation level correlated with RNA level, but only in a very small group of genes. Diuron exposure also displayed genotoxicity through the evidence of primary DNA damages (strand breaks, 8-oxodGuo lesions) in somatic, germinal and reproductive cells of diuron-exposed genitors (Barranger et al. 2014; 2015, 2016). During this latter work, the vertical transmission of modified genetic materials with DNA damage (strand breaks, base oxidation, and chromosome abnormalities) and changes in DNA methylation level (hypermethylation) was demonstrated for the first time in a bivalve mollusc. Such a transmission could explain the differences in gene expression profiles (Rondon et al. 2016) and physiological traits (development, growth, field survival) that were observed in F1 at different life stages (Barranger et al. 2014).

In this context, we further investigated the epigenetic effect of diuron on oysters. Global DNA methylation in whole oyster tissue was measured in the same samples as those subjected to the gene expression analysis for which change in DNMT1 gene expression was observed (Akcha et al. 2016). For these samples, total DNMT activity was also assessed *in vitro* using a colorimetric ELISA-like reaction. We hypothesised that gene expression is related to a change in DNMT activity and DNA methylation level. Considering the possibility for the diuron effect to be tissue-specific, DNA methylation was also measured in different tissues.

We also studied *in vitro* the possible link between diuron genotoxicity and DNA methylation activity by using different substrates for purified human DNMT1. We used oyster gonad DNA from control and diuron-exposed genitors as diuron-exposure has been shown to result in 8-oxodGuo (Barranger et al. 2016) and DNA adduct formation (present paper) in this tissue. We also used modified synthetic oligonucleotides presenting a different number of 8-oxodGuo lesions at different relative position towards unmethylated deoxycytidine. These results will help to better assess the consequences of genotoxicity on methylation mechanisms and long-term responses to chemical stress.

2. Material and methods

2.1 Chemical reagents

Diuron (Pestanal, analytical standard), acetonitrile (anhydrous, 99.8%), Tris, Trizma base, Tris hydrochloride, sodium chloride, sodium phosphate, sodium dodecyl sulfate (SDS), diammonium hydrogen orthophosphate, potassium acetate, lithium formate, urea, lithium chloride, acetonitrile, isopropanol, proteinase K, alkaline phosphatase, nuclease P1 and desoxyribonucleotides were purchased from Sigma Aldrich Chemicals (France). 5-mdCMP and synthetic 30-mers with and without 8-oxodGuo lesions were respectively bought from CliniSciences (France) and Eurogentec (France). EpiQuik™ Nuclear Extraction Kit (reference OP-0002-1), EpiQuik™ DNA Methyltransferase Activity/Inhibition Assay Kit (reference P-3001-2) and human DNMT1 protein (GenBank Accession No. NM_001130823, reference E15000-1) were products from Epigentek purchased from Euromedex (France). Bradford reagent and Pierce DNA Coating solution were respectively purchased from Bio-Rad (France) and ThermoFisher Scientific (France). [γ-^{32}P] ATP was purchased from Amersham (United-Kingdom) and Polyethyleneimine (PEI)-cellulose-coated sheets from Macherey Nagel (France).

2.2 Exposure of oyster genitors to the herbicide diuron

The oysters *Crassostrea gigas* were obtained in the frame of the GIMEPEC project funded by the French National Agency for Research (ANR-CESA-01601) (Barranger et al. 2014). Briefly, wild adult oysters from Marennes-Oléron Bay (France) were transferred from the field to the experimental hatchery of Ifremer La Tremblade. 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 oxygenated flow system. Oysters were fed daily with a

mixture of four marine microalgae (*Skeletonema costatum*: 0.6 × 10^6 cells. mL^{-1}, *Isochrysis galbana*: 4.0 × 10^6 cells. mL^{-1},*Tetraselmis suecica*: 0.8 × 10^6 cells. mL^{-1}, *Chaetoceros gracialis*: 4.0 × 10^6 cells. mL^{-1}); the water temperature was maintained at 8 ± 1°C throughout the one-month acclimatization period. In order to initiate the oyster gonad maturation process, the temperature was then raised by 2 °C day^{-1} for one week, to reach 19.8 ± 0.3°C at the start of the experiment. Then, individuals were divided into three experimental groups (3 replicate tanks per group, 240 individuals per group): a diuron-exposed group (D), a solvent-exposed group with diuron prepared in 0.005% acetonitrile (SC) and a seawater control group (SWC). At the start and the mid-course of gametogenesis, the oysters were exposed during two seven-day periods to 0.2 and 0.3 μgL^{-1} of diuron. Diuron was measured with Polar Organic Chemical Integrative Samplers (POCIS) in the assay tanks (Barranger et al., 2014). This exposure scenario is expected to be environmentally realistic with an exposure time that mimics rain events at diuron concentrations previously reported in coastal waters. The day after the end of the second herbicide exposure period, individuals from each genitor lots (SWC, SC and D) were recovered and sacrificed for analysis.

2.3 Measurement of global DNA methylation in whole and target oyster tissues

2.3.1 DNA extraction

DNA methylation level was measured in whole oyster tissue (10 individuals per experimental group) using genomic DNA (gDNA) previously isolated for gene expression analysis (Akcha et al. 2016). At the end of the experiment, whole oyster tissues were individually frozen in liquid nitrogen and ground with a Mixer Mill MM 400 (Retsch) under liquid nitrogen conditions. Then DNA was extracted from individual frozen oyster powders. Samples were incubated overnight at 55 °C in 500 µL of DNA extraction buffer (0.1 M Tris-HCl pH 8.5, 0.1 M NaCl, 0.05 M EDTA pH 7, 1% SDS, proteinase K at 5 $\mu g\ mg^{-1}$ of tissue). Following a 30-min extraction step with 200 µL of 3 M potassium acetate, the gDNA samples were precipitated overnight at −20 °C by addition of 250 µL of isopropanol. The quantity and quality of the gDNA samples were assessed by spectrophotometry (NanoDrop ND1000 Thermo Scientific) and stored at −20°C prior to DNA methylation analysis.

To compare DNA methylation levels between oyster tissues, another set of samples was used for which digestive gland and gill tissue were individually sampled from the same individuals and frozen in liquid nitrogen until analysis. For each experimental group, DNA was extracted from digestive gland and gills from eight individuals. For three out of these eight individuals, a

piece of the gonad was also available for analysis. Following a speed defrost, tissues were homogenised in the DNA extraction buffer using a potter. Then, DNA was extracted as previously described. After quantification, DNA samples were stored at −20°C prior to DNA methylation analysis.

For the gonad tissue, additional data were obtained by analysis the DNA samples previously extracted by the chaotropic method for 8-oxodGuo analysis (Barranger et al. 2016).

2.3.2 DNA digestion and HPLC-UV analysis

The global DNA methylation level was analysed as previously described in Bachère et al. (2017). Each gDNA sample (2.5 µg in duplicate) were digested into deoxyribonucleosides with nuclease P1 (1.5 U μg^{-1} DNA, 2 h, 37 °C) and alkaline phosphatase (2.5 U μg^{-1} DNA, 1h, 37 °C) prior to analysis by HPLC coupled to UV detection (Agilent 1200 series). Separation was made at 10°C on a Phenomenex Security Guard ULTRA Cartridges UHPLC C18 pre-column and a Phenomenex Kinetex 2.6 µm C18 100 A 100×4.6 mm column. The mobile phase was 50 mM diammonium hydrogen orthophosphate (diammonium hydrogenophosphate), 3% acetonitrile and pH 4.1 using the isocratic mode. The elution flow rate was set at 1.3 mL min^{-1}, and the pressure in the system was 330 bars. Commercially available nucleotides (dAMP, dTMP, dGMP, dCMP and dUMP) were injected individually and in mixture (total injection volume of 13 µL) to determine their respective retention time following UV detection at 280 nm. Standard curves were also generated for dCMP (0.5–2.0 nmoles) and 5-mdCMP (0.015–0.100 nmoles). For the described conditions, the run time was 10 min and the retention times for dCMP and 5-mdCMP were 1.4 and 2.2 min, respectively.

2.4 Measurement of total DNMT activity of whole tissue protein extracts

2.4.1 Preparation of nuclear protein extracts from individual whole tissue of control and diuron-exposed genitors

For each genitor group, nuclear protein extracts were prepared for the same samples (10 per experimental group) as those used for DNA methylation and gene expression analysis. Protein extracts were prepared from aliquots of individual frozen oyster powders using the EpiQuik™ Nuclear Extraction Kit from Epigentek following manufacturer instructions. For each sample, 60 mg of powder were homogenised on ice in diluted DTT containing NE1 buffer (5 mL per g) using a Potter grinder with PTFE pestle. Following 15 min incubation on ice, samples were centrifuged (10 min, 12,000 rpm, 4°C) and supernatants were removed. Pellets were

resuspended in NE2 buffer containing DTT and PIC (5 µL mg^{-1} of tissue) and incubated on ice for 15 min. The extracts were sonicated for 1 min to increase nuclear protein extraction. Following centrifugation, the supernatants were recovered; for each sample a 10 µL-fraction was collected and kept on ice for protein concentration measurement by a standard Bradford protein assay. The rest of the samples were stored below –80°C prior to DNMT activity analysis.

2.4.2 Total DNMT activity assay

The total DNMT activity was measured in whole tissue nuclear protein extracts using the EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit from Epigentek. The assay is based on unique cytosine-rich DNA substrate stably coated on microplate strip wells. DNMT enzymes contain in tested extracts transfer a methyl group from Adomet to cytosine of DNA substrate. The methylated DNA is recognised with an anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, is then colorimetrically quantified through an ELISA-like reaction. The DNMT activity was measured in nuclear protein extracts prepared from the oyster genitor lots (SWC, SC, D) following manufacturer instructions.

For each sample, three wells were used for the positive control and blank. For the samples, the assay was realised by adding 15 µg of nuclear proteins per well. For the positive control, we used 2 µL of DNMT positive control solution. The incubation period at 37 °C was set at 2 hr. Then the capture and detection antibodies were used following dilution at a 1:1000 ratio in 1X M1 buffer. Following the addition of the developer solution, colour was let to develop in the dark for two min. Stop solution was added and absorbance of each well was read on a TECAN microplate reader at 450 nm.

For each assay well, the total DNMT activity was calculated using the following formulas: DNMT Activity (OD/H/mg) = [(sample OD – blank OD)/protein amount (µg) * time (hr)] *1000.

2.5. Investigating *in vitro* the link between the presence of DNA damage and human DNMT1 activity

2.5.1 Qualifying the integrity of oyster gonad DNA by DNA adduct analysis

Oyster gonad DNA from control- and diuron-exposed genitors (10 males and 10 females per group, oyster sex was determined by histological analysis) was previously extracted by the

chaotropic method for 8-oxodGuo analysis (Barranger et al. 2016). These DNA samples were available for the ^{32}P post-labelling analysis of DNA adducts. For each experimental group, analysis was realised in duplicate on pooled DNA samples of gDNA from both male and female genitors and of male gDNA exclusively. Male gametes are known to be more sensitive to genotoxicants compare to female (Aitken and Curry 2010).

DNA adducts were analysed by applying the nuclease P1-enhanced post-labelling technique (Le Goff et al. 2005). For each sample, 5 µg of DNA were digested to 3-monophosphate deoxyribonucleosides (dNps). Following dephosphorylation of unadducted dNps, adducted dNps were radiolabelled by incubation with ^{32}P ATP. For each sample, DNA adduct separation was achieved by thin-layer chromatography (TLC) on PEI cellulose plates, using contact transfer procedure after the first migration (D1) in a solvent system consisting of four solvents: D1) 1 M sodium phosphate, pH 6.8; D2) 4.5 M lithium formate, 8.5 M urea, pH 3.5; D3) 1.6 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0; D4) 1 M sodium phosphate, pH 6.8. Adduct profiles were analysed qualitatively and semi-quantitatively using autoradiography of the plates. DNA adduct levels were expressed as the number of adducts per 10^9 normal nucleotides.

2.5.2 Measurement of human DNMT1 activity using gonad DNA from control and diuron-exposed genitors as substrates

For each experimental group, gonad DNA samples were used as substrates for the activity measurement of human DNMT1 protein (GenBank Accession No. NM_001130823; Epigentek) using the EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit. First, the DNA samples were coated into microplate wells. For each DNA sample, three wells were coated with 10 µg of DNA each using Pierce DNA Coating solution (Thermo Scientific). For each sample, one volume of DNA (about 150 µl containing 30 µg of DNA) was mixed with one volume of Pierce solution for 10 min in a glass tube. Then, 100 µL were deposited into each of the three assay wells and left under light agitation at room temperature overnight. After removing the excess of solution in each well and a washing step in phosphate buffered saline, the microplate was used in combination with the EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit (Epigentek) replacing nuclear protein extracts by pure human DNMT1 (100 ng $well^{-1}$).

2.5.3 Measurement of human DNMT1 activity using synthetic DNA with 8-oxodGuo lesions

DNA methylation occurs in bivalves but DNMTs have not been purified yet. It is the reason why human DNMT1 was used for this *in vitro* approach. Five different oligonucleotides (30-

mers, 100 µM, Eurogentec) were used as substrates for measuring human DNMT1 activity (Table 1). Oligos 2–5 have one methylated dC to make DNA methylation possible. Oligo 1, containing no dC, and oligo 2, presenting no unmethylated dC, were used as negative controls. Oligos 4 and 5 have unmethylated dC but also 8-oxodGuo lesions at adjacent and unadjacent positions respectively. Because this oxidative DNA lesion is present in the genome of all living organisms (Cadet and Davies, 2017), these short oligonucleotides were considered as a representative part of what could be observed in the genome. The oligonucleotides were coated in microplate wells depositing 10 µg well^{-1} (4 wells per condition). Then, the activity of human DNMT1 activity was measured as described previously for oyster gonad DNA.

2.6 Statistics

Raw data were analysed using Statistica 8.0 (Statsoft, Inc.). Depending on the number of tested factors, we conducted either a main effects ANOVA or a one-Way ANOVA. Data normality (Shapiro test) and homogeneity of variances (Levene test) were previously checked for testing of the ANOVA's assumptions. Significant differences were considered at $p<0.05$ and when required a Tukey's pairwise comparison test was performed.

For the comparison of the DNA methylation level between tissues from the same individuals, t tests for dependent samples were applied. The regression analysis between 8-oxodGuo level and human DNMT1 activity was also realized with Statistica 8.0.

Graphs were realized with R software (Version 1.2.1335) (R Core Team, 2019).

3. Results:

3.1 DNA methylation level in whole oyster tissue

No statistical difference in the methylation level of whole oyster DNA was observed between the different experimental groups (one-way ANOVA, $p = 0.33$), despite a downward trend observed in genitors exposed to the herbicide (1.67 ± 0.26 for D vs 1.93 ± 0.28 for SWC and 1.81 ± 0.34 for SC; Fig. 1).

3.2 Comparison of DNA methylation level in different oyster tissues using dependent samples

Data obtained in gills and digestive glands from the same individuals were first separately analysed by a one-way ANOVA to study a possible effect of diuron exposure on tissue

methylation level. Diuron had no significant effect on the DNA methylation level in gills ($p = 0.17$), but significantly increased DNA methylation in the digestive gland ($p = 0.045$). To a lesser extent in the digestive tissue, DNA methylation was also higher in the group exposed to the solvent vehicle acetonitrile (Fig. 2). A T-test for dependent samples showed a significantly higher level of DNA methylation in the digestive gland than in the gills ($p < 0.0001$).

Gonad tissue was also available for analysis but only for a limited number of individuals (three per experimental group). Results of T-test for dependent samples showed that methylation levels for gonad and digestive gland were not statistically different ($p = 0.11$, % DNA methylation of 2.00 ± 0.04 and 2.14 ± 0.013, respectively); both were significantly higher than those measured in gill tissue ($p < 0.001$, % DNA methylation in gills of 1.48 ± 0.08). In the gonad, diuron exposure did not change the percentage of DNA methylation ($p = 0.97$). It was confirmed by the analysis of the other set of DNA samples previously prepared for 8-oxodGuo analysis in this tissue for both sexes ($p = 0.33$) or males exclusively ($p = 0.81$).

3.3. Total DNMT activity of whole tissue protein extracts

A one-way ANOVA showed a significant decrease in DNMT activity in group of oysters exposed to either the solvent vehicle or diuron ($p = 0.043$; Fig. 3).

3.4 Human DNMT1 activity using gonad DNA as substrates

The post-labelling analysis showed that diuron exposure resulted in the formation of only one detectable adduct in gonad DNA from genitors exposed to the herbicide. The radioactivity associated to this adduct was quantified on the TLC plates of both control and diuron-exposed samples (Fig.4).

By using gonad DNA samples from the different experimental groups and presenting a different level of DNA lesions, significant differences in human DNMT1 activity level were observed (one-way ANOVA, $p < 0.0001$; Figure 5). Result from a Tukey's test showed a significantly higher activity in DNMT1 activity when using gonad DNA from genitors exposed to diuron ($p = 0.001$ and $p = 0.008$ for D vs SWC and SC respectively; Fig. 5).

Considering the level of 8-oxodGuo previously measured in this tissue (Barranger et al. 2016), the regression analysis demonstrated a significant positive correlation between the presence of oxidative DNA damage as measured by the level of 8-oxodGuo and DNA methylation as measured by human DNMT1 activity (Number of 8-oxodGuo x 10^6 dGuo = 11.0456 + 1.4737 x DNMT1 activity in OD hr^{-1} mg^{-1}; $p = 0.019$).

3.5 Human DNMT1 activity using synthetic DNA with 8-oxodGuo lesions

Using the same amount of oligonucleotides and human DNMT1 per well, differences were observed in the methylation activity depending on the nature of the oligonucleotides coated on the microplate for the assay reaction. In the absence of dC available for methylation, a basal signal is detected by spectrocolorimetry with oligo 2 due to the presence of one 5-mdC in the DNA sequence compared to oligo 1 with no 5-mdC (T-test for independent samples, $p = 0.019$; Fig. 6). By coating a sequence with more sites available for methylation (4 for oligo 3), a slight but significant increase in the absorbance (nearly x5) was detected corresponding to an increase in the methylation activity (T-test for independent samples, $p = 0.05$). To study whether the presence of 8-oxodGuo lesions and their position towards unmethylated dC can modify DNMT1 activity, we compared results obtained with oligo 3, 4 and 5. The presence of 8-oxodGuo lesions in the 30-mers oligonucleotide increased DNMT1 activity (one-way ANOVA, $p < 0.001$), but only when lesions are adjacent to an unmethylated dC (*a posteriori* Tukey test, $p < 0.001$; Fig. 6).

4. Discussion

4.1 Diuron exposure has an effect on whole individual DNMT1 gene expression and total DNMT activity

In the present study, significantly lower total DNMT activity was observed *in vitro* when using nuclear protein extracts from whole tissue genitors exposed to either diuron or its solvent vehicle. This result is consistent with a previous observation of lower DNMT1 gene expression (Akcha et al. 2016), which could explain the trend in DNA hypomethylation observed during this study. Diuron exposure appeared to be able to disrupt DNA methylation mechanism, much like the solvent vehicle, but to a lower extent.

Recently, research has documented the possibility for chemical pollutants to change DNA methylation level in humans and a high diversity of organisms (Chatterjee et al. 2018; Gurbanov et al. 2019; Hu and Yu 2019). DNA methylation changes were observed either following direct or indirect exposure (e.g., parental exposure) to diverse inorganic and organic pollutants; exposure led to either hypermethylation or hypomethylation of the DNA, depending on the pollutants or species. In aquatic species, few papers dealt with the effect of pesticide exposure on DNA methylation. Similar to our results, exposure of medaka and zebrafish embryo to atrazine resulted in a significant reduction of DNA methylation level (Cleary et al. 2019, Wirbisky-Hershberger et al. 2017). DNMT1 activity was also significantly depressed in the

zebrafish (Wirbisky-Hershberger et al. 2017). Atrazine exposure was responsible for a significant decrease in both DNMT1 gene expression and DNA methylation (Wang et al. 2014; Xing et al. 2015) in different tissues (liver, kidney, gill, brain, gonad) of the common carp. By using a bacterial methyltransferase (*M. SssI*) able to methylate cytosine similarly to mammalian DNMT1, Wirbisky-Hershberger et al. (2017) put forward the possibility for atrazine to be a non-competitive inhibitor of DNMT1 activity. They suggest that atrazine or its residues can bind to the enzyme at a site different from the catalytic centre, decreasing then the activity. Atrazine and diuron belongs to different herbicide families, namely triazines and phenylurea. Despite difference in chemical structure, they can share similar modes of action. For example, both types of herbicide are known inhibitors of plant photosystem-II and are able to block electron transport by binding to protein D1 (Wilkinson et al., 2015). It could be possible for diuron to have the same effect as atrazine to be a non-competitive inhibitor of DNMT1 activity. Given these findings and because total DNMT activity was measured in our study, it would be worth looking at the effects of diuron exposure on DNMT1 and DNMT3 proteins, and S-adenosylmethionine content as well.

It is worth mentioning that exposure to pesticides may also result in hypermethylation of the DNA. It is the case for goldfish exposed to 2, 4-dichlorophenol (2, 4-DCP); this chlorinated phenolic pollutant in aquatic environments is due to contamination by pesticides and their degradation products, such as the herbicide 2, 4-dichlorophenoxyacetic acid (2, 4 D) (Zhang et al. 2014). Hypermethylation was partially explained by a significant increase in the content of S-adenosylmethionine providing the methyl group for addition to cytosine by DNMTs.

The solvent vehicle, acetonitrile at 0.005%, was also responsible for a change in DNMT1 activity (this study) and gene expression (Bachère et al. 2017). This emphasises the necessity to have both a seawater and a solvent control experimental group when studying the effect of hydrophobic pollutants. By exposing 3D cardiac and hepatic microtissues to medium with or without 0.1% DMSO, Verheijen et al. (2019) showed extreme changes in microRNAs and alterations in epigenetic marks such as DNA methylation. Solvent vehicles used for toxicity testing are not inert and that must be taken into account for a good interpretation of the results.

4.2 Diuron exposure results in tissue-dependent change in DNA methylation level

In the present study, significant differences were first observed in the basal methylation level of oyster tissues. The digestive gland and the gonads have a higher level of DNA methylation than the gills. Moreover, a tissue-specific effect of diuron exposure on DNA methylation was observed; herbicide exposure increased DNA methylation in the digestive gland only. The

tissue-specificity of pollutant exposure on DNA methylation was already investigated in some studies. Xing et al. (2015) and Wang et al. (2014) reported basal DNA methylation level in different tissues of common carps from the same experiment. In carp, DNA from brain, gonad and liver presented the highest methylation levels (between 12.5% and 11%), followed by kidneys and gills (around 9%). Following exposure to chlorpyrifos, a significant effect on DNA methylation was observed from the lowest tested concentration of 1.16 $\mu g.L^{-1}$ in brain, gonad and liver; a higher concentration was required for an effect in kidneys and gills. Both *tissue*- and sex-specific effects on DNA methylation were observed in adult zebrafish exposed for 24 days to environmentally relevant concentrations of depleted uranium (Gombeau et al. 2016). In this study, the sex-specific differences were attributed to the endocrine system as differential pattern of hormone secretion between males and females is known to modulate DNA methylation.

Concerning the tissue-specific effect, it may be attributed to differences in chemical exposure due to biodistribution and potential bioaccumulation. The organotropism of some metal trace elements and persistent organic pollutants in the Greenland shark pointed out differences in accumulation depending on both tissue type and substance (Corsolini et al. 2014). Tissue specificity in accumulation has also been observed in several freshwater fish species for some phenolic endocrine- disrupting compounds (Peng et al. 2018). Concerning bivalves, different tissue bioaccumulation patterns were also observed in *Mytilus galloprovincialis* and *Dreissena polymorpha* for ^{32}Phosphorus radionucleid and metals (Mikolaczyk et al., 2016, Vernon et al. 2018). Tissue-specific effect could also be related to organ function. In the present study, diuron exposure was associated to increased DNA methylation in the digestive gland, which is the major tissue involved in pollutant biotransformation in marine bivalves. There could be a link between increased DNA methylation in the digestive gland and expression of genes involved in biotransformation and detoxification.

During the present study, no effect was observed on the methylation level of male gonad tissue, but hypermethylation of sperm and resulting offspring was previously reported (Bachère et al. 2017). During spermatogenesis in the gonad tissue, the male germ cell or spermatocyte undergoes meiosis to produce haploid spermatozoa. Changes in DNA methylation patterns may have occurred during meiosis, disturbing meiotic epigenetic inheritance (Skvortsova et al. 2018). Research in epigenetics are still in its infancy in aquatic species including bivalves, and there is no knowledge on the possibility for reprogramming epigenetic marks during embryonic development and primordial germ cells formation as previously described in mammals (Cantone and Fischer 2013).

438 **4.3 Diuron is genotoxic in oyster, producing both 8-oxodGuo and DNA adducts**

439 The genotoxic effects of diuron have been observed in several aquatic organisms. In fish, *in*
440 *vivo* chronic exposure to environmental concentrations of diuron resulted in an increase in the
441 level of DNA strand breaks in blood cells of European topminnow and zebrafish (Bony et al.
442 2008; 2010). Diuron and its 3,4 DCA metabolite were responsible for a concentration-
443 dependent increase in the level of DNA damage as measured by the comet assay and its Fpg-
444 modified version in different fish cell lines (RTL-W1, RTG-W1, and PLHC-1), (Kienzler et al.
445 2012). Regarding *C. gigas*, exposure to environmentally realistic concentrations of diuron
446 during gametogenesis increased the level of DNA strand breaks in the haemocytes (Barranger
447 et al. 2014). DNA fragmentation was also detected in sperm of exposed male genitors, such as
448 the presence of the oxidative DNA lesion 8-oxodGuo in the germinal cells from both sexes
449 (Barranger et al. 2016). In oyster embryos, diuron was responsible for DNA strand breaks;
450 genotoxicity was proposed to be one of the probable molecular events involved in its
451 embryotoxicity (Akcha et al. 2012; Mai et al. 2013; Behrens et al. 2016; Sussarellu et al. 2018).
452 DNA strand breaks and 8-oxodGuo are oxidative DNA lesions that are thought to be produced
453 during the oxidative stress occurring following diuron exposure. An increase in reactive oxygen
454 species (ROS) production has been observed in oyster haemocytes following a four-week
455 exposure to 0.3 and 3.0 μgL^{-1} of diuron (Bouilly et al. 2007). In Behrens et al. (2016), flow
456 cytometric analysis revealed a steady concentration-dependent increase in ROS-associated
457 fluorescence when exposing trochophore larvae for 16hr to 0.5 μgL^{-1} of diuron. Moreover, the
458 involvement of ROS production in the genotoxicity and embryotoxicity of diuron in the Pacific
459 oyster has been demonstrated by decreased toxicity during co-exposure with ascorbic acid, a
460 known ROS scavenger.

461 In the present study, diuron exposure resulted in the formation of one DNA adduct in oyster
462 with a low relative adduct labelling level. To our knowledge, no previous research has
463 demonstrated DNA adduct formation following diuron exposure. Adduct formation has only
464 been reported for the haemoglobin protein, for which binding by one of the metabolite of
465 diuron, namely 3-4 DCA, was demonstrated in rats chronically exposed to the herbicide (Wang
466 et al. 1993). However, several papers pointed out the possibility for pesticides to produce DNA
467 adducts. By using a DNA-electrochemical biosensor, Oliveira-Brett and da Silva (2002)
468 showed that the metabolites of different triazine herbicides (atrazine, propazine, terbutylazin,
469 cyanazin, ametryn, prometryn, terbutryn, simetryn, prometon and terbumeton) could interact
470 with DNA. DNA adduction was also demonstrated for the herbicide, pendimethalin (Ahmad et
471 al. 2016), and the carbamate-pesticides 1-naphthyl-*N*-methylcarbamate and methyl

benzimidazol-2-ylcarbamate (Macii et al. 2019) by using multispectroscopic and molecular docking approaches and spectroscopy coupled to quantum chemistry, respectively. Several papers also put forward the formation of DNA adducts following occupational exposure to pesticides *in vivo* (Le Goff et al. 2005; Marcelino et al. 2019).

4.4 The presence of structural DNA lesions and their position towards unmethylated cytosine change significantly the ability of human DNMT1 to methylate the DNA

Using an *in vitro* approach, we demonstrated that human DNMT1 was able to carry out *de novo* methylation of short single-strand DNA those activity was depending on the presence of DNA damage. Despite DNMT1 activity has still not been specifically measured in the oyster, several findings support the existence of a similar activity in this invertebrate species. A sequence with a high homology to DNMT1 was reported in the oyster genome those expression was detected during the all life cycle of the oyster, from gametes to embryo cells and adult tissues (Wang et al. 2014a, 2014b). Moreover, conserved domain organization of the DNMT1, DNMT2 and DNMT3 proteins among oyster and human were reported by Wang et al. (2014b) that stated that the oyster genome preserved a complete and functional genetic toolkit for DNA methylation, encoding conserved enzymes capable of both maintenance and de novo methylation. Finally, significant changes in both DNMT1 gene expression and total DNA methylation level were observed during the early development of *C. gigas* (Rivière et al. 2013, Wang et al., 2014b, Sussarellu et al., 2018) that is consistent with methylation maintenance required by active cell divisions at these earlier developmental stages. Regarding these findings, we can legitimately make the hypothesis of the existence of interactions between DNA damage and DNA methylation activity in the oyster as suggested by results obtained with human DNMT1 in the present paper.

By using hemimethylated DNA extracted from eukaryotic cells as a substrate for mouse spleen methyltransferase, Wilson and Jones (1983) previously demonstrated that DNA lesions induced by several chemical carcinogens could inhibit the methyltransferase activity. The formation of DNA alkylation lesions, DNA adducts, apurinic sites and single-strand breaks had an inhibitory effect on the activity, whereas the presence of thymine dimers or double-strand breaks was much less effective. A similar decrease in the *Hpa* II prokaryotic DNA methyltransferase activity was also observed *in vitro* using annealed pairs of oligonucleotides containing the enzyme recognition site and one 8-oxodGuo lesion in presence or absence of a 5-methylcytosine at the CpG site (Weitzman et al. 1994). They showed that the presence of 8-oxo-dGuo reduced the methylation activity of adjacent cytosines, an observation they later

confirmed using a fraction containing total human methyltransferase activity (Turk et al. 1995). Whereas the presence of 8-oxodGuo is associated to an inhibition of methyltransferase activity in these papers, the opposite was observed during the present study. The difference in the results could be explained by the fact that different substrates and enzymes were used during these studies. The conformation of the DNA sequence used may be of primary importance for enzyme activity. Moreover, we used purified human DNMT1 protein for our assay compared to a mix of human methyltransferases in the study of Turk et al. (1995). Effects can so be different depending on DNA lesions and sequences, and nature of the DNMT enzyme used for these experiments.

On the other hand, other authors demonstrated *in vitro* that methylated CpG constituted preferential targets for the DNA adduction of benzo[a]pyrene-7, 8-dihydrodiol-9,10-epoxide and mutations induced by benzo[a]pyrene exposure in human p53 gene (Denissenko et al. 1997; Yoon et al. 2001). Methylation could favour DNA adduction, which has been demonstrated in the case of a prenatal exposure to polycyclic aromatic hydrocarbons in humans (Herbstman et al., 2012); a clear positive correlation was established in human cord blood between the level of global DNA methylation and the level of benzo[a]pyrene-DNA adducts. As suggested by Denissenko et al. (1997), the hydrophobic effects or increased molecular polarisability and base stacking derived from the methyl group may facilitate the creation of an intercalation site for BPDE.

There are so complex interactions between DNA damage, their repair mechanisms and DNA methylation; this has been reported in oxidative stress involved in carcinogenesis (Wu and Ni 2015; Ding et al. 2016). The presence of structural DNA lesions disturbs methylation activity, but this must be more deeply investigated. Altered methylation patterns can lead to aberrant gene expression due to the demonstrated rule of DNA methylation in gene silencing in human and higher vertebrates, although this was not clearly demonstrated in invertebrate species such as in oyster (Rondon et al. 2017).

5. Conclusion

Due to growing availability of genomic resources and to the development of high-throughput DNA sequencing and bioinformatics methods, epigenetic analysis brings new opportunities to assess the long-term consequences of chemical pollution exposure on human and other living organisms. Epigenetics is receiving growing attention; it can bring knowledge on the way exposed organisms can adapt to chronic chemical stress due to the presence in the environment of complex pollutant mixtures.

Diuron exposure seems to interfere with DNA methylation by different mechanisms. Diuron could potentially bind directly to the DNMT enzyme as demonstrated for other herbicides such as atrazine. This mechanism was not investigated during the present study requiring modelling of DNMT protein/herbicide interactions. However as demonstrated in the present study, it can modulate the expression of DNMT genes. Following exposure to environmentally realistic concentrations of the diuron herbicide, the decrease in DNA methylation level and DNMT activity of whole genitor tissue was consistent with a significant decrease in DNMT1 gene expression. Another mechanism could rely on complex interactions between genotoxicity and methylation activity, the presence of DNA damage favoring either methylation or its inhibition depends on the type of DNA lesions. By using *in vitro* approaches, the presence of 8-oxodGuo and DNA adducts known to be produced in oyster by diuron exposure was shown to modulate human DNMT1 activity and potentially the enzymes involved in DNA methylation in bivalves. The epigenetic effect of diuron seemed to be tissue-specific in oyster, hypermethylation was observed in the digestive gland but not in gill and gonad tissue. Further investigations are required to investigate this point and to determine precisely by which mechanisms the herbicide impacts methylation in the Pacific oyster.

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Ethical Approval

All procedures performed in the invertebrate Pacific oyster *C. Gigas* were in accordance with the ethical standards.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Authors' contributions

F. Akcha, A. Barranger and E. Bachère designed the experiment and participated to the experimentation and sampling operations. E. Bachère performed the gene expression analysis. F. Akcha measured global DNA methylation level and activity, and human DNMT1 activity using different substrates. A. Barranger measured 8-oxodGuo levels. F. Akcha wrote the manuscript which was read and approved by the two co-authors.

Competing Interests

The authors declare that they have no conflict of interest.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figure 1

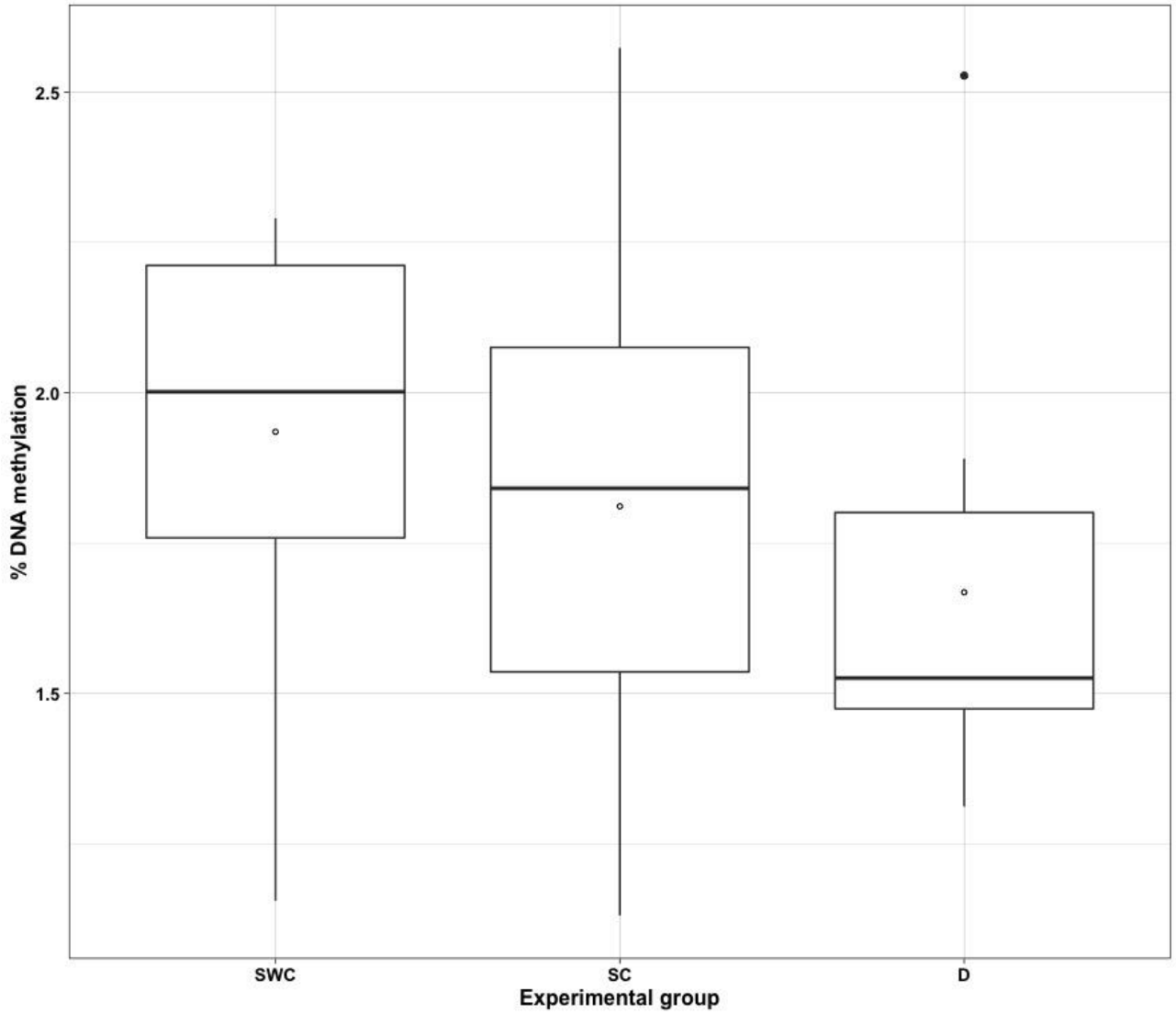

Figure 1. Global DNA methylation level measured by HPLC-UV analysis in whole oyster tissue from controls and diuron-exposed group. The five elements of the boxplot are the minimum, first quartile, median, third quartile, and maximum. The dot represents the mean.

Figure 2

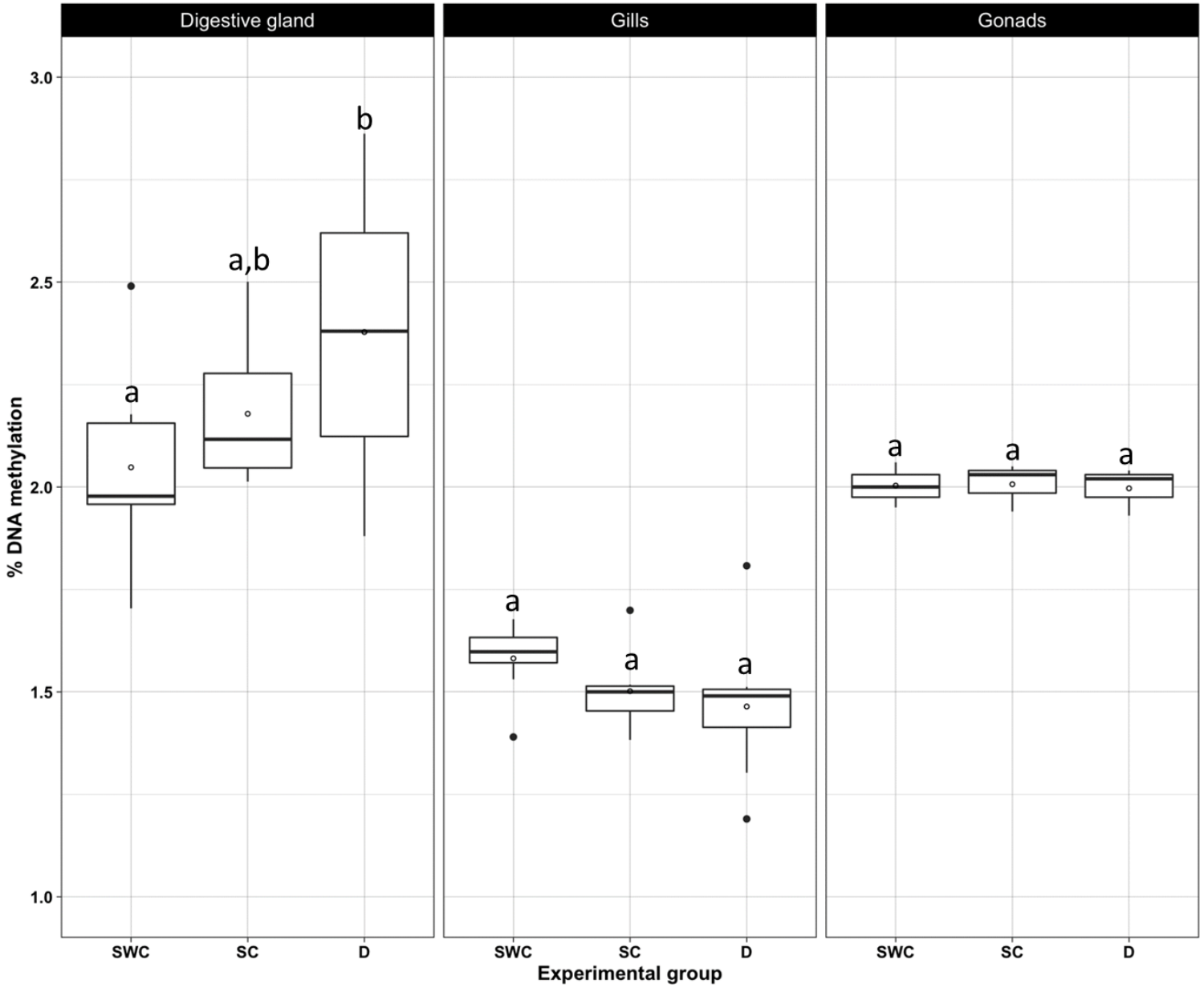

Figure 2: Percentage of global DNA methylation measured in dependent samples of digestive gland, gills and gonads from the different experimental groups. Data with different letters are significantly different ($p<0.05$). The five elements of the boxplot are the minimum, first quartile, median, third quartile, and maximum. The dot represents the mean.

Figure 3

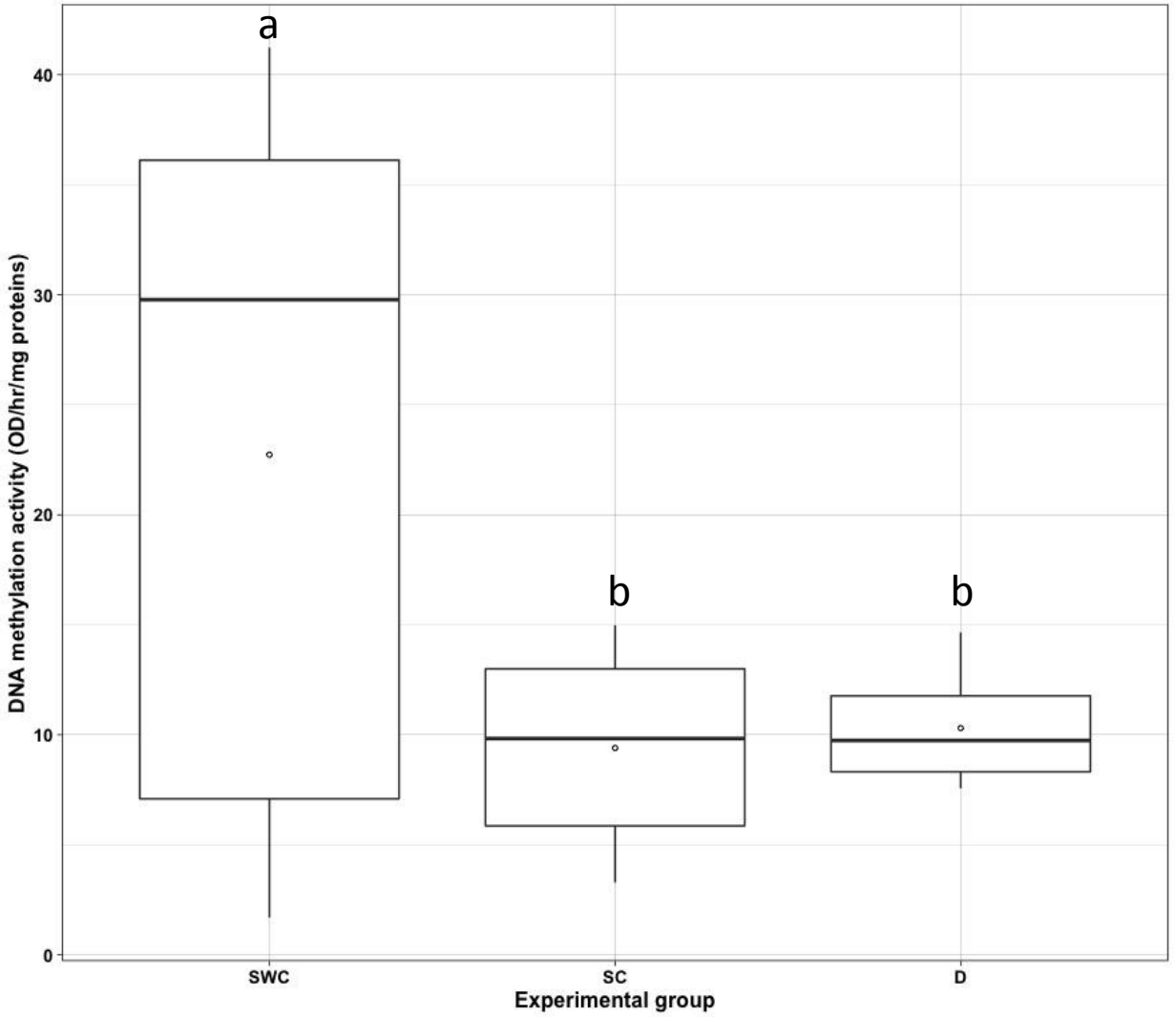

Figure 3: Total DNMT activity in nuclear protein extract of whole oyster tissue from the different experimental groups. Data with different letters are significantly different ($p<0.05$). The five elements of the boxplot are the minimum, first quartile, median, third quartile, and maximum. The dot represents the mean.

Figure 4

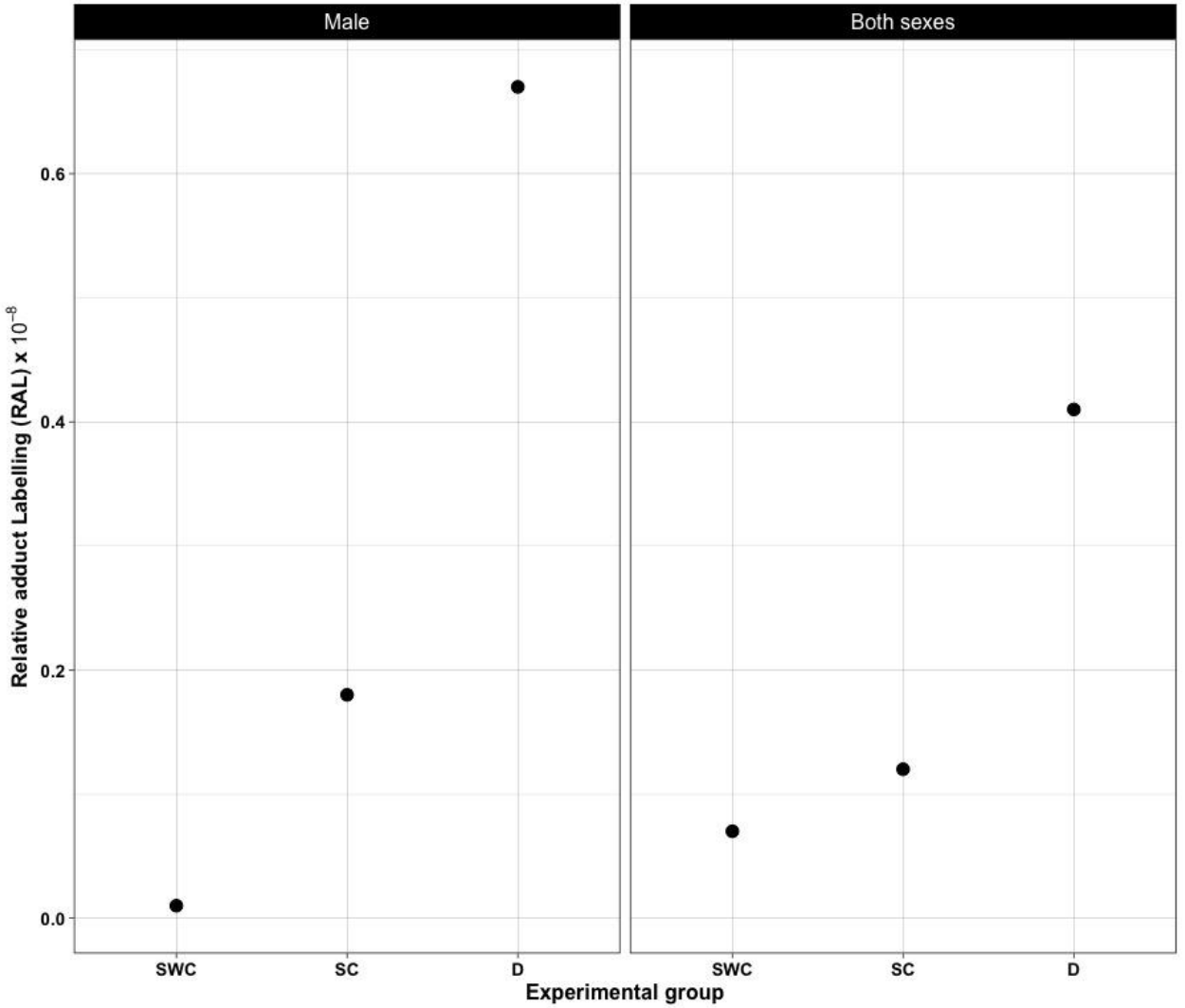

Figure 4: DNA adduct levels measured in the TLC plates of gonad DNA from control- and diuron-exposed groups of genitors. For each experimental group and sample type (male vs both sexes), the RAL data is the mean calculated from two duplicate measurement.

Figure 5

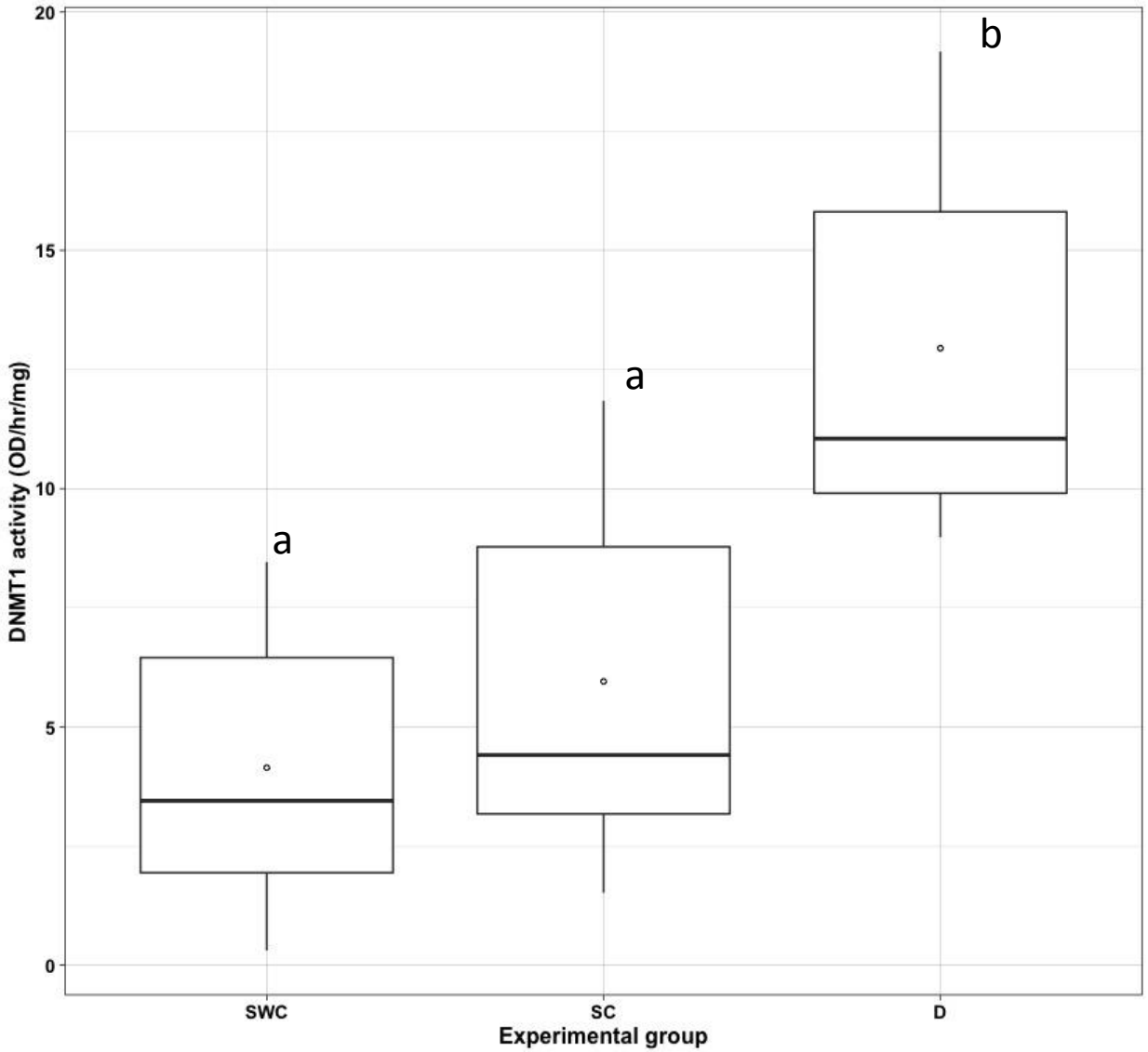

Figure 5: Level of human DNMT 1 activity measured *in vitro* using gonad DNA from the different experimental groups of oyster genitors. Data with different letters are significantly different ($p<0.05$). The five elements of the boxplot are the minimum, first quartile, median, third quartile, and maximum. The dot represents the mean.

Figure 6

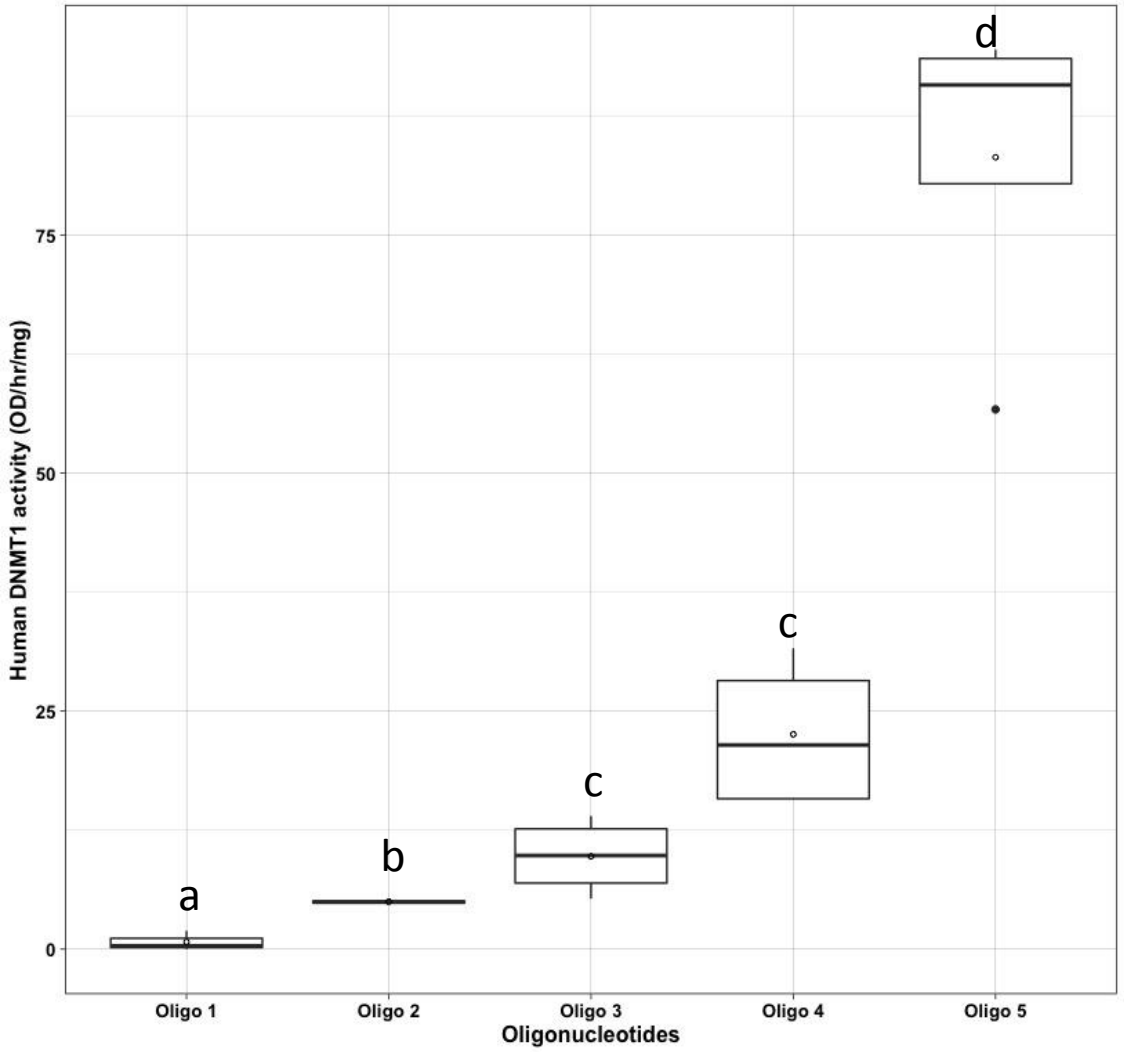

Figure 6: Human DNMT1 activity by using oligonucleotides presenting different levels of unmethylated dC, unadjacent (UA) and adjacent (A) 8-oxodGuo lesions. Oligo 1: 5 mdC (0), dC (0), 8- oxodGuo (0 UA, 0 A), Oligo 2: 5 mdC (1), dC (0), 8-oxodGuo (0 UA, 0 A), Oligo 3: 5 mdC (1), dC (4), 8-oxodGuo (0 UA, 0 A), Oligo 4: 5 mdC (1), dC (4), 8-oxodGuo (2 UA, 0 A), Oligo 5: 5 mdC (1), dC (4), 8-oxodGuo (0 UA, 2 A). Data with different letters are significantly different ($p<0.05$). The five elements of the boxplot are the minimum, first quartile, median, third quartile, and maximum. The dot represents the mean.

Table 1: Custom oligonucleotides used as substrates for human DNMT1 activity

	DNA sequence	Nr of methylated dC	Nr of unmethylated dC	Nr of unadjacent 8-oxodGuo	Nr of Adjacent 8-oxodguo
Oligo 1	AATTTTAAATATATAGTTATGGAAAAATAT	0	0	0	0
Oligo 2	GGTA**C**TTAGTTATAAGATAGTTAAATTGGA	1	0	0	0
Oligo 3	GGTA**C**TTAGTT**C**TAAG**C**TAGTT**C**AATTG**C**A	1	4	0	0
Oligo 4	GGTA**C**TTA**G**TT**C**TAAG**C**TA**G**TT**C**AATTG**C**A	1	4	2	0
Oligo 5	GGTA**C**TTAGTT**C**TAA**G****C**TAGTT**C**AATT**G****C**A	1	4	0	2