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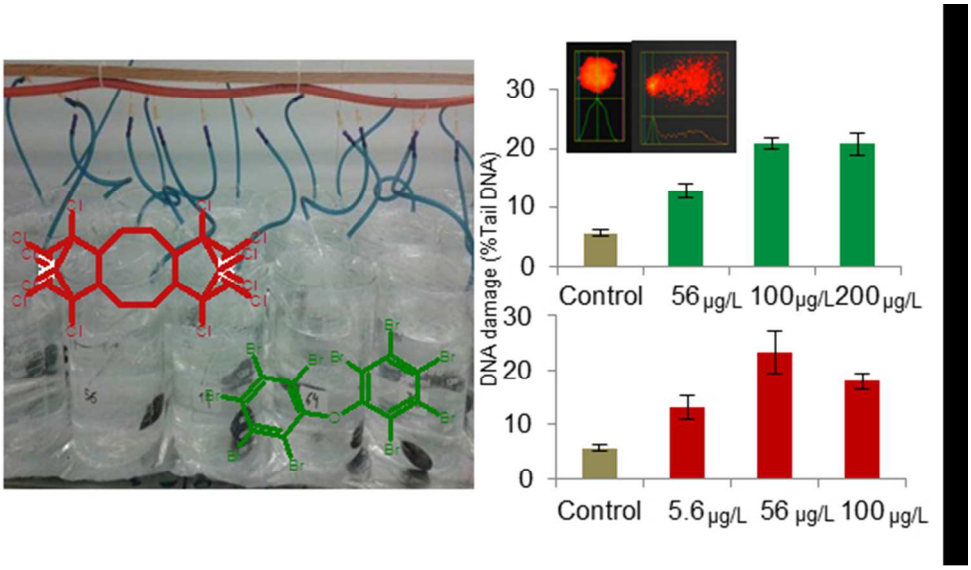

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Evaluation of the genotoxic and physiological effects of decabromodiphenyl ether (BDE-209) and dechlorane plus (DP) flame retardants in marine mussels (*Mytilus galloprovincialis*)

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Abstract

Dechlorane plus (DP) is a proposed alternative to the legacy flame retardant Decabromodiphenyl ether (BDE-209), a major component of Deca-BDE formulations. In contrast to BDE-209, toxicity data for DP are scarce and often focused in mice. Validated dietary *in vivo* exposure of the marine bivalve (*Mytilus galloprovincialis*) to both flame retardants did not induce effects at the physiological level (algal clearance rate), but induced oxidative DNA damage, as determined by the comet assay, at all the concentrations tested. Micronuclei formation was induced by both DP and BDE-209 at the highest exposure concentrations (100 and 200 μg/L, respectively, at 18% above controls). DP caused similar effects to BDE-209 but at lower exposure concentrations (5.6, 56 and 100 μg/L for DP and 56, 100 and 200 μg/L for BDE-209). Moreover, bioaccumulation of DP was shown to be concentration dependent, in contrast to BDE-209. The results described suggest that DP poses a greater genotoxic potential than BDE-209.

Introduction

Polybrominated diphenyl ethers (PBDEs) were one of the most used halogenated flame retardants (HFRs) worldwide, available as three main commercial mixtures: Penta-BDE, Octa-BDE and Deca-BDE.[1] However, this situation has changed due to recent restrictions over PBDEs. Within the European Union (EU), Penta- and Octa-BDE mixtures were banned in 2004, while Deca-BDE mixture was banned in 2008.[2] PBDEs have been found in a wide range of environmental matrices such as sediment, water, fish or cetaceans, and also in humans.[3-8] Nonetheless, environmental behavior and effects of BDE-209 have been studied to a lesser extent to those of lower brominated PBDEs. This might be due to the limitations in the analytical methodologies for the analysis of this compound in the past, due to its high $\log K_{ow}$ and molecular weight.[9] Despite the bioaccumulation potential being lower than other low brominated PBDEs such as BDE-47, BDE-209 has been found in different vertebrate and invertebrate species worldwide.[2, 10, 11] In fact, BDE-209 was the main PBDE found in species with terrestrial diet[2, 10, 12] and also in mussels.[11, 13] BDE-209 has shown thyroid and endocrine disruption properties[14, 15] and it could affect the liver of fish and mice.[15, 16] Most of the studies are focused in vertebrate models such fish or mice,[17] thus studies in invertebrates such as mussels are scarce.

Dechlorane plus (DP) was selected as an alternative to Mirex when it was banned as a FR, and currently it has been proposed as an alternative to the Deca-BDE mixture. It is considered a novel HFR and is still barely regulated.[18-20] Similar to BDE-209, DP has been found a wide range of biological matrices such as fish, mussels or cetaceans and also in humans, showing its bioaccumulation capacity.[18-21] Toxicity data for DP are still very scarce.[22, 23] In fish, DP affected protein responses in the liver and induced

apoptosis,[24] while it showed genotoxical potential in bacteria[22] as well as histopathological changes in mice liver.[25]

Mussels have proven to be a good tool to evaluate the environmental behavior of organic pollutants.[26] Furthermore, effects of organic pollutants in mussels have been correlated with effects of the same pollutants in humans[27] which shows that these contaminants can affect the whole food chain. Thus, the study of the effects of FRs in mussels could provide useful information concerning the potential for effects of these contaminants in other biota and ecosystems. Consequently, the aim of this study was to evaluate the genotoxic and physiological effects of one classical FR (BDE-209, which represents about the 98% of Deca-BDE commercial mixture) and one alternative FR commercial mixture (DP) in *Mytilus galloprovincialis* through an *in vivo* exposure via the dietary pathway. To our knowledge, this is the first time that the toxicity of DP has been evaluated in this way. *Mytilus galloprovincialis* is predominately native to the Mediterranean coast and the Black and Adriatic Seas, however, has established itself as a global invader. This species has highly conserved gene sequences shared by higher organisms including humans as described by us in previous studies.[28] Effects reported in this model invertebrate would therefore have significance for higher-level impacts in coastal environments and could be translated to other species.

Materials and methods

Sample collection

M. galloprovincialis (5-6 cm length) were collected during the last week of July 2014 from Trebarwith Strand (North Cornwall, UK), one of the most pristine sites in the UK, and were immediately transported to the laboratory, rinsed with sea water and acclimatised in an aerated tank with 50 L of filtered seawater (0.8 μm), where they were

maintained at 15 ºC ± 1 ºC with a photoperiod of 12h Light:12h Darkness for 10 days and fed every two days with *Isochrysis galbana* (Liquifry, Interpet, Dorking, UK). Stocking density was 3 mussels per L. Water was changed 2-3 hours after feeding. Any spawning animals were removed from the holding conditions and no animals spawned during the experiments.

Chemicals and reagents

Triton X-100, Sodium chloride, Normal Melting point Agarose (NMPA), Low melting point agarose (LMPA) and N-lauryl sarcosine were purchased from Sigma-Aldrich (UK). BFR-PAR solution, containing BDE-28, BDE-47, BDE-99, BDE-100, BDE-154, BDE-183 and BDE-209, together with *syn*- and *anti*- DP were purchased from Wellington Laboratories (Guelph, ON, Canada), as well as the internal standard ^{13}C-BDE-209. ^{13}C-*syn*-DP, used also as internal standard, was obtained from Cambridge Isotope Laboratories (Andover, MA).

Experiment

To assess whether the feeding route was a valid exposure pathway for filter-feeding organisms when exposed to high Log K_{ow} organic contaminants, a preliminary experiment using benzo(a)pyrene (B(α)P) was performed. Genotoxic potential of this polycyclic aromatic hydrocarbon (PAH) is well known and it is often used as genotoxic model.[28] Individual mussels were placed in 2 L beakers containing 1.8 L of filtered seawater and exposed to B(α)P at either 100 or 200 μgL^{-1} for six days, each concentration dosed either by spiking algae *Isochrysis galbana* or directly into the aqueous media (n=6 per concentration treatment, including a solvent carrier (acetone, 0.05 % v/v) control with only acetone). Both exposure pathways were conducted

following a semi-static model where water was changed every day and mussels fed daily. B(α)P was chosen as a model organic contaminant as it is relatively insoluble in water (Log K_{OW} = 6.04), is known to cause genetic damage and is a priority pollutant.[28] After the dietary pathway proved to be a valid exposure route, mussels were exposed to three different concentrations of BDE-209 (56, 100 and 200 μgL^{-1}) and DP (5.6, 56 and 100 μgL^{-1}), following the procedure described above, *i.e.* individual mussels (n=7 for each concentration treatment) were placed in 2 L beakers containing 1.8 L of filtered seawater. Algae mortality was evaluated before the first exposure by exposing an aliquot of the algae to seawater, acetone, acetone + DP and acetone + BDE-209. No changes in cell size were observed. Concentrations of DP found in the environment and specifically in mussels are considerably lower than concentrations found for BDE-209. Thus, exposure concentrations of DP were settled in lower scale, although 2 common concentrations were maintained for comparisons. Environmental concentrations of these contaminants in mussels depend in a great extent on the sampling area. For instance, DP has been found at levels up to 190 ng/g lw (lipid weight) in an industrial area of China, but concentrations in rural areas were considerably lower, 4.1 ng/g lw.[29, 30] These concentrations are of the same magnitude (parts per billion, ppb) as concentrations selected in this study. Concerning BDE-209, concentrations reported worldwide vary substantially. BDE-209 has been reported at concentrations up to 812 ng/g dw in sediments[31] and it is detected consistently in wild mussels.[32] Hence, it is present at important levels in the environment and bioconcentrates. The selected exposure concentrations are higher than some reported globally, but are in the same order as others. A B(α)P exposure at 100 μgL^{-1} as positive *in vivo* control together with a negative control (acetone, 0.05 %, v/v final volume) were also performed (n=7 per

129 treatment). H_2O_2 was also used as positive *in vitro* control (1 mM and 30 min of
130 exposure time).
131 In both experiments, after the six days of exposure mussel haemolymph was extracted
132 from the posterior adductor muscle using an ice-chilled 1 mL syringe and 21G needle
133 and transferred into individual Eppendorf tubes held on ice, following the protocol
134 described by Brown *et al.* (2004).[33]
135
136 *Water quality*
137 Water quality (temperature, salinity, dissolved oxygen and pH) was measured every day
138 for each beaker and three water samples of each treatment were taken immediately after
139 dosing and prior to water change (*i.e* after 23 h of exposure).
140 Water temperature during the exposure was 16.0 ± 0.5 ºC, salinity was 36.3 ± 0.2‰,
141 dissolved oxygen was 7.93 ± 0.2 mg/L, and pH was 7.92 ± 0.08. No intra- or inter-day
142 variations among treatments were observed (ANOVA and post-hoc Tukey's test) and
143 these values were considered optimal for the exposures.
144
145 *Clearance rate*
146 Clearance rate (CR) was determined prior to haemolymph collection as described
147 previously.[34] Mussels were placed in separate 400 mL beakers containing 350 mL
148 seawater (filtered to 0.8 μm) and a stirring bar. They were allowed to acclimatise at 15
149 °C for 15 min. *Isochrysis galbana* was added in a concentration of 10,000 cells/mL,
150 including several procedural blanks (beaker plus 300 mL of seawater). Aliquots of 20
151 mL were removed immediately after the addition and after 10, 20 and 30 minutes. These
152 aliquots were analysed on a Beckman Coulter Particle Size and Count Analyser set to
153 count particles between 4 and 10 μm. Three separate counts per mussel were made. CR

was calculated using the equation CR = V($\log C_1$-$\log C_2$)/t, where V is the volume of water, C_1 and C_2 are the cell concentrations at the beginning and end of each increment, and t corresponds to the time interval.[35]

Comet assay

Determination of DNA strand breaks using haemocytes was evaluated following a previously optimized protocol.[36, 37] Slides were pre-coated with normal melting point (NMP) agarose and kept overnight at 20 ºC to dry. 150 µL of haemolymph were centrifuged at ~350 g at 4 ºC for 2 min and then mixed with 150 µL of molten low melting point (LMP) agarose. Two separate drops of 75 µL were placed on the slide and immediately covered with a coverslip. Prior to performing the comet assay, cell viability was determined using Eosin Y staining;[38] viability was deemed >95 %. Slides were kept at 4 ºC and in the dark for one hour to allow the gel to solidify. In the case of the H_2O_2 *in vitro* positive control, after one hour 1 mL of H_2O_2 (1 mM) was added dropwise and incubated at 4º C for 30 min. Slides were incubated in lysis solution for one hour and in the dark at 4 ºC, placed in the electrophoresis chamber, filled with electrophoresis buffer, and incubated for 20 min to unwind. Afterwards, the chamber was turned on (25 V, 400 mA) and electrophoresis performed for 20 min. Following on, slides were neutralised with cold neutralization buffer. All the steps in the electrophoresis procedure were performed at 4 ºC and in the dark. Slides were stained with ethidium bromide (20 µL of a 20 µg/mL solution in each drop) and scored under an epifluorescense microscope (Leica, DMR) using the Komet 5 software (Kinetic Imaging, Nothingam). 50 cells in each drop, thus a total of 100 cells per slide, were scored and % tail DNA was used for the evaluation of DNA strand breaks, since it has been validated through inter-laboratory comparisons.[39, 40] In total, 7 slides per treatment for a total number of

63 slides (3 DP treatments, 3 BDE-209 treatments, 1 B(α)P treatment, 1 negative control and 1 H_2O_2 treatment) were analysed. Abnormal comets were excluded from the scoring following the criteria proposed previously.[41] In short, cells outside the gel, double cells or comets in contact with other comets were not scored, and only comets with one round head on the back most side in the direction of the analysis were scored.

Mn assay

Induction of micronuclei (Mn) in haemocytes was evaluated as described by Jha *et al*.[37] Slides were previously coated with 10% poly-L-lysine solution and dried overnight. 200 µL of haemolymph was spread gently onto the slide and left at 15 ºC for 30 min and then fixed with MeOH for 15 min. Afterwards, slides were stained using Giemsa stain (5%, v/v) for 20 min; excess stain was removed with Milli-Q water and once the slides were air dried, a coverslip was mounted using DPX. Slides were scored randomly under the microscope for the induction of Mn. Approximately 1000 cells from each slide were scored following the criteria described in previous works.[37] In total, 63 slides (7 per treatment) were analysed. Only agranular cells were scored, and apoptotic and necrotic haemocytes were excluded from the analysis. Moreover, haemocytes with induced MN were carefully distinguished from haemocytes with nuclear buds; the latter were not counted.[42]

Chemical analysis

Regarding water and algae analysis, the methodology described by Di *et al.* was adopted.[28] Hexane (1 mL) was added to 9 mL of the exposure water samples and internal standards (^{13}C-BDE-209 and ^{13}C-*syn*-DP) were added. Samples were manually shaken and then centrifuged at 3500 rpm for 10 min. The aqueous phase was discarded

and the organic phase was evaporated to dryness and was reconstituted to a final volume of 500 µL with toluene.

Mussel samples were extracted using a previously described methodology.[43, 44] Briefly, samples were spiked with 100 ng of ^{13}C-BDE-209 and ^{13}C-*syn*-DP and kept overnight to equilibrate prior to extraction by pressurized liquid extraction (PLE). Afterwards, lipid content was determined gravimetrically and re-dissolved in hexane prior to acid treatment ($H_2SO_{4(c)}$). A solid phase extraction (SPE) using Al-N cartridges (Biotage, 5 g and 20 mL) was performed to complete the clean-up and resulting extracts were concentrated to a final volume of 40 µL.

Instrumental analysis was carried out using gas chromatography coupled to mass spectrometry working in negative chemical ionization mode (GC-NCI-MS) using an Agilent Technologies 7890A GC system coupled to 5890A GC/MS Single Quadrupole, following previously optimized protocols.[45, 46] BDE-209 was analysed using NH_3 as reagent gas, whereas DP was analysed using CH_4 as reagent gas. Selected ion monitoring (SIM) was used to enhance sensitivity. Two ions were monitored for each compound: the most intense was used for quantification and the second for confirmation. Ions monitored were *m/z* 487 and 489 for BDE-209 (497 and 499 for ^{13}C-BDE-209) and *m/z* 654 and 656 for DP (664 and 666 for ^{13}C-*syn*-DP). Recoveries, method detection limits (MDLs) and method quantification limits (MQLs) are shown in Table 1. Recoveries were determined by spiking 1 g of individual mussel samples with 10 ng of syn- and anti-DP and 50 ng of BDE-209. Five replicates were made, together with 3 blank samples. MDLs and MQLs were determined as the concentrations which gave a signal to noise ratio (S/N) of 3 and 10, respectively.

Statistical analysis

Data were tested for normality and homogeneity of variances using the Shapiro–Wilks test of normality and an F test. Statistical significance between different treatments was determined using analysis of variance (ANOVA), post-hoc Tukey's test and t-test; a p value ≤ 0.05 was used to determine significant differences. Statistical analyses were conducted using the open-source statistical programming language R v.3.1.1 (http://cran.r-project.org).

Results and discussion

H_2O_2 in vitro control validation

Various concentrations (0.2, 0.5 and 1 mM) and time points (10 and 30 min) were explored in order to validate H_2O_2 doses to promote DNA damage. Results show that DNA damage due to H_2O_2 exposure *in vitro* is time-dependant with significantly more DNA damage apparent at the longer time point (ANOVA, $p < 0.001$). Based on these data, both in the pathway validation and in the main experiment *in vitro* controls were performed using a concentration of 1 mM and 30 min of exposure time.

Dietary pathway validation

DNA damage was observed in all B(α)P-exposed mussels, irrespective of exposure route (diet or aqueous), and was significantly different from control mussels (ANOVA, $p < 0.001$), (Fig. 1). The solvent control exhibited a small amount of DNA damage (<10 %) and DNA damage levels of B(α)P were similar in all B(α)P-exposed mussels (*ca.* 30 ± 6 %, mean ± standard deviation), approximately 20 % higher than in controls. DNA damage observed in the positive *in vitro* control, H_2O_2, was fivefold greater than observed in the controls (at 50 ± 9 %). DNA damage was not concentration-dependent. Results showed that the dietary pathway and the direct aqueous exposure did not affect

the results. B(α)P is a known genotoxin and our results are in agreement with previous studies.[28, 47, 48] For instance, Di et al. report 60% damage following a 12 days *in vivo* exposure *Mytilus edulis*.[28] However, DNA strand breaks in control mussels were 30% and thus, DNA relative damage induced by B(α)P was up to 30%, similar to our reported values.

Clearance rate

It has been previously demonstrated that CR in mussels can be affected by several chemical contaminants.[34] In this experiment, CR ranged from 0.49 to 0.90 L/h in the first time increment (10 min) both for BDE-209 and DP, while it was 0.46 and 0.69 L/h for B(α)P and control treatments, respectively. No statistical differences were found among the treatments, although all of them were significantly different than the seawater control (ANOVA, $F_{2.78}$=3.196, p <0.05). The same scenario occurred in the second time increment (20 min), where the CR value increased to 1.64-2.16 for BDE-209 and DP, to 1.29 for B(α)P and to 1.57 in controls. Even though values for BDE-209 (100 μg/L) and DP (56 μg/L) increased faster than other treatments, differences were not significant with any treatment with FR. Finally, after 30 min CR reached values ranging from 1.98 to 2.92 L/h both for BDE-209 and DP, 1.77 L/h for B(α)P and 2.09 for control mussels. Again, even if BDE-209 (100 μg/L) and DP (56 μg/L) showed higher values than the other treatments, these differences were not significant (Figure 2). Thus, we can summarize that mussels are not significantly affected by these FRs at a physiological level, at least with the endpoint chosen in this study. This fact was described for B(α)P in a similar experiment[28] and suggests that mussels can take up these types of compounds without showing significant physiological changes.[38]

279 *Comet assay*

280 In all cases, DNA strand breaks observed were significantly higher than the negative
281 control (ANOVA and Tukey's test, $p < 0.001$) (Figure 3). *In vivo* positive control,
282 B(α)P, caused an effect of 35 ± 6% (mean % tail ± SD), while the *in vitro* positive
283 control, H_2O_2, resulted in 56 ± 10%. Damage induced by BDE-209 was 13 ± 3%, 21 ±
284 3% and 21 ± 6% for 56, 100 and 200 μg/L exposure concentrations, respectively.
285 Damage induced by DP was 13 ± 4%, 23 ± 3% and 18 ± 6% for 5.6, 56 and 100 μg/L
286 exposure concentrations, respectively. For BDE-209, DNA damage displayed a
287 significant increase from 56 to 100 μg/L treatments, but no increase was observed from
288 100 to 200 μg/L treatments. Concerning DP, DNA damage induced by the 56 μg/L was
289 higher than the 5.6 μg/L treatment. However, damage induced by the highest
290 concentration (100 μg/L) was less than that induced by 56 μg/L. It has been described
291 that DNA repair mechanisms can affect the response of the mussels to organic
292 contaminants, since the simple breaks mainly produced by these compounds might be
293 repaired by base excision (BER).[49] Furthermore, reduction of the DNA damage in the
294 most concentrated treatments could be caused by the exclusion of the apoptotic cells of
295 the cell count.[50] Comparison between BDE-209 and DP exposures at 56 and 100 μg/L
296 showed that DP at 56 μg/L induced oxidative damage at the same level as BDE-209 at
297 100 μg/L (23% and 21%, respectively), while DNA strand breaks induced by BDE-209
298 at 56 μg/L were in the same level as the low level of DP (13% and 13%, respectively).
299 Surprisingly, oxidative damage induced by DP at 100 μg/L (18%) was lower than at 56
300 μg/L (or following BDE-209 exposure at 100 and 200 μg/L) (Figure 3). This difference
301 might be attributed to possible differences in BDE-209 and DP metabolization. In
302 contrast, de-bromination products of BDE-209 are often more toxic than parent BDE-
303 209.[52] Furthermore, BDE-209 presents a more complex metabolism since low-

304 brominated OH-PBDEs could also be formed.[53] However, this has not been studied in
305 mussels and the % of PBDEs metabolized to OH-PBDEs seems to be low even in
306 mammals.[54-56]
307 Hence, results presented demonstrate that BDE-209 and DP can both induce DNA
308 strand breaks in mussels. This is in agreement with what previously reported effects in
309 zebra mussel (*Dreissena polymorpha*) where, similar to this study, BDE-209 caused
310 non-dose dependant DNA damage after an *in vivo* exposure of 7 days to 0.1, 2 and 10
311 μg/L.[57] *In vivo* exposures of BDE-47, BDE-100 and BDE-154, also in zebra mussel,
312 caused significant DNA damage up to 5, 11 and 12% respectively (expressed as % tail
313 DNA; controls up to 5%). These values are lower than those reported in this study, but
314 exposure concentrations (0.1, 0.5 and 1 μg/L) and exposure time (4 days) were also
315 lower.[58] To our knowledge, this is the first study reporting the oxidative capacity of DP
316 in mussels.
317
318 *Mn assay results*
319 Mn induced in the negative control were 1.7 ± 0.6, while in the positive B(α)P control
320 were 2.9 ± 1%, representing a significant 2 fold increase (ANOVA and post-hoc
321 Tukey's test, $p<0.05$). Concerning BDE-209, inductions were 1.6 ± 0.9, 1.7 ± 0.6 and
322 2.7 ± 0.7 for 56, 100 and 200 μg/L treatments, respectively. The first two concentrations
323 did not cause significant Mn induction compared to controls, but Mn induced by 200
324 μg/L exposure was significantly higher (ANOVA and post-hoc Tukey's test, $p<0.05$).
325 Furthermore, DP caused Mn inductions of 2.0 ± 0.8, 2.0 ± 1 and 2.5 ± 0.8 at 5.6, 56 and
326 100 μg/L treatments, respectively (Figure 4). In this case, BDE-209 and DP showed the
327 same pattern, *i.e*, Mn induction was only significant at the highest level of exposure.
328 Consequently, DP showed an effect at a lower concentration than BDE-209 (100 and

329 200 μg/L, respectively) which implies that DP is more capable of causing this kind of
330 damage. However, no other studies are available to corroborate this statement.
331 Mn induced by BDE-47, BDE-100 and BDE-154 in zebra mussel were up to 2, 2 and
332 2.5, respectively, but inductions were not significantly different than negative controls.[58]
333 Furthermore, both exposure concentrations (0.1 μg/L, 0.5 μg/L and 1 μg/L) and
334 exposure time (4 days) were lower than our conditions. This is in agreement with our
335 study, where Mn induction was only found at the highest exposure concentrations. Riva
336 et al. (2007) also reported that BDE-209 can induce DNA strand breaks, but not Mn
337 induction.[57] Oxidative stress induced by reactive oxygen species (ROS) has been
338 described as one the most plausible mechanism of the toxicity of BDE-209.[59] As a
339 result, de-bromination of BDE-209 was also considered, since less brominated BDEs
340 present higher oxidative capacity.[60] In this case, no other brominated congeners were
341 detected (see results below), probably because metabolic/enzymatic capacity of mussels
342 is not as high as in fish. Comet and Mn assay results were not correlated either for BDE-
343 209 or DP. This might indicate that these compounds induce primary and repairable
344 lesions rather than permanent ones[57] since their genotoxic induction can arise through
345 several pathways. However, this topic still requires further work in order to truly
346 understand how these pollutants induce oxidative DNA damage.
347
348 *Chemistry results*
349 Water analysis: Concentrations found in water samples taken immediately after dosing
350 were, expressed as mean ± SE (μg/L): 0.02 ± 0.01, 0.03 ± 0.02 and 0.3 ± 0.2 in BDE-
351 209 treatments (56, 100 and 200 μg/L, respectively). Compared to values found after 23
352 h of exposure, concentrations in water decreased 92, 97 and 90%, respectively; in all
353 cases concentrations after 23 h were lower (One-way ANOVA, $p<0.05$). Similarly,

354 concentrations of DP immediately after dosing were 0.4 ± 0.3, 0.3 ± 0.2 and 0.7 ± 0.5
355 μg/L in 5.6, 56 and 100 μg/L treatments, respectively. These concentrations decreased
356 significantly (one-way ANOVA, p<0.05) up to 77%, 79% and 86%, respectively, after
357 23 h. Levels in control water were below MDL for both compounds in all cases (Figure
358 5A). Concentrations used in this study exceeded the estimated solubility of these
359 compounds (< 1 μg/L).[61] However, it has been demonstrated that presence of dissolved
360 organic matter enhances solubility.[28] BDE-209 and DP rapidly distributes between
361 particulates and mussels, thus concentrations in the aqueous phase are expected to be
362 low.
363
364 Mussel analysis: Levels of BDE-209 found in the exposed mussels at the end of the
365 treatment were always substantially higher than those in the controls, proving that
366 mussels bioaccumulated BDE-209 through the *in vivo* exposure (ANOVA and post-hoc
367 Tukey's test, p < 0.05). Values were 1.9 ± 1.3, 1.7 ± 1.1 and 1.6 ± 1.2 μg/mussel,
368 corresponding to the 56, 100 and 200 μg/L exposures. No differences were observed
369 between the three exposures (ANOVA and Tukey's test, p>0.05). This could be due to
370 BDE-209 de-bromination, but while it has been described in fish[62] to the best of our
371 knowledge there are no studies in mussels. During the instrumental analysis no other
372 peaks with *m/z* 79 and *m/z* 81 were observed. Hence, no lower brominated PBDEs or
373 MeO-PBDEs were present in the mussels above the limits of detection. On the other
374 hand, values found in mussels exposed with DP were 4.7 ± 3.1, 8.8 ± 2.1 and 21 ± 9.1
375 μg/mussel, corresponding to the 5.6, 56 and 100 μg/L treatments, respectively. As for
376 BDE-209, DP values were significantly higher than in the controls in all cases (ANOVA
377 and post-hoc Tukey's test, p<0.05). Furthermore, in the case of DP a concentration
378 dependant increase was found (ANOVA and post-hoc Tukey's test, p<0.05). These

379 results show that DP is bioaccumulated by mussels, as has been previously reported.[11,
380 29] Moreover, the ratio between the anti-isomer and the total DP burden was also
381 evaluated. F_{anti} is defined as the concentration of anti-DP with respect to the total DP
382 concentration, both lipid-normalized. It has been described as a good indicator of the
383 different behaviour of the two isomers in the environment, since the initial F_{anti} in the
384 commercial mixture (~0.7) can change when analysing complex organisms such as
385 dolphins.[18] F_{anti} values found in mussels from the three different exposures (0.74 ± 0.02,
386 0.69 ± 0.03 and 0.73 ± 0.02 for low, medium and high levels, respectively) were similar
387 and significantly lower than values found in the control mussels, which were up to 0.79
388 ± 0.04 (ANOVA and post-hoc Tukey's test, $p<0.05$). The commercial mixture of DP
389 used in the exposure was also analysed (n=3, 0.72 ± 0.02). Even if values of the exposed
390 mussels were different than controls, values are still in the range described for
391 commercial DP mixtures. Thus, no *syn*-DP enrichment was observed, which is in
392 agreement with other studies of DP in mussels.[11] It has been described that the
393 particulate matter in the gastro-intestinal tract can affect BDE-209 determinations in
394 mussels.[63] However, since mussels were sampled 24 h after the last feeding, influence
395 of ingested food in BDE-209 analysis was considered to be minimal, as has been
396 suggested previously.[57]
397
398 Overall, these data confirm the use of *M. galloprovincialis* as a suitable biological
399 model for *in vivo* exposures to FRs. In addition, data for DP represents the first evidence
400 of a genotoxic capacity of this compound in mussels. Both DP and BDE-209 induced
401 significant DNA damage even at the lowest selected concentrations, whereas Mn
402 induction was only significant in the highest doses. Other factors such as the timeframe
403 needed to induce micronuclei require further investigation. In general, further studies

using longer exposure times are recommended. In contrast, the feeding rate was not significantly altered by exposure to either compound.

Associated content

Supporting information: Concentrations in exposure water (Table S1). Concentrations in control mussels (Table S2). Individual concentrations of DP and BDE-209 in mussels (Tables S3 and S4). Individual Mn and %Tail DNA for each treatment (Tables S5-S7).

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Notes

The authors declare no competing financial interest

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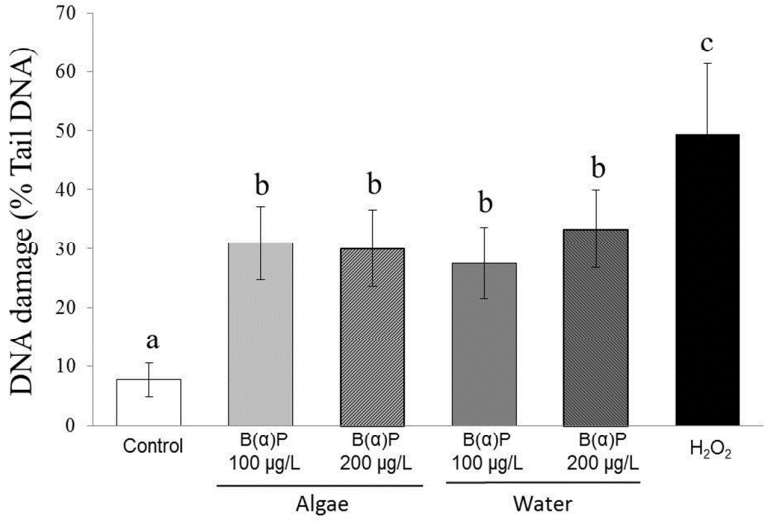
632 in SPMDs and blue mussels (Mytilus edulis). *Chemosphere* **2002,** *46*, (5), 683-688.
633
634

635 **Table 1**: Recoveries (%), RSD (%), MDL and MDL of BDE-209 and DP in water
636 (ng/mL) and mussel (pg/g lw)

Compound	Water				Mussel			
	R	RSD	MLOD	MLOQ	R	RSD	MLOD	MLOQ
BDE-209	75	11	0.40	1.30	68	6	200	330
syn-DP	67	8	0.60	2.00	85	7	5.50	18.3
anti-DP	73	12	0.10	0.30	88	5	4.30	14.3

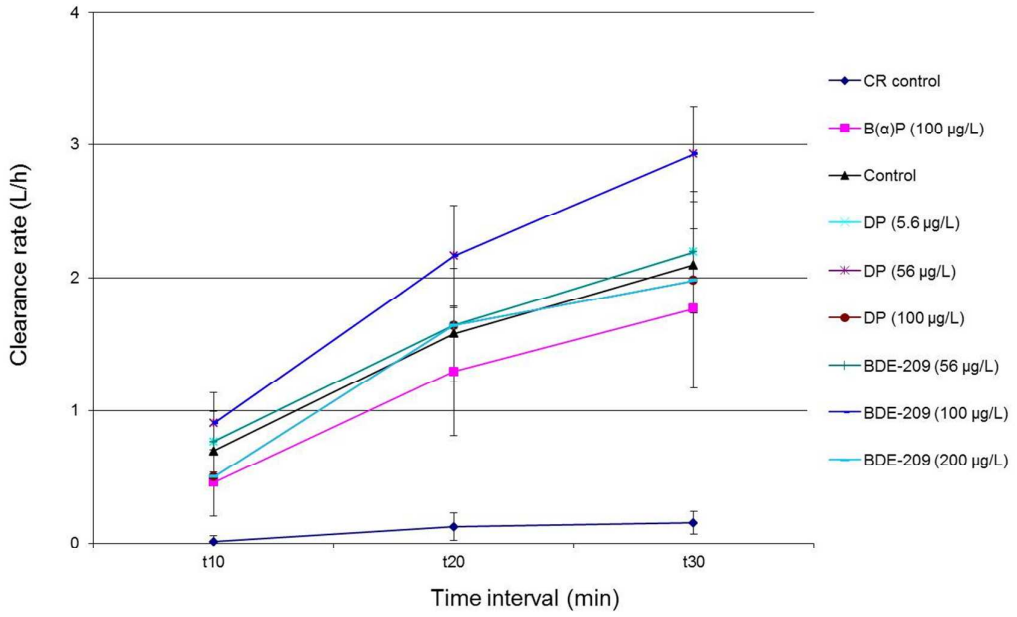
637



638

639 **Figure 1**: DNA damage (mean ± SD; n = 6 per treatment) in benzo(α)pyrene-exposed
640 mussels. Treatments with the same letter are not significantly different; where
641 significant differences occur between treatments, $p < 0.001$.

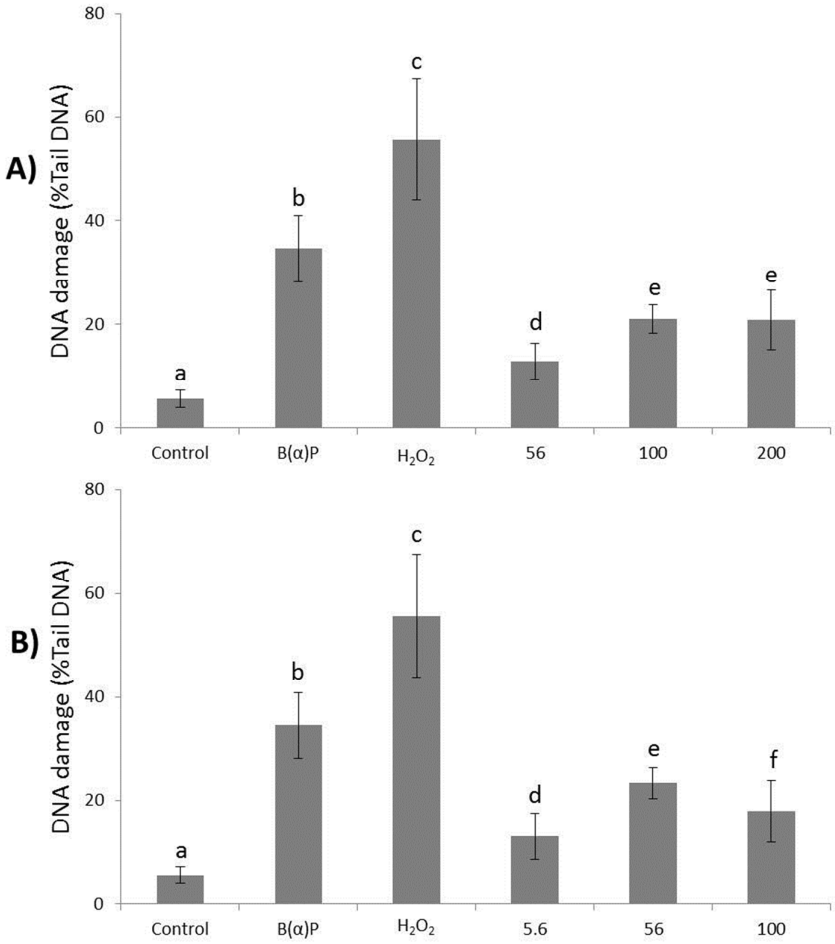
642



643

644 **Figure 2:** Clearance rate (L/h) of the different treatments. Error bars represent SD. n=7

645 CR control = seawater. Control = control mussel exposed to acetone (0.05 %, v/v)



646

647 **Figure 3**: Induction of DNA strand breaks (represented as % Tail DNA ± SD) in
648 *Mytilus galloprovincialis* haemolymph after 6 days of exposure to FRs compared to
649 control mussels, exposure to B(α)P (100 µg/L) and H_2O_2 (1 mM, *in vitro*). **A**) BDE-209.
650 **B)** DP. Treatments with the same letter are not significantly different; where significant
651 differences occur between treatments, $p < 0.05$.

652

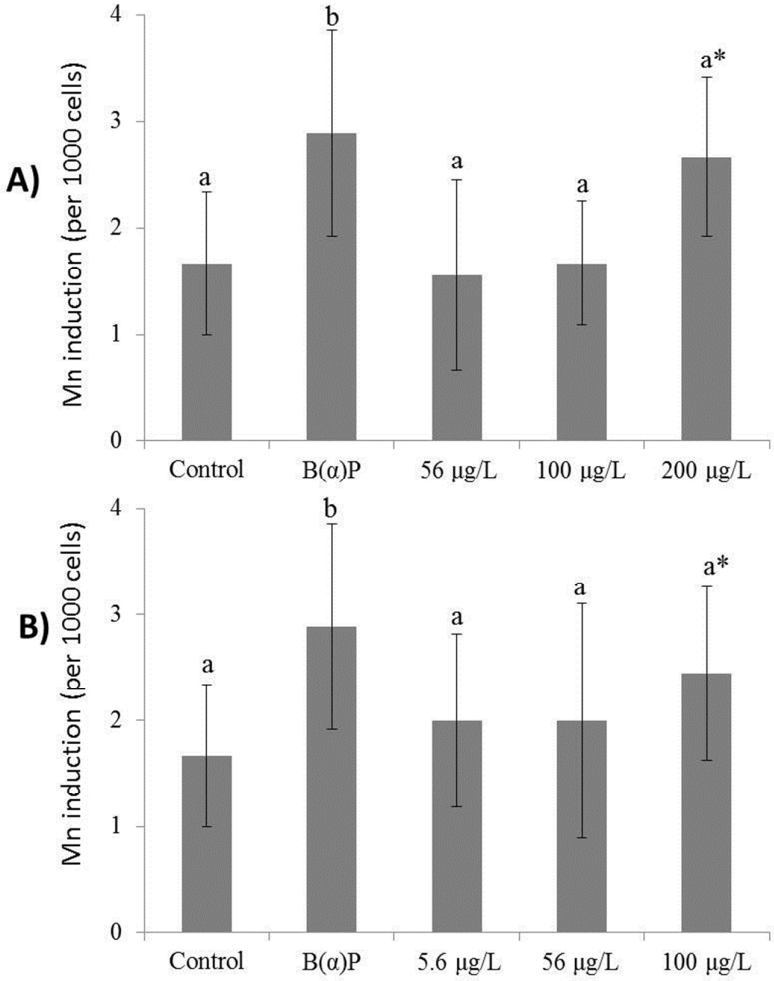


Figure 4: Mn induction (represented as mean ± SD) in *Mytilus galloprovincialis* haemolymph after 6 days of exposure to FRs compared to control mussels and exposure to B(α)P (100 µg/L). **A**) BDE-209. **B)** DP. Treatments with the same letter are not significantly different; where significant differences occur between treatments, $p < 0.05$.

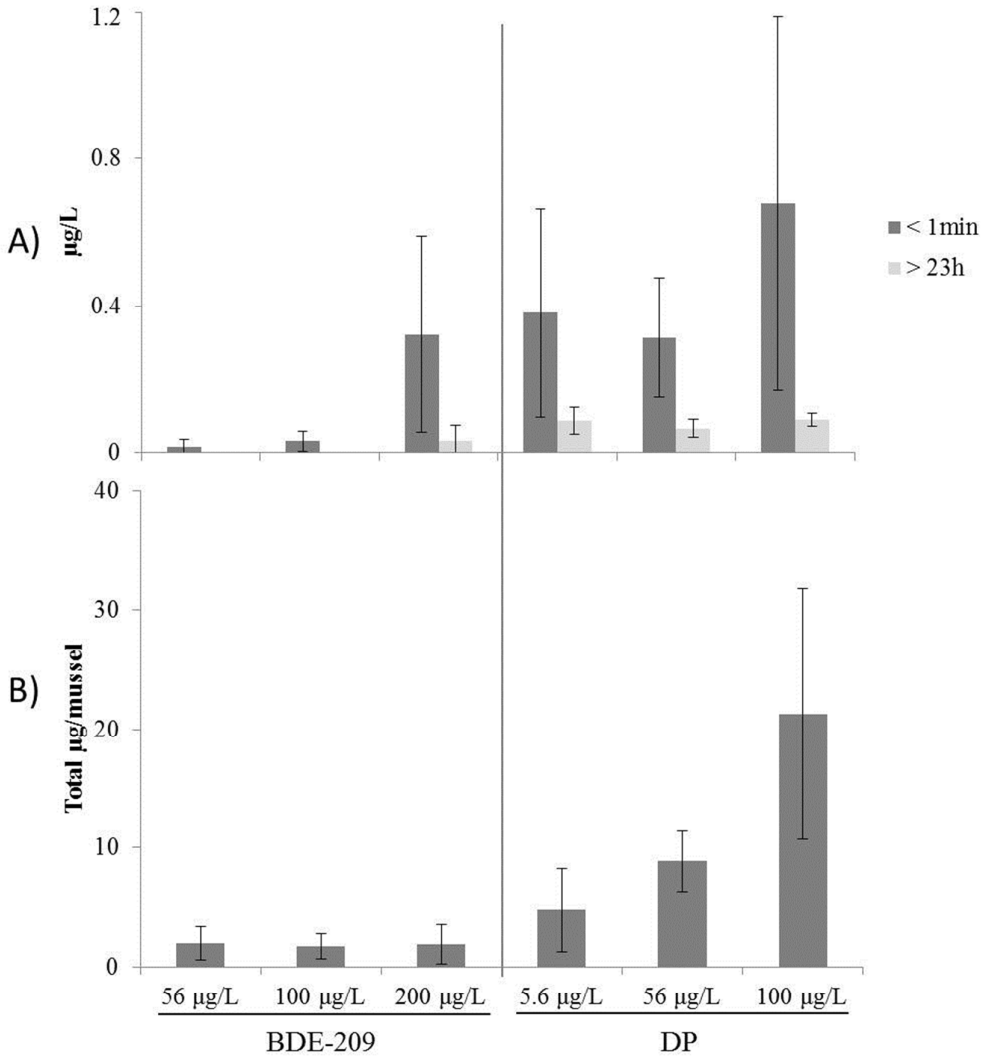


Figure 5: **A)** Concentrations of BDE-209 and DP found in water samples corresponding to the exposures (n=3 per treatment) after dosing and immediately before the water change. Concentrations in control samples were below the MDL in both cases. **B)** Levels of BDE-209 and DP found in exposed mussels (n=7). Control levels were 0.04 ± 0.02 µg for BDE-209 and 0.11 ± 0.06 µg for DP.