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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. 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. PBDEs have been found in a wide range of environmental matrices such as sediment, water, fish or cetaceans, and also in humans. 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 logKow and molecular weight. 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. In fact, BDE-209 was the main PBDE found in species with terrestrial diet and also in mussels. BDE-209 has shown thyroid and endocrine disruption properties and it could affect the liver of fish and mice. Most of the studies are focused in vertebrate models such fish or mice, 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. 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. Toxicity data for DP are still very scarce. In fish, DP affected protein responses in the liver and induced
apoptosis, while it showed genotoxical potential in bacteria as well as histopathological changes in mice liver.

Mussels have proven to be a good tool to evaluate the environmental behavior of organic pollutants. Furthermore, effects of organic pollutants in mussels have been correlated with effects of the same pollutants in humans 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. 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 °C ± 1 °C 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(a)P) was performed. Genotoxic potential of this polycyclic aromatic hydrocarbon (PAH) is well known and it is often used as genotoxic model. Individual mussels were placed in 2 L beakers containing 1.8 L of filtered seawater and exposed to B(a)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
sediments31 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
treatment). \( \text{H}_2\text{O}_2 \) was also used as positive \textit{in vitro} control (1 mM and 30 min of exposure time).

In both experiments, after the six days of exposure mussel haemolymph was extracted from the posterior adductor muscle using an ice-chilled 1 mL syringe and 21G needle and transferred into individual Eppendorf tubes held on ice, following the protocol described by Brown \textit{et al.} (2004).

\textit{Water quality}

Water quality (temperature, salinity, dissolved oxygen and pH) was measured every day for each beaker and three water samples of each treatment were taken immediately after dosing and prior to water change (\textit{i.e} after 23 h of exposure).

Water temperature during the exposure was 16.0 ± 0.5 \(^\circ\text{C}\), salinity was 36.3 ± 0.2‰, dissolved oxygen was 7.93 ± 0.2 mg/L, and pH was 7.92 ± 0.08. No intra- or inter-day variations among treatments were observed (ANOVA and post-hoc Tukey’s test) and these values were considered optimal for the exposures.

\textit{Clearance rate}

Clearance rate (CR) was determined prior to haemolymph collection as described previously.\textsuperscript{34} Mussels were placed in separate 400 mL beakers containing 350 mL seawater (filtered to 0.8 \(\mu\text{m}\)) and a stirring bar. They were allowed to acclimatise at 15 \(^\circ\text{C}\) for 15 min. \textit{Isochrysis galbana} was added in a concentration of 10,000 cells/mL, including several procedural blanks (beaker plus 300 mL of seawater). Aliquots of 20 mL were removed immediately after the addition and after 10, 20 and 30 minutes. These aliquots were analysed on a Beckman Coulter Particle Size and Count Analyser set to count particles between 4 and 10 \(\mu\text{m}\). Three separate counts per mussel were made. CR
was calculated using the equation $CR = \frac{V(\log C_1 - \log C_2)}{t}$, 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.\textsuperscript{35}

Comet assay

Determination of DNA strand breaks using haemocytes was evaluated following a previously optimized protocol.\textsuperscript{36, 37} Slides were pre-coated with normal melting point (NMP) agarose and kept overnight at 20 °C to dry. 150 µL of haemolymph were centrifuged at ~350 g at 4 °C 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;\textsuperscript{38} viability was deemed >95 %. Slides were kept at 4 °C and in the dark for one hour to allow the gel to solidify. In the case of the $H_2O_2$ \textit{in vitro} positive control, after one hour 1 mL of $H_2O_2$ (1 mM) was added dropwise and incubated at 4° C for 30 min. Slides were incubated in lysis solution for one hour and in the dark at 4 °C, 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 °C 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 epifluorescence 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.\textsuperscript{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 H₂O₂ treatment) were analysed. Abnormal comets were excluded from the scoring following the criteria proposed previously. 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. 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 °C 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. 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.

*Chemical analysis*

Regarding water and algae analysis, the methodology described by Di et al. was adopted. Hexane (1 mL) was added to 9 mL of the exposure water samples and internal standards (¹³C-BDE-209 and ¹³C-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. 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 ($\text{H}_2\text{SO}_4(c)$). 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. 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.\textsuperscript{28, 47, 48} For instance, Di et al. report 60% damage following a 12 days \textit{in vivo} exposure \textit{Mytilus edulis}.\textsuperscript{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.

\textit{Clearance rate}

It has been previously demonstrated that CR in mussels can be affected by several chemical contaminants.\textsuperscript{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\textsuperscript{28} and suggests that mussels can take up these types of compounds without showing significant physiological changes.\textsuperscript{38}
Comet assay

In all cases, DNA strand breaks observed were significantly higher than the negative control (ANOVA and Tukey’s test, \( p < 0.001 \)) (Figure 3). \textit{In vivo} positive control, B(α)P, caused an effect of 35 ± 6\% (mean % tail ± SD), while the \textit{in vitro} positive control, H\(_2\)O\(_2\), resulted in 56 ± 10\%. Damage induced by BDE-209 was 13 ± 3\%, 21 ± 3\% and 21 ± 6\% for 56, 100 and 200 µg/L exposure concentrations, respectively. Damage induced by DP was 13 ± 4\%, 23 ± 3\% and 18 ± 6\% for 5.6, 56 and 100 µg/L exposure concentrations, respectively. For BDE-209, DNA damage displayed a significant increase from 56 to 100 µg/L treatments, but no increase was observed from 100 to 200 µg/L treatments. Concerning DP, DNA damage induced by the 56 µg/L was higher than the 5.6 µg/L treatment. However, damage induced by the highest concentration (100 µg/L) was less than that induced by 56 µg/L. It has been described that DNA repair mechanisms can affect the response of the mussels to organic contaminants, since the simple breaks mainly produced by these compounds might be repaired by base excision (BER). Furthermore, reduction of the DNA damage in the most concentrated treatments could be caused by the exclusion of the apoptotic cells of the cell count. Comparison between BDE-209 and DP exposures at 56 and 100 µg/L showed that DP at 56 µg/L induced oxidative damage at the same level as BDE-209 at 100 µg/L (23\% and 21\%, respectively), while DNA strand breaks induced by BDE-209 at 56 µg/L were in the same level as the low level of DP (13\% and 13\%, respectively). Surprisingly, oxidative damage induced by DP at 100 µg/L (18\%) was lower than at 56 µg/L (or following BDE-209 exposure at 100 and 200 µg/L) (Figure 3). This difference might be attributed to possible differences in BDE-209 and DP metabolization. In contrast, de-bromination products of BDE-209 are often more toxic than parent BDE-209. Furthermore, BDE-209 presents a more complex metabolism since low-
brominated OH-PBDEs could also be formed.\(^5^3\) However, this has not been studied in mussels and the % of PBDEs metabolized to OH-PBDEs seems to be low even in mammals.\(^5^4,^5^6\)

Hence, results presented demonstrate that BDE-209 and DP can both induce DNA strand breaks in mussels. This is in agreement with what previously reported effects in zebra mussel (\textit{Dreissena polymorpha}) where, similar to this study, BDE-209 caused non-dose dependant DNA damage after an \textit{in vivo} exposure of 7 days to 0.1, 2 and 10 \(\mu\)g/L.\(^5^7\) \textit{In vivo} exposures of BDE-47, BDE-100 and BDE-154, also in zebra mussel, caused significant DNA damage up to 5, 11 and 12% respectively (expressed as % tail DNA; controls up to 5%). These values are lower than those reported in this study, but exposure concentrations (0.1, 0.5 and 1 \(\mu\)g/L) and exposure time (4 days) were also lower.\(^5^8\) To our knowledge, this is the first study reporting the oxidative capacity of DP in mussels.

\textit{Mn assay results}

Mn induced in the negative control were 1.7 ± 0.6, while in the positive B(\(\alpha\))P control were 2.9 ± 1\%, representing a significant 2 fold increase (ANOVA and post-hoc Tukey’s test, \(p<0.05\)). Concerning BDE-209, inductions were 1.6 ± 0.9, 1.7 ± 0.6 and 2.7 ± 0.7 for 56, 100 and 200 \(\mu\)g/L treatments, respectively. The first two concentrations did not cause significant Mn induction compared to controls, but Mn induced by 200 \(\mu\)g/L exposure was significantly higher (ANOVA and post-hoc Tukey’s test, \(p<0.05\)). Furthermore, DP caused Mn inductions of 2.0 ± 0.8, 2.0 ± 1 and 2.5 ± 0.8 at 5.6, 56 and 100 \(\mu\)g/L treatments, respectively (Figure 4). In this case, BDE-209 and DP showed the same pattern, \textit{i.e}, Mn induction was only significant at the highest level of exposure. Consequently, DP showed an effect at a lower concentration than BDE-209 (100 and
200 µg/L, respectively) which implies that DP is more capable of causing this kind of
damage. However, no other studies are available to corroborate this statement.

Mn induced by BDE-47, BDE-100 and BDE-154 in zebra mussel were up to 2, 2 and
2.5, respectively, but inductions were not significantly different than negative controls. Furthermore, both exposure concentrations (0.1 µg/L, 0.5 µg/L and 1 µg/L) and
exposure time (4 days) were lower than our conditions. This is in agreement with our
study, where Mn induction was only found at the highest exposure concentrations. Riva
et al. (2007) also reported that BDE-209 can induce DNA strand breaks, but not Mn
induction. Oxidative stress induced by reactive oxygen species (ROS) has been
described as one the most plausible mechanism of the toxicity of BDE-209. As a
result, de-bromination of BDE-209 was also considered, since less brominated BDEs
present higher oxidative capacity. In this case, no other brominated congeners were
detected (see results below), probably because metabolic/enzymatic capacity of mussels
is not as high as in fish. Comet and Mn assay results were not correlated either for BDE-
209 or DP. This might indicate that these compounds induce primary and repairable
lesions rather than permanent ones since their genotoxic induction can arise through
several pathways. However, this topic still requires further work in order to truly
understand how these pollutants induce oxidative DNA damage.

Chemistry results

Water analysis: Concentrations found in water samples taken immediately after dosing
were, expressed as mean ± SE (µg/L): 0.02 ± 0.01, 0.03 ± 0.02 and 0.3 ± 0.2 in BDE-
209 treatments (56, 100 and 200 µg/L, respectively). Compared to values found after 23
h of exposure, concentrations in water decreased 92, 97 and 90%, respectively; in all
cases concentrations after 23 h were lower (One-way ANOVA, p<0.05). Similarly,
concentrations of DP immediately after dosing were 0.4 ± 0.3, 0.3 ± 0.2 and 0.7 ± 0.5 µg/L in 5.6, 56 and 100 µg/L treatments, respectively. These concentrations decreased significantly (one-way ANOVA, \( p < 0.05 \)) up to 77%, 79% and 86%, respectively, after 23 h. Levels in control water were below MDL for both compounds in all cases (Figure 5A). Concentrations used in this study exceeded the estimated solubility of these compounds (< 1 µg/L). However, it has been demonstrated that presence of dissolved organic matter enhances solubility. BDE-209 and DP rapidly distributes between particulates and mussels, thus concentrations in the aqueous phase are expected to be low.

Mussel analysis: Levels of BDE-209 found in the exposed mussels at the end of the treatment were always substantially higher than those in the controls, proving that mussels bioaccumulated BDE-209 through the \textit{in vivo} exposure (ANOVA and post-hoc Tukey’s test, \( p < 0.05 \)). Values were 1.9 ± 1.3, 1.7 ± 1.1 and 1.6 ± 1.2 µg/mussel, corresponding to the 56, 100 and 200 µg/L exposures. No differences were observed between the three exposures (ANOVA and Tukey’s test, \( p > 0.05 \)). This could be due to BDE-209 de-bromination, but while it has been described in fish to the best of our knowledge there are no studies in mussels. During the instrumental analysis no other peaks with \( m/z \) 79 and \( m/z \) 81 were observed. Hence, no lower brominated PBDEs or MeO-PBDEs were present in the mussels above the limits of detection. On the other hand, values found in mussels exposed with DP were 4.7 ± 3.1, 8.8 ± 2.1 and 21 ± 9.1 µg/mussel, corresponding to the 5.6, 56 and 100 µg/L treatments, respectively. As for BDE-209, DP values were significantly higher than in the controls in all cases (ANOVA and post-hoc Tukey’s test, \( p < 0.05 \)). Furthermore, in the case of DP a concentration dependant increase was found (ANOVA and post-hoc Tukey’s test, \( p < 0.05 \)). These
results show that DP is bioaccumulated by mussels, as has been previously reported.\textsuperscript{11} Moreover, the ratio between the anti-isomer and the total DP burden was also evaluated. $F_{\text{anti}}$ is defined as the concentration of anti-DP with respect to the total DP concentration, both lipid-normalized. It has been described as a good indicator of the different behaviour of the two isomers in the environment, since the initial $F_{\text{anti}}$ in the commercial mixture (~0.7) can change when analysing complex organisms such as dolphins.\textsuperscript{18} $F_{\text{anti}}$ values found in mussels from the three different exposures (0.74 ± 0.02, 0.69 ± 0.03 and 0.73 ± 0.02 for low, medium and high levels, respectively) were similar and significantly lower than values found in the control mussels, which were up to 0.79 ± 0.04 (ANOVA and post-hoc Tukey’s test, $p<0.05$). The commercial mixture of DP used in the exposure was also analysed (n=3, 0.72 ± 0.02). Even if values of the exposed mussels were different than controls, values are still in the range described for commercial DP mixtures. Thus, no syn-DP enrichment was observed, which is in agreement with other studies of DP in mussels.\textsuperscript{11} It has been described that the particulate matter in the gastro-intestinal tract can affect BDE-209 determinations in mussels.\textsuperscript{63} However, since mussels were sampled 24 h after the last feeding, influence of ingested food in BDE-209 analysis was considered to be minimal, as has been suggested previously.\textsuperscript{57}

Overall, these data confirm the use of \textit{M. galloprovincialis} as a suitable biological model for \textit{in vivo} exposures to FRs. In addition, data for DP represents the first evidence of a genotoxic capacity of this compound in mussels. Both DP and BDE-209 induced significant DNA damage even at the lowest selected concentrations, whereas Mn induction was only significant in the highest doses. Other factors such as the timeframe 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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Table 1: Recoveries (%), RSD (%), MDL and MDL of BDE-209 and DP in water (ng/mL) and mussel (pg/g lw)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Water</th>
<th>Mussel</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>R</td>
<td>RSD</td>
</tr>
<tr>
<td>BDE-209</td>
<td>75</td>
<td>11</td>
</tr>
<tr>
<td>syn-DP</td>
<td>67</td>
<td>8</td>
</tr>
<tr>
<td>anti-DP</td>
<td>73</td>
<td>12</td>
</tr>
</tbody>
</table>
Figure 1: DNA damage (mean ± SD; n = 6 per treatment) in benzo(α)pyrene-exposed mussels. Treatments with the same letter are not significantly different; where significant differences occur between treatments, p < 0.001.
Figure 2: Clearance rate (L/h) of the different treatments. Error bars represent SD. n=7

CR control = seawater. Control = control mussel exposed to acetone (0.05 %, v/v)
Figure 3: Induction of DNA strand breaks (represented as % Tail DNA ± SD) in *Mytilus galloprovincialis* haemolymph after 6 days of exposure to FRs compared to control mussels, exposure to B(α)P (100 µg/L) and H₂O₂ (1 mM, *in vitro*). A) BDE-209. B) DP. Treatments with the same letter are not significantly different; where significant differences occur between treatments, *p* < 0.05.
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.