Study of the toxic effects of flame retardant PBDE-47 on the clam Chamelea gallina *(Linnaeus, 1758)*

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Bioaccumulation, *Chamelea gallina*, Clam, Flame retardant, Food chain, PBDE-47, *Venus gallina*.

Summary
The purpose of the study is to evaluate the effects of 2,2',4,4'-tetrabromodiphenylether (PBDE-47) on the *Chamelea gallina* clam (according to current commercial regulations: *Venus gallina*). PBDEs, which are used as flame retardants in various industrial products, are classed as hazardous substances by Directive 2011/65/EU. They are bioaccumulative compounds, considered to be endocrine disruptors, genotoxic, neurotoxic and practically ubiquitous, and their concentration in the environment has considerably increased in recent years. The aim of this study is to establish the effects of PBDE-47 on *Chamelea gallina*: toxic power and any harmful effects on the gonads, bioaccumulation capacity in the tissues, and possible entry into the food chain. The research used 96-hour and 21-day experimental tests on clams housed in filtered seawater. The tests were preceded by a period of acclimatisation of the molluscs lasting five to seven days. The clams were fed on seaweed (*Dunaliella tertiolecta*). The choice of the toxic compound PBDE-47 was based on the high concentration, among the congeners of PBDE, found in some aquatic species. The study demonstrated that the concentration of the contaminant used did not alter the vital functions, cause significant levels of mortality or lead to evident alteration in the gonads of *Chamelea gallina*. However, the research demonstrated the bioaccumulation capacity of the bivalve mollusc, allowing PBDE-47 to enter the food chain.

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Introduction

The flame retardants known as polybromodiphenylethers (PBDE) are hydrophobic chemical compounds included among the hazardous substances contemplated by Directive 2011/65/EU (26). They are commonly used in the textile and electronics industries, and in the manufacture of plastic packaging and construction materials, because they possess the ability to delay the spread of flames in case of fire. Their use has increased in recent years, with a consequent increase in their concentration in the environment (1, 22). PBDEs are additives that are mixed with polymers of various kinds, and are not chemically bonded to plastics or tissues. This characteristic can cause their gradual release over time, which reduces the flameproofing properties of the product, and also increases the risk of environmental contamination (22). These substances can be released into the environment at the stage of manufacture, use, disposal by tipping, during incineration operations and through industrial wastewater (7, 27).

These environmental pollutants have become ubiquitous (in water, air and soil) (4, 6, 22, 24). They have been found in fish, birds, bivalve molluscs, marine mammals, and in human milk, adipose tissue, human blood and serum (13, 22, 24). PBDEs are considered to be persistent, stable, not very sensitive to chemical and biological degradation processes, and able to perform their contaminant action even at a considerable distance from the place of emission (22, 24). They are known as bioaccumulative (14, 22, 24), endocrine disrupting (1), neurotoxic (17, 24) and genotoxic compounds with the ability to induce chromosomal aberrations (3, 7). Harmful effects on the gonads of bivalve molluscs (mussels) are reported in the literature (1).

The clam *Chamelea gallina* (Figure 1), an endobenthic bivalve mollusc, which lives in high-density shoals on sandy or sandy/muddy sea beds, has a good power of bioaccumulation of environmental contaminants. Its diet, based on microphagy by means of a filtering system (15, 18), makes the clams particularly sensitive to water quality, particulate matter in suspension and chemical stresses. In general, the health of fish

Figure 1. Chamelea gallina. *lavalledelcesano.it.*

stocks reflects the conditions of the environment in which they live. This yields the possibility of using ecotoxicology studies on bivalve molluscs to determine the health of fish stocks (namely fish products destined for human consumption) and the quality of aquatic ecosystems.

The present study was designed to evaluate, by simulating a polluting event in the aquarium, the toxic power and possible harmful effects of flame retardant 2,2',4,4'-tetrabromodiphenylether (PBDE-47) on the reproductive apparatus of clams. The ability to bioaccumulate the toxic compound in the tissues of the mollusc was also evaluated in order to establish whether it could enter the food chain. PBDE-47 was selected in view of the high concentration of this substance found in aquatic species (11, 19). The present study, relating to project IZSAM 09/07 RC - Study of the toxic effects of PDBE-47 flame retardants on the *Chamelea gallina* clam - was funded by the italian Ministry of Health.

Materials and methods

Sampling plan and sample taking

The study was conducted at the Water Biology Centre of the 'G. Caporale' Institute. Specimens of *Chamelea gallina* (Superkingdom: Eukaryota, Kingdom: Animalia, Phylum: Mollusca, Class: Bivalvia, Order: Veneroida, Family: Veneridae, Genus: *Chamelea*, Species: *Gallina*) were used. Clam and seawater specimens for the aquariums were taken monthly from a sampling point (Latitude: 42° 27' 279 N and Longitude: 14° 15' 865 E) situated approximately 500 m from the coast of Abruzzo, to the south of Pescara harbour, at a depth of approximately 6 m (Figure 2).

Ecotoxicology tests

The molluscs sampled were transferred to a portable refrigerator, immediately conveyed to the laboratory under refrigeration, and selected on the basis of minimum marketable size (25 mm ± 10%). The specimens were divided between ten aquariums made of toughened glass (40x25x28 cm), and fitted with oxygenation and mechanical water filtration devices. 16 litres of seawater, prefiltered by a device consisting of three polypropylene fibre cartridges arranged in succession (25 µm, 10 µm and 1 µm), were used for each aquarium. The aquariums were located in a thermostat-controlled chamber with a constant temperature of 19 ± 1°C, light intensity of 65 ± 30 lux, and photoperiod of 16 hours' light and 8 hours' darkness.

The clams were housed for an acclimatisation period of five to seven days (Figure 3). The molluscs were fed on *Dunaliella tertiolecta*, a unicellular seaweed (Figure 4) grown in the laboratory on Guillard's-F/2 50X concentrated (20 ml x 1 L of seawater) culture

Figure 2. *Sampling point.* Latitude: 42° 27' 279 N, Longitude: 14° 15' 865 E. *Source*: Google maps.

Figure 3. *Acclimatisation phase of* Chamelea gallina.

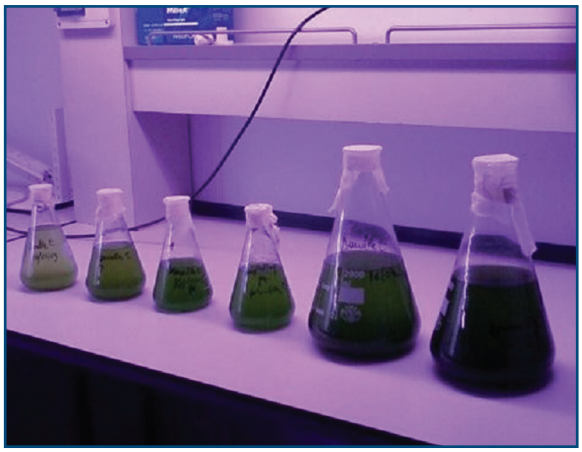

Figure 4. *Coltivation of seaweed* (Dunaliella tertiolecta).

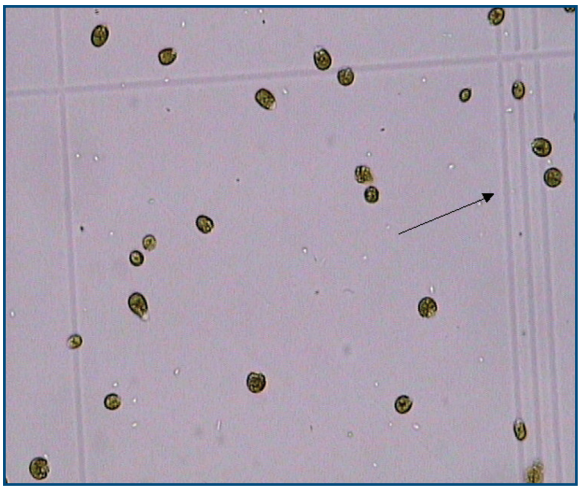

Figure 5. Dunaliella tertiolecta *(20X)) in Fuchs Rosenthal chamber.* A detail of the counting grid, arrow on the right.

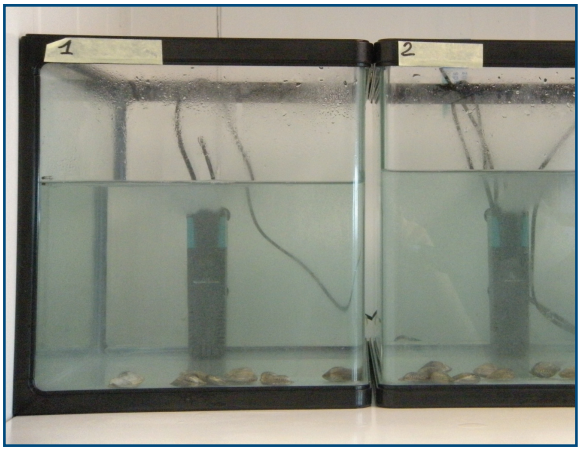

Figure 6. *Ecotoxicology assay with PBDE-47.*

medium (Marine Water Enrichment Solution SIGMA Aldrich®, USA) (2). Before the administration of *Dunaliella tertiolecta*, the seaweed cells were recovered from the culture medium by centrifugation (400 RCF x 10 min at 4°C) (RCF: *relative centrifugal force*), counted in a Fuchs Rosenthal chamber with an optical microscope (10-40X) (Figure 5) and used at the concentration of 5-15 x 10^3cells/ml (2). As described in the literature, the clams were fed on the first and fourth days (2, 23).

The study used seven ecotoxicology tests, each at a different PBDE-47 concentration (0.25, 0.50, 1.0, 3.0, 9.0, 15.0 and 30.0 µg/L) (Figure 6). Each test involved a control test without the addition of the toxic compound. For the first test only (0.25 µg/L) a "solvent control" test with the sole addition of acetone (<10 mg/l), a diluent of PBDE-47 was used. All the tests involved five replications, conducted with the same number of molluscs (12 clams) under the same environmental conditions. The number of dead molluscs, the chemico-physical parameters of the water (temperature, salinity, pH and dissolved oxygen) and the ammonia concentration were recorded every day. PBDE-47 (ChemService, Inc., West Chester, PA, USA) dissolved in acetone was added to the aqueous medium at known concentrations. The range of concentrations of the toxic compound and the exposure times were chosen according to the criteria reported in the literature and on the basis of the preliminary results obtained during the study (1, 10). The concentration of acetone in water was less than 10 mg/L in all the tests, ie. below the No Observed Effect Concentration (NOEC) (1).

For each PBDE-47 concentration the toxic effects were evaluated after 96 hours (mortality), and the harmful effects on the reproductive apparatus were evaluated after 21 days. The test at the PBDE-47 concentration of 0.5 µg/L was terminated early, after 14 days, due to a malfunction in the aeration system. During the 96-hour ecotoxicology tests the molluscs were not fed (2, 23), whereas in the 21-day tests, food was administered twice a week. At the end of each 21-day test, for each concentration of toxic substance, histological tests were conducted on the gonad tissues of the exposed molluscs and the controls. The PBDE-47 concentration in the tissues was also determined, to establish whether bioaccumulation took place.

Histological tests

The samples of clams, shelled and preserved in 10% formalin (v/v), underwent histological tests of the gonads, in order to investigate any alteration, at the Veneto Experimental Animal Disease Prevention Institute's National Reference Laboratory for mollusc diseases. A statistically representative number of clams (20-30 specimens) was examined for each test. The test was performed as reported in the literature (5).

Analytical determination of PBDE-47

To determine the levels of PBDE-47 in the clam tissue, a validated method for the determination of 9 polybromodiphenylethers (PBDE-28-47-66-85-99-100-153-154-183) in food matrices was optimised. The method involved the use of the isotope dilution technique, high-resolution gas chromatography (HRGC) separation and high-resolution mass spectrometry (HRMS) detection.

Samples (5-10 grams) were mixed with a quantity of diatomaceous earth two-to-three times greater than the weight of the aliquot and kept overnight in a stove at 40°C. The mixture of 'internal standards', consisting of seven PBDEs labelled with carbon 13, representative of the analytes being tested, was added to each sample. The samples were then extracted with an acetone/hexane (20:80, v/v) mixture by accelerated extraction with solvent, using an ASE® 200 extraction system (Dionex, Sunnyvale, California, USA) at the pressure of 1,500 psi and the temperature of 125°C.

After careful solvent evaporation, gravimetric lipid determination was performed. The sample purification was performed in two steps. Firstly, the extract dissolved in hexane was subjected to liquid-liquid partitioning (passage through concentrated H_2SO_4 and saturated aqueous solution of NaCl). After this, the extract was purified by a multilayer chromatography column manually packed with 3 grams of neutral silica gel, 4 grams of silica gel impregnated with 44% (w/w) H_2SO_4 and 1 gram of anhydrous sodium sulphate. Interfering substances were removed with hexane, and PBDEs were eluted with a mixture of dichloromethane/hexane (10:90, v/v).

The final extract was evaporated to dryness under nitrogen stream and immediately dissolved with an injection of standard solution constituting of two PBDEs labelled with carbon 13 (different from the preceding ones). The solution obtained was injected into a high-resolution gas chromatograph and the test compounds were detected by a mass spectrometer operating with a resolution (R) higher than 9,500 in single ion monitoring.

The instrumental analysis was conducted using an HRGC-HRMS system constituting of a Trace Series 2000 capillary gas chromatograph (ThermoQuest CE Instruments, Milan, Italy) coupled to a MAT 95 XP mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The analysis was conducted on a DB-5 MS capillary column (60 m x 0.25 mm, 0.10 µm, J&W Scientific, California, USA). All the reference standard solutions were obtained from Wellington Laboratories Inc. (Ontario, Canada).

Results and Discussion

The values of the chemico-physical parameters considered remained within the following ranges:

- ammonia concentration (NH3): < 0.25 mg/L;
- water temperature: 18.5 – 20.7°C;
- salinity: 34.4 – 39.6 g/L;
- pH: 8.22 – 8.37;
- dissolved oxygen: 8.69 – 9.27 mg/L.

During the acclimatisation stage in the aquarium, a mortality rate of less than 10% was recorded for the clams (Figure 7). In the 96-hour toxicity test, it always remained below 10% for all the concentrations specified.

The specimens used showed no apparent signs of suffering; feeding normally and showing half-closed valves with the siphons clearly visible on the exterior (Figure 8). Specifically, observation of the molluscs

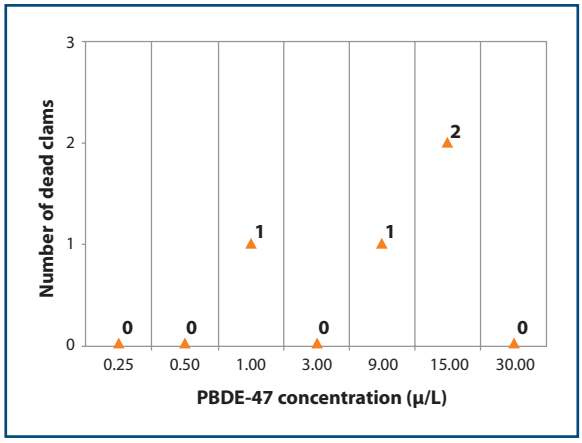

Figure 7. *Number of clams that died at the acclimatisation stages for each of the 7 toxicity tests.*

Figure 8. *Clams in aquarium: specimen with the siphons on the exterior.*

showed one dead clam at PBDE concentrations of 0.50, 1.0, 9.0 and 15.0 µg/L (mortality = 1.67%). Univariate analysis of variance (ANOVA) did not reveal any statistically-significant differences (F = 0.500; p = 0.808) between the mortality values observed at the different concentrations (Table I). The mortality rates observed at all concentrations were not statistically different from expected (0%); in fact, all the confidence intervals included 0% (Table II). No dead molluscs were found at any of the other concentrations used (Figure 9).

The histological tests did not detect any alteration in the gonad tissue at the end of each toxicity test (21 days). Figures 10 and 11 show, by way of example, at the value of 15 µg/L, histological preparations of male and female gonads seen under the optical microscope.

Mortality among the bivalve molluscs exposed to the toxic compound was always below 10%, even when the tests continued for 14 and 21 days, with three dead clams at PBDE concentrations of 0.50 µg/L and two deaths at PBDE concentrations of 1, 9 and 15 µg/L (Figure 12) (mortality rates of 5% and 3.33% respectively). These values are not significant, because univariate ANOVA did not detect any statistically-significant differences (F = 1.250; p = 0.279) between the mortality values observed at the different concentrations (Table III). The mortality rates observed at all concentrations were not statistically different from expected (0%); in fact, all the confidence intervals included 0% (Table IV).

Tissue contamination levels

Table V shows the results of PBDE-47 levels found in the tissue of clams exposed to increasing concentrations of the toxic substance in the 21-day tests. The results of the tests at PBDE-47 concentrations of 9 and 15 µg/L are not shown, due to analytical problems at the sample extraction step; low recovery of the internal standard prevented a reliable determination of the analyte tested, and as the sample was unique, the test could not be repeated.

The PBDE-47 levels in the tissue were reported for wet weight, dry weight and lipid basis. For the effects of concentration on the dry weight, reference was made to a mean moisture value of 80.6%, obtained from a study conducted on samples of *Chamelea gallina* obtained in the Adriatic Sea (21). This approach was used because the small number of specimens constituting the sample was insufficient for simultaneous determination of moisture and PBDE-47.

The lipid fraction was determined in all samples. The values obtained did not show any significant differences according to sampling period. The lipids values were between 0.73% and 1.16%, with a mean value of 0.98%.

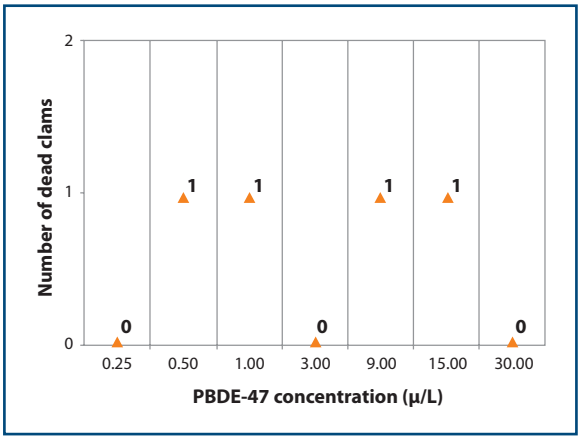

Figure 9. *Number of dead clams for each PBDE-47 concentration used in the 96-hour toxicity tests.*

Table I. *Univariate analysis of variance (Anova) method (96-h Test).*

Source	Sum of squares	Degree free	Mean of squares	Fisher's F	*P* value
Concentration	0.029	6	0.005	0.500	0.808
Residues	3.933	413	0.010		
Total	3.962	419			

Table II. *Estimation of expected mortality of the bivalve molluscs used in the study (96-h Test).*

Concentration (PBDE-47 µg/L)	Mortality	Student's t test	*P* value	Confidence interval (95%)	
				Lower limit	Upper limit
0.25	0.00%	0.000	1.000	-3.50%	3.50%
0.5	1.67%	0.935	0.350	-1.84%	5.17%
1	1.67%	0.935	0.350	-1.84%	5.17%
3	0.00%	0.000	1.000	-3.50%	3.50%
9	1.67%	0.935	0.350	-1.84%	5.17%
15	1.67%	0.935	0.350	-1.84%	5.17%
30	0.00%	0.000	1.000	-3.50%	3.50%

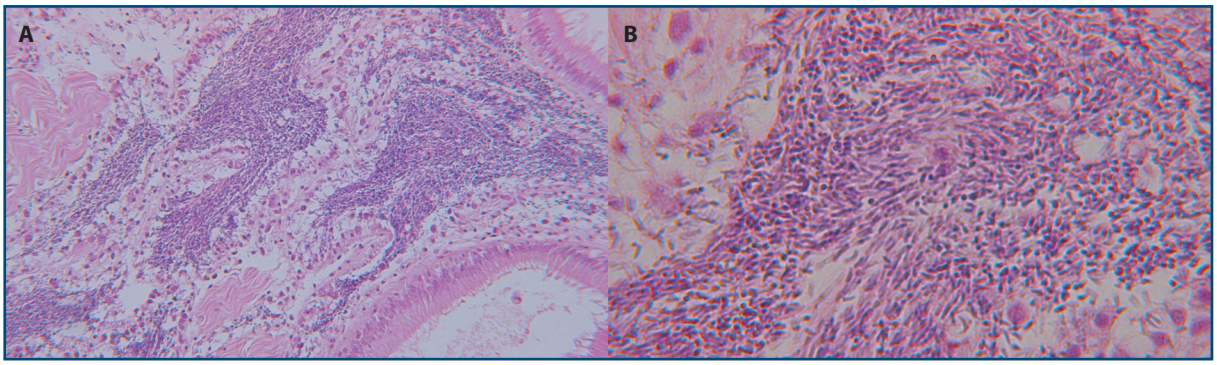

Figure 10. *Histological section of the male gonad without alteration and with good production of gametes of* Chamelea gallina *exposed to 15 µg PBDE-47/L.* (A) 100X; (B) 400X.

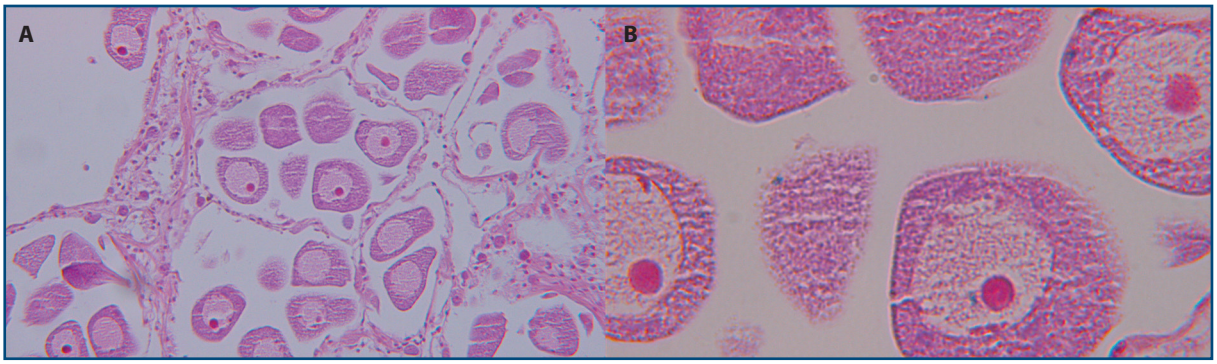

Figure 11. *Histological section of female mature gonad without alteration of* Chamelea gallina *exposed to 15 µg PBDE-47/L.* (A) 100X; (B) 400X.

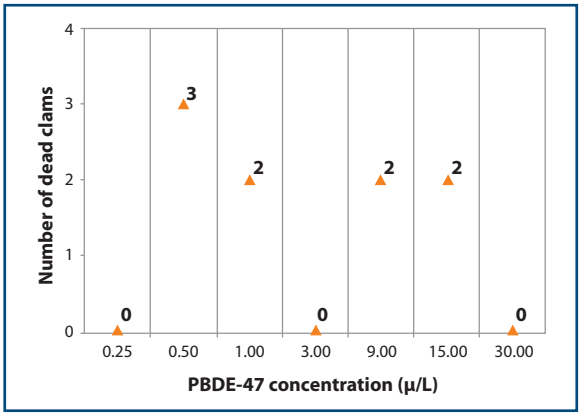

Figure 12. *Number of dead clams for each PBDE-47 concentration in the 21-day toxicity tests.* The study conducted at the concentration of 0.5 µg/L was terminated after 14 days.

Table III. *Univariate analysis of variance (Anova) method (21day -Test).*

Source	Sum of squares	Degree free	Mean of squares	Fisher's F	*P* value
Concentration	0.157	6	0.026	1.250	0.279
Residues	8.650	413	0.021		
Total	8.807	419			

Table IV. *Estimation of expected mortality of the bivalve molluscs used in the study (21-day test*).*

Concentration (PBDE-47 µg/L)	Mortality	Student's t test	*P* value	Confidence interval (95%)	
				Lower limit	Upper limit
0.25	0.00%	0.000	1.000	-5.19%	5.19%
0.5	5.00%	1.892	0.059	-0.19%	10.19%
1	3.33%	1.262	0.208	-1.86%	8.53%
3	0.00%	0.000	1.000	-5.19%	5.19%
9	3.33%	1.262	0.208	-1.86%	8.53%
15	3.33%	1.262	0.208	-1.86%	8.53%
30	0.00%	0.000	1.000	-5.19%	5.19%

* The study conducted at the concentration of 0.5 µg/L was terminated after 14 days

Table V. *Analytical values of the lipid fraction and the PBDE-47 concentrations in the tissues at the end of each 21-day toxicity test*.*

PBDE-47 H_2O (ng/L)	Control lipids (%)	Control PBDE-47 (ng/kg)	Control PBDE-47 (ng/kg dry weight)	Control PBDE-47 (ng/kg fat)	Exposed lipids (%)	Exposed PBDE-47 (ng/kg)	Exposed PBDE-47 (ng/kg dry weight)	Exposed PBDE-47 (ng/kg fat)
250	1.07	119	613	11,200	0.97	110×10^3	566×10^3	$11,300 \times 10^3$
500	0.84	130	670	15,500	0.73	97.3×10^3	501×10^3	$13,300 \times 10^3$
1,000	1.03	118	608	11,500	1.14	122×10^3	627×10^3	$10,700 \times 10^3$
3,000	1.08	118	608	10,900	1.14	394×10^3	$2,030 \times 10^3$	$34,600 \times 10^3$
9,000	1.16	96.0	495	8,300	1.05	-	-	-
15,000	0.77	116	598	15,200	0.90	-	-	-
30,000	0.90	120	619	13,300	0.95	$5,870 \times 10^3$	$30,300 \times 10^3$	$618,000 \times 10^3$

* The study conducted at the concentration of 500 ng/L was terminated after 14 days.

The PBDE-47 levels found at the end of the toxicity test in the control groups were negligible compared with those detected in the exposed individuals, with values of between 96 ng/kg and 130 ng/kg. These values are comparable to, and sometimes lower than, those found in monitoring studies conducted on some species of bivalve molluscs in the European and Asian regions (8, 16, 19, 20, 25). Analysis of the clams not exposed to the contaminant showed a contamination profile (ratios of the nine congeners determined) comparable with those found in other aquatic organisms and reported in the literature (11, 19). In a bioaccumulation study conducted on mussels, it was found that the abundance of congeners 47 and 99 in the tissues may be associated with more efficient mechanisms of intake and accumulation of the two congeners compared with other PBDEs (20).

The PBDE-47 levels in the tissue of the clams were 1,000 to 50,000 times higher in the exposed groups than the corresponding controls (Table V). These levels showed a slight, though non-linear, increase as the concentration of the toxic substance in water increased. On a wet-weight basis, the contamination levels were between 110×10^3 ng/kg in the clams exposed to the minimum concentration of 250 ng/L of PBDE in water, and $5,870 \times 10^3$ ng/kg for those exposed to the maximum concentration of 30,000 ng/L.

In different exposure studies relating to aquatic organisms and persistent organic contaminants such as PCB and PBDE, a linear ratio was identified between the quantity of contaminant determined in the tissue and the hydrophobic characteristics of the compound tested, the latter being described by the logarithm of its octanol-water partition coefficient ($\log k_{ow}$). The data in the literature give a log kow value between 6.0 and 6.8 (10, 12) for PBDE-47. A high log kow value indicates that the compound has marked hydrophobic characteristics and is potentially hazardous because it easily accumulates in the adipose tissues of clams by means of passive diffusion mechanisms.

Although the PBDE-47 concentrations used in the toxicity tests were much higher than those found in particularly polluted areas (the maximum values reported in the literature are approx. 0.5 ng/L), the study demonstrated the ability of the clam to bioconcentrate the contaminant in question, with the consequent possibility of its introduction into the food chain (16). Bioconcentration is the result of direct intake of a chemical substance by an organism through water. The bioconcentration factor (BCF) is defined as the ratio, at the steady state (absorption rate equal to elimination rate), between the concentration of contaminant in the aquatic organism and the corresponding concentration in the aquarium water.

$$BCF = C_o/C_w$$

where:

C_o = concentration of contaminant (ng/kg) in the aquatic organism;

C_w = concentration of contaminant (ng/L) in water.

The C_o value can be expressed on a wet-weight, dry-weight and lipid basis, giving rise to three factors (BCF_W, BCF_L and BCF_D) which can differ considerably in numerical terms. In Europe, chemical substances with a BCF_W value greater than 100 are considered to have high potential to bioaccumulate and are classified as 'dangerous to the environment', because they could impair the health of an aquatic organism or of predators feeding on that organism (9). In the present study, the experimental data on the PBDE-47 levels determined in the clam tissues were used to calculate a lower estimate of the value of BCF. The lower estimate was calculated because the experimental protocol used in the toxicity tests did not allow evaluation of whether the steady state was reached. In fact, the experimental protocol involved a single analytical determination of PBDE-47 in the tissue of the clams after a maximum exposure time of 21 days. Table VI shows the BCF values calculated at the different exposure concentrations. In all cases the value of BCFw was greater than 100.

Table VI. *BCF values calculated at the different exposure concentrations.*

Concentration PBDE-47 H_2O (ng/L)	BCFW (L/kg)	BCFD (L/kg)	BCFL (L/kg)	log BCF_W	log BCF_D	log BCF_L
250	439	2,260	45,300	2.6	3.4	4.7
500	195	1,000	26,600	2.3	3.0	4.4
1,000	122	630	10,700	2.1	2.8	4.0
3,000	131	680	11,500	2.1	2.8	4.1
30,000	196	1,010	20,600	2.3	3.0	4.3

BCF_W = bioconcentration factor on wet weight basis;
BCF_L = bioconcentration factor on fat basis;
BCF_D = bioconcentration factor on dry weight basis.

No experimental studies of bioaccumulation of PBDE-47 in *Chamelea gallina* are reported in the literature, but such studies have been conducted on other bivalve molluscs, such as *Mytilus edulis* (10, 19). The BCF values in these organisms are higher than those found in the present study. This difference may be associated with:

- ammonia concentration (NH3): < 0.25 mg/L;
- failure to reach the steady state due to the short exposure time;
- inappropriate comparison between biologically different organisms.

To confirm this hypothesis, a mathematical model (16) was used which relates the log kow of a given substance to the BCF value in *Mytilus edulis*:

$\log BCF_W = 0.858 \log k_{ow} - 0.808$

The equation enables the bioaccumulation factor of the mussel to be estimated once the log kow value of a substance is known. The linearity equation used would lead to a log BCF result of 4.3, a higher value than that experimentally estimated in this study for *Chamelea gallina*.

Conclusion

Under the operating conditions optimised in this study, *Chamelea gallina* showed good adaptability in the aquarium, allowing the correct conduct of ecotoxicology tests in the laboratory.

The results of the 96-hour and 21-day toxicity tests did not indicate any correlation between the concentration of PBDE-47 in the seawater in the aquariums and the mortality of *Chamelea gallina*. In fact, exposure to the maximum concentration of PBDE-47 did not give rise to significant mortality. The results of the histological tests did not indicate any evident alterations in the tissue of the reproductive apparatus under the experimental conditions used.

The chemical analyses indicated that clams exposed to PBDE-47 presented levels of contaminant in the tissue thousands of times higher than unexposed clams, depending on the concentration in the aquatic ecosystem, thus demonstrating the ease with which *Chamelea gallina* bioconcentrates the contaminant. The entry of PBDE-47 into the food chain may constitute a risk to food safety, the quality of marine ecosystems and the health of clam stocks.

Acknowledgements

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