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Uptake and depuration kinetics of dicofol metabolite 4,4′-dichlorobenzophenone, in the edible Asiatic clam *Meretrix meretrix*

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ABSTRACT

Uptake and depuration kinetics of 4,4′-dichlorobenzophenone (main metabolite of dicofol) in the edible clam *Meretrix meretrix* were evaluated through a mesocosm experiment. *M. meretrix* was exposed to different dicofol concentrations (environmental concentration, D1 = 50 ng/L; supra-environmental concentration, D2 = 500 ng/L) for 15 days, followed by the same depuration period. To accomplish this goal, an analytical method was successfully optimized for 4,4′-DCBP using QuEChERS as extraction method with a range of concentrations 0.3–76.8 ng/g ww quantified by gas chromatography coupled to tandem mass spectrometry. Our results demonstrated different kinetics of accumulation depending on the two dicofol treatments. For D1, the uptake kinetic was best fitted using a plateau followed by one phase association kinetic model, while for D2 a one phase association kinetic model suited better.

Similar bioconcentration factors were obtained for both concentrations but only animals exposed to D2, showed 4,4′-DCBP levels above the limits of quantification after 24 h exposure. These animals also showed lower uptake rate (k_u) than organisms exposed to D1.

During the depuration period, only organisms exposed to D1 successfully depurated after 24 h. On the other hand, although animals exposed to D2 presented higher elimination factor, they did not reach the original levels after depuration. Moreover, values detected in these clams were higher than the Maximum Residue Level (10 ng/g) established by the European legislation. This indicates that longer periods of depuration time than the ones used in this study, may be needed in order to reach safe levels for human consumption.

This work also demonstrated that studies on metabolite kinetics during uptake/depuration experiments, could be a new alternative to understand the impact and metabolism of pesticides in the marine environment.

1. Introduction

Organochlorine pesticides (OCPs), are a classical example of persistent organic pollutants (POPs) of worldwide concern due to their persistence in the environment, bioaccumulation ability, and potential negative impacts on biota and human health (Guan et al., 2009; Guo et al., 2008). Among OCPs, dicofol is of special interest due to its high biomagnification potential, similarity with dichlorodiphenyltrichlorethane (DDT), and extensive use, predominantly in Southeast Asia (Guo et al., 2008; United Nations Environmental Programme, 2016). It is true that the global production of DDT and dicofol have shown a significant decline since the Stockholm Convention adoption, however these pesticides are still being used, i.e. DTT is used in response to the development of resistance in malaria vectors (mainly in Asia and Africa) (Berg,V.H., et al., 2017) and dicofol as a pesticide (mainly in Asia). Moreover, these prohibited compounds could also be used in Europe in case of emergency situations that pose a danger to plant production and ecosystems (SANCO, 2013). Therefore, despite the pesticides ban from different countries, dicofol could be a global problem affecting not only China, but also other countries where this compound has been or is still being used in special situations. Dicofol is an organochlorine acaricide that has been used in agriculture since the late 1950s to protect mainly citrus and cotton cultivations from mites (Thiel et al., 2011; WHO/FAO, 1996). In a previous work, dicofol was quantified as the most frequent OCP in water and sediment samples collected in 2009 from Jiulang River (North East China) (Zheng et al., 2016). It is also identified as a potential "endocrine disrupting compound" due to its animal toxicity, cancerogenic and negative estrogenic effects (Liu et al., 2004; Reynolds et al., 2005; Thiel et al., 2011). The technical product is usually synthesized from DDT via chlorination and subsequent hydrolysis and consists of approximately 80% and 20% of 4,4′- and 2,4′-dicofol isomers, respectively (Qiu et al., 2005).

Owing to the instability and easy degradation of dicofol in water — when exposed to higher pH (85 days, 64–99 h or 26 min of half-life at pH 5, pH 7 and pH 9, respectively), light (sensitive to sun light) and higher temperature (3.3 days of aqueous half-life photoly-

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sis at 20 °C and pH 7)— 4,4′-dichlorobenzophenone (DCBP) is the main metabolite and probably the most available form in surface waters (Fujii et al., 2011; Thiel et al., 2011; Yin et al., 2017; FOOTPRINT PPDB). In fact, 4,4′-DCBP, was quantified in surface waters (2.79–29.87 ng/L) from the mouth of the Pearl River Delta in a previous study (Ivorra et al., 2019).

Some metabolites are often more persistent than their corresponding parent compounds and exhibit similar or even greater toxicity, e.g. the major biodegradation product of nonylphenol ethoxylates (nonylphenol) or endosulfan I/II (endosulfan sulfate) (Jahan et al., 2007; Stanley et al., 2009). In some cases, metabolites were quantified in aquatic environments in even higher levels than those of the parent compounds (Farré et al., 2008). Therefore, it is crucial to study the effect of metabolites in aquatic organisms.

Bivalves, as filter-feeding organisms, have been widely used to monitor pollutants in aquatic ecosystems due to their wide geographical distribution, sessile lifestyle, resistance to stress and high and rapid accumulation of toxic substances (Goldberg et al., 1978; Suárez et al., 2013; Walker and Livingstone, 1992), and also because of their economic interest and their implications in the food chain (Cardoso et al., 2013; Metian et al., 2008). For this study, we selected a common bivalve, *Meretrix meretrix*— known as Asiatic hard clam— which is widely consumed around the world and widespread in the Indo-West Pacific region (Poutiers, 1998).

Considering the chemical instability of dicofol, we assume that 4,4′-DCBP is possibly the most persistent form in the aquatic environment. Therefore, and regarding the lack of information about metabolites, the main goal of this work is to study the pattern of bioaccumulation and elimination kinetics of the metabolite 4,4′-DCBP in clams exposed to environmental and supra-environmental concentration (10x more) of dicofol. Thus, this work investigated if edible bivalves have the ability to accumulate and depurate 4,4′-DCBP, if the kinetics of these organisms will be different between both dicofol concentrations and if depurated clams will reach acceptable levels for human consumption.

2. Materials and methods

2.1. Sample description

Bivalves, originally collected in Guangzhou province, were acquired from a local market and transported immediately to the lab. During acclimation period (approximately 4 days), animals (ca. 700) were distributed in two containers (15 L each) and kept under oxic conditions ensured by air-bubbling the water. Temperature and salinity were gradually adjusted (1 °C/day and 2 ppt/day, respectively) until a final temperature of 27 °C and salinity of 16–18 ppt. Animals were fed daily with 600 µL (1:10 dilution) of a commercial mixture of spirulina and kelp (Kent Marine Microvert) under a photoperiod regime of 12:12 light/dark cycle.

2.2. Experimental set up

The experimental set-up included a total of 60 sub-experiments (3 replicates*4 treatments*5 sampling times) corresponding to 60 different glass aquaria. The conditions for each treatment were: 1) control (C) only with seawater, 2) solvent control (SC, methanol 0.1%), 3) dicofol at environmental concentration (D1, 50 ng/L), and 4) a supra-environmental dicofol concentration (D2, 500 ng/L).

The experiment ran for 30 days and was divided into the exposure phase (15 days exposed to dicofol) and the decontamination phase (15 days free of dicofol). During each phase, five sampling times were established: day 1, 2, 3, 7 and 15. Fig. 1 shows the schematic representation of the experimental design.

After the initial acclimation period, 10 clams were distributed per aquarium, 24 h before the beginning of the experiment (to ensure the stability of the system). All aquaria were placed randomly into water baths (8 aquaria/water bath) with heater and aeration to assure a stable and homogeneous temperature. Each glass aquarium, containing 1 kg of pre-washed commercial sand (Xin Jing aquarium gravels) and

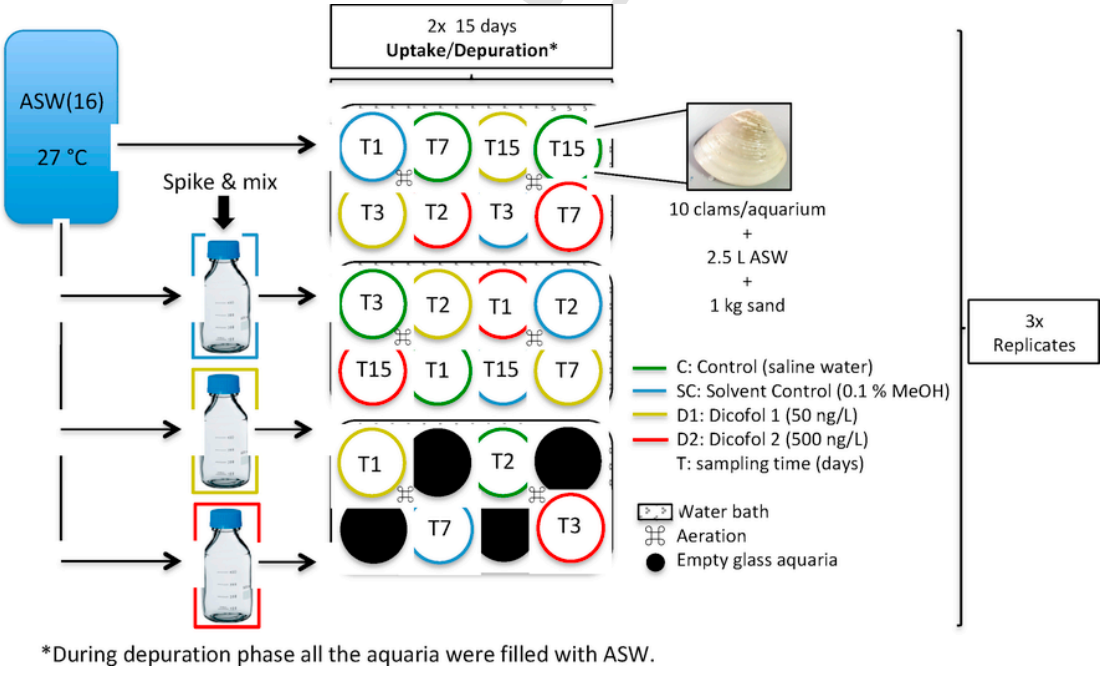

Fig. 1. A schematic representation of the experiment set-up with 4 different treatments (C/SC/D1/D2) at 5 different sampling times (T1/T2/T3/T7/T15), randomly distributed in 8 water baths.

2.5 L of artificial seawater (ASW), was maintained at the same oxic conditions as described above.

Owing to the instability and easy degradation of dicofol mentioned above, the medium was renewed daily. For this purpose, a peristaltic water pump (BT100 M, Generic) was used to remove and replace the water, completely. Moreover, to ensure a homogenous concentration in the spiked aquaria, an aliquot of the water from the aquarium (250 mL approx.) was taken, spiked and mixed previously. 4,4′-DCBP quantification in water was performed regularly (right after spiking and 24 h later) to control the concentration levels throughout the experiment. The same food proportion was kept as in the acclimation period. All aquaria were individually covered with a glass to avoid cross-contaminations. Moreover, at the pre-defined sampling times, three organisms were removed and placed in constantly aerated clean seawater for 24 h depuration (to remove pseudo-fecal and fecal material from the digestive tract) (Coelho et al., 2006; Metian et al., 2008). After this period, clams were measured, weighed (with and without shell), cut opened and the soft tissue frozen (−80 °C) for later 4,4′-DCBP quantification. Condition index (CI) was also calculated according to CI = (fresh weight/shell weight) x 100, as complementary information about the health status of the organisms (Hyötyläinen et al., 2002). Survival rate (%) of the organisms was also controlled during the whole experiment.

Physical parameters were measured daily for temperature and weekly for pH and dissolved oxygen (DO). The water temperature in the aquaria was 26.92 ± 0.17 °C, and pH and DO were 8.45 ± 0.14 and 106 ± 4.01%, respectively.

2.3. 4,4′-DCBP quantification by GC-MS/MS

2.3.1. Reagents

LC/GC grade solvents such as, methanol (CH_3OH), acetonitrile (CH_3CN), ethyl acetate ($C_4H_8O_2$), and dichloromethane (CH_2Cl_2) were purchased from Merck Limited Company (Germany). Ultrapure water was obtained from a Milli-Q water system (resistance = 5.1 μΩ/cm at 25 °C).

Anhydrous magnesium sulfate ($MgSO_4$), sodium acetate ($C_2H_3NaO_2$), and Supelclean PSA SPE Bulk Packing (polymerically bonded, ethylenediamine-*N*-propyl phase that contains both primary and secondary amines), were acquired from Sigma-Aldrich (Steinheim, Germany); $MgSO_4$ was preheated (5 h at 500 °C) to eliminate residual water and phthalates.

Dicofol-d_8 (used as surrogate and internal standard (IS)), dicofol and 4,4′-DCBP with purity > 98%, were purchased from Sigma-Aldrich (Steinheim, Germany). All compounds were individually prepared in CH_3OH with 0.1% acetic acid (CH_3COOH; Sigma-Aldrich, USA) to produce the final stock solution of 1000 μg/L and kept in the dark at −20 °C. D-sorbitol and 3-ethoxy-1,2-propanediol (used as protectants) were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solution of 182 mg/mL in 70% CH_3OH:H_20 and 800 000 mg/L in 100% CH_3OH were prepared for D-sorbitol and 3-ethoxy-1,2-propanediol, respectively. Protectants were used as 0.1:1 mg/mL (D-sorbitol:3-ethoxy-1,2-propanediol). Stock solutions of 3-ethoxy-1,2-propanediol were kept at 4 °C, and D-sorbitol and the protectant mixture were stored in the same conditions as the surrogate and standard. For quantification purposes, an aliquot of each sample (195 μL) was taken and mixed with a protectants' solution (5 μL) at a final concentration of 0.0025:0.025 g/mL, respectively.

2.3.2. Bivalve and water samples preparation

Biological samples: 4,4′-DCBP extraction was performed using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique as was previously described by Cruzeiro et al. (2016). First, the frozen bivalve tissue was thawed, chopped, and then ground with a high-speed disintegrator model number FW80 (Faithful). A homogenate sample of 5 g was transferred into a 50 mL Teflon centrifuge tube (Nalgene Oak Ridge High-Speed, Thermo-Fisher, NY, USA), and spiked with 50 μL of the surrogate (0.5 μg/L) and/or calibration curve concentrations. The fortified sample was settled for 5 min and vortexed, then 5 mL of CH_3CN was added and vortexed again. The rest of the extraction was done by adding subsequently a combination of different salts followed by vortex and centrifugation (4 °C, 4024 rcf, 5 min) between steps; 1) 2 g $MgSO_4$ and 500 mg $C_2H_3NaO_2$; 2) collect upper layer (2.5 mL) and add 125 mg PSA and 375 mg $MgSO_4$; 3) collect the final extract (2 mL).

Water samples: 2 replicates of 500 mL from each treatment group were collected in amber flasks, just after the dicofol addition (T0) and before the water renewal (T24). Samples were filtered (0.45 μm glass fibre filter; Sartorious, Germany) and acidified to pH 5 with CH_3COOH for higher sample stability.

The compound was extracted by solid-phase extraction (SPE) using the OASIS HLB cartridges (200 mg, 6 cc; Waters, Ireland) following Ivorra et al. (2019) protocol. Briefly, fortified water samples were loaded into pre-conditioned cartridges (5 mL CH_3OH followed by 5 mL ultrapure water), allowed to dry, and eluted (2.5 mL $C_4H_8O_2$ followed by 2.5 mL of CH_2Cl_2 and 2.5 mL more of a 1:1 mix of CH_2Cl_2 and $C_4H_8O_2$ (v/v)). The extracts were evaporated to dryness, under N_2 stream (99.995%) and then reconstituted into 200 μL of CH_3OH.

2.3.3. Method validation and quality assurance

The validation procedure followed the European guidance document on pesticide residue analytical methods SANTE/11813/2017 rev 0 (SANTE, 2017). Linearity was evaluated using three independent calibration curves, each with seven nominal standard concentration of 4,4′-DCBP, (ranging from 0.06 to 3.84 μg/L) spiked (50 μL) into 5 g of homogenate organism matrix with the surrogate (0.5 μg/L). Curves were plotted using the ratio between the standard (4,4′-DCBP) and the IS area (dicofol-d_8). The limits of detection (LOD) and quantification (LOQ) were determined with the same curves, using the following formulas: LOD = 3.3 α/S and LOQ = 10 α/S, where α is the standard deviation of the response and S is the average slope of the calibration curves.

Recoveries, accuracy and precision were evaluated by analysing three independent replicates of each quality control samples (QCs) at two levels of concentration (low and medium) calculated as, QC_{low} = LOQ (4.01 μg/L) and QC_{medium} = 4LOQ (16.04 μg/L). Recoveries were determined by comparing the area ratio in spiked matrix with the area ratio of the same concentration in a matrix blank spiked after extraction. Precision was expressed as the relative standard deviation (% RSD) of the replicate measurements, and the accuracy was evaluated as the percentage of agreement between the methods results and the nominal amount of added compound.

As part of the validation, the matrix effect (ME) was also evaluated at both concentrations (LOQ), where matrix samples were spiked after extraction ($A_{standard\ in\ matrix}$) and compared with those of injected standards ($A_{standards}$), as indicated in the following equation: $ME = -((A_{standard} - A_{standard\ in\ matrix})/A_{standard}) * 100$.

The ions selection and the collision energies for quantification purposes were obtained from the auto selected reaction monitoring. Information from published methods, regarding the target ions were also taken into consideration (de Kok et al., 2005; EU Reference Laboratories for Residues of Pesticides, 2013; Pereira et al., 2014). The software Xcalibur (version 4.0.27.10, Thermo Scientific), to-

gether with the NIST library, were used for ion products confirmation and quantification (Table S1).

For the water samples, the validation procedure followed the European guidance document on pesticide residue analytical methods SANCO/825/00 rev 8.1 (SANCO, 2010). In this matrix, the range of concentrations used were 3–400 ng/L, and three different QCs were included during validation (QC_{low} = 2LOQ (1.6 ng/L), QC_{medium} = 20LOQ (16.48 ng/L) and QC_{high} = 100LOQ (82.4 ng/L). More details can be found in Ivorra et al. (2019).

2.3.4. Instrumental methods

Analyses were carried out using a gas chromatograph (Trace 1310 GC, Thermo Scientific), coupled with a triple quadrupole mass spectrometer detector (TSQ 8000 EVO, Thermo Scientific), an autosampler (Thermo ScientificTriPlus™) and a Trace Pesticides column (TR-pesticides II, 30 m × 0.25 mm × 0.25 µm + 5 m Guard).

For the animal samples, column oven temperatures were programmed for a 35 min period using several ramps: a) from 80 °C with an initial equilibrium time of 2 min to b) 180 °C at 20 °C/min until c) 290 °C at 5 °C/min, where the temperature was maintained for 7 min. The injector port temperature was set to 200 °C, and both ion source and MS transfer line were at 290 °C.

For the water samples, column oven temperatures were programmed for a 14 min period instead using several ramps: a) from 75 °C with an initial equilibrium time of 3 min to b) 180 °C at 30 °C/min until c) 280 °C at 5 °C/min, where the temperature was maintained for 1 min. The injector port temperature was set to 250 °C, and both ion source and MS transfer line were at 280 °C.

In both analyses, helium (99.999% purity) was used as carrier gas and was maintained at a constant flow rate of 1.2 mL/min. Sample injection (2 and 1.5 µL for animal and water samples, respectively) was in the split-less mode (4 mm straight liner, 453A1925), using a 50 mm long needle. New liners were used every 200 injections.

2.4. Data analyses

Uptake and depuration kinetics of the soft tissues were expressed in terms of change of 4,4′-DCBP concentration over time. The data obtained for 4,4′-DCBP uptake or depuration per unit of time was modelled by nonlinear regression analysis, using GraphPad Prism version 6.00, that uses the least-squares fitting method: the plateau followed by one phase association (eq. (1)) and the one-phase association kinetic model (eq. (2)) were applied for the uptake data for D1 and D2, respectively. In addition, the one-phase exponential decay model (eq. (3)) was used to fit data from 4,4′-DCBP depuration for D2 concentration.

$$C_t = \mathrm{IF}\left(t < t_0, C_0, C_0 + \left(C_{ss} - C_0\right) * \left(1 - \exp\left(-K_u * \left(t - t_0\right)\right)\right)\right) \quad (1)$$

$$C_t = C_0 + \left(C_{ss} - C_0\right) * \left(1 - \exp\left(-k_u t\right)\right) \quad (2)$$

$$C_t = \left(C_0 - C_{ss}\right) * \exp\left(-\mathrm{ket}\right) + C_{ss} \quad (3)$$

where C_t and C_{ss} are the concentrations at time t (d) and at steady-state, respectively; k_u is the uptake rate constant (d^{-1}) and k_e is the depuration rate constant (d^{-1}); C_0 is the concentration at time 0 (Hédouin et al., 2011).

In order to assess the experimental data goodness of the fit, the coefficient of determination (R^2) and the standard deviation of residues ($S_{y.x}$) were determined. A relatively high R^2 and low value of $S_{x/y}$ were used as criteria for good fit. For each case, the fitting was tested using the mean 4,4′-DCBP concentration at each studied time.

In addition, a biological half-life (the time it takes to reach half of the equilibrium value) was calculated ($T_{b1/2}$) from the corresponding uptake (k_u) and depuration (k_e) rate constants, according to the relation $T_{b1/2} = \ln 2/k_u$ and $T_{b1/2} = \ln 2/k_e$, respectively.

Bioconcentration factors (BCFs) were generally calculated as the ratio of internal biota concentration (ng/kg) to the water exposure concentration (ng/L).

The elimination of 4,4′-DCBP was expressed in percentage of lost 4,4′-DCBP concentration. Elimination factor was described by equation EF = 100 - [(C_e/C_t) x 100], where EF is the percentage of lost 4,4′-DCBP concentration, C_e is the 4,4′-DCBP concentration in the bivalve tissue after depuration period, C_t is the 4,4′-DCBP concentration in the tissue of transplanted bivalves after 15 days exposure.

To infer differences between treatments and sampling times, all data were initially checked for normality (Kolmorgorov-Smirnov test) and homogeneity of variances (Levene's test). In order to determine differences between treatments and sampling times a 2-way ANOVA was applied. Transformations of the data were needed to fit the assumptions for the analysis. The Tukey post-hoc test was applied, to assess differences in sampling times for each treatment; and Dunnett's test to assess differences between the solvent control (SC) and the treatments (D1 and D2). Finally, for each treatment, comparisons between uptake and depuration phase were done using Wilcoxon matched-pairs signed rank test. All statistical analyses were done using GraphPad Prism version 6.00.

3. Results

3.1. Bivalves QuEChERS validation

LOD and LOQ were quantified with a final value of 1.33 and 4.02 µg/L, respectively. All validation criteria were successfully established, with final average percentages of 108.95%, 93.17% and 3.98%, for LOQ, and 108.75%, 90.15% and 4.11%, for 4LOQ (Fig. S1), respectively for recovery, accuracy and precision.

Regarding the matrix effect results, an enhancement of the signal of 40.07% was observed for LOQ.

3.2. 4,4′-DCBP uptake and depuration rates

4,4′-DCBP was not detected in control aquaria and control clams, thus indicating the absence of contamination. Survival rate of the organisms presented average values higher than 85% for all the treatments, except for D2, which showed an average value of 82.2% during uptake. Moreover, no differences were observed between control (C) and solvent control (SC) treatments, therefore SC was chosen for graphical representation and data comparison. Concentrations between LOD and LOQ were transformed as LOQ/2, as described by Beal (2001), and estimated as if all the values were real; values < LOD were not included in the analysis.

Generally, the bivalves exhibited an increase on 4,4′-DCBP concentration, in relation to solvent control, through the entire exposure period (Fig. 2), however the kinetics of accumulation were different for the two dicofol treatments. For D1, the uptake kinetic was best fitted using a plateau followed by one phase association kinetic model, while for D2 a one phase association kinetic model suited better. In addition, for the lowest concentration (D1), the bivalves

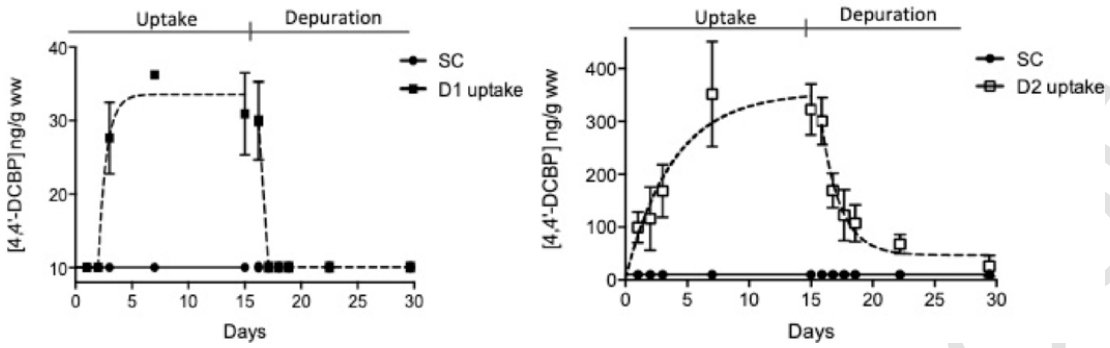

Fig. 2. Kinetics of accumulation (days 0–15) and depuration (days 15–30) of 4,4′-DCBP in *M. meretrix* (ng/g ww) exposed to 50 ng/L (D1, left) and 500 ng/L (D2, right). Results are expressed by mean ± standard error (n = 3 per sampling time).

showed a faster accumulation than when exposed to the highest concentration (D2), since for the first case, after three days of exposure they reached 89% of the final concentration while for the second case, for the same period of time they just reached 52% of the final concentration. This pattern was corroborated by the kinetic parameters that indicated an uptake rate constant (k_u) of 1.35 d^{-1} for D1 and 0.25 d-1 for D2 (Table 1). A stabilization of 4,4′-DCBP accumulation was attained after 5–7 days and 12–15 days for D1 and D2, respectively. After 15 days of exposure, final concentrations of 30.93 ng/g ww and 322.53 ng/g ww of 4,4′ DCBP were detected for D1 and D2, respectively. Finally, and considering the kinetic model, the biological half-life ($T_{b1/2}$) was also determined for D2 with a value of 2.79 d during uptake. For D1 it was not possible to estimate the biological half-life. After 15 days of exposure, the log BCFs of 4,4′-DCBP were slightly higher in animals exposed to higher concentrations (3.86 for D2) than those exposed to lower concentration (3.79 for D1) (Table 2).

Significant differences between sampling times (2-way ANOVA, $F_{(5,36)} = 81.87$, $p < 0.0001$) and treatments (2-way ANOVA, $F_{(2,36)} = 724$, $p < 0.0001$) were observed. For D1, SC group was significantly different from it ($p < 0.0001$) from day 2 to day 15, while no significant differences ($p > 0.05$) were found for D1 between T3, T7 and T15. In the case of D2, significant differences ($p < 0.001$) from SC group were observed after 24 h exposure (T2) and initial accumulation (T1 - T3) showed significant differences from T7 and T15. Moreover, interaction between both factors (sampling time x treatment) was also significant ($F_{(10,36)} = 36.32$, $p < 0.0$).

On the other hand, the depuration kinetics for D2 treatment was best fitted using one-phase exponential decay model, with 43.7% decay after 24 h. D1 treatment did not match any kinetics model due to its rapid decay (67.5%) after 24 h transfer to a clean system. D2 treatment showed an elimination rate constant (k_e) of 0.57 d^{-1}. For D1 it was not possible to estimate this parameter. After 15 days of depuration, 27.16 ng/g ww of 4,4' DCBP was detected in organisms from D2 treatment, in contrast to D1, which presented values < LOQ. The biological half-life ($T_{b1/2}$) for D2 depuration was 1.20 d, and an EF of 67.14% and 92.63% (Table 2) was calculated for D1 and D2, respectively.

In this case, D2 treatment also presented significant differences between sampling times (2-way ANOVA, $F_{(5,23)} = 11.99$, $p < 0.001$) and treatments (2-way ANOVA, $F_{(1,23)} = 540.75$, $p < 0.001$). D2 treatment was significantly different from SC during the whole depuration except for T15. At this sampling time no significant differences were observed with T0, which corresponds to the initial point of the experiment. Initial sampling times (T1 to T3) showed significant difference from T15; and T1 was also significantly different from T7. Moreover, interaction between both factors (sampling time x treatment) was also significant ($F_{(5,23)} = 11.99$, $p < 0.05$). Finally, results from Wilcoxon test showed that uptake and depuration kinetics for each treatment did not have a significant difference ($p > 0.05$).

Considering the CI, no significant differences ($p < 0.05$) were observed between SC and both dicofol treatments during the whole experiment (Fig. S2).

4. Discussion

4.1. Biological method validation

The validated method accomplished all the criteria (i.e., evaluation of linearity, accuracy, precision and recoveries) established by SANTE/11813/2017 rev 0 (SANTE, 2017), demonstrating to be valid for 4,4′-DCBP extraction and quantification. This was only possible because all calibration curves were done in matrix, avoiding overestimation during the quantification due to higher ME. The target metabolite was quantified at very low range of concentrations (0.3–76.8 ng/g ww) indicating that this method is acceptable to detect the Maximum Residue Level (MRL) of pesticides in food (10 ng/g) established by the European Union (European Commission, 2019), and therefore it can be used for future studies regarding food safety.

Table 1
Estimated uptake and depuration parameters of 4,4 DCBP in the bivalve M. meretrix exposed for 15 days to the pesticide and then kept for 15 days in clean water. C_0, concentration at time 0; C_{ss}, concentration at steady state; K_u: uptake rate constant (d−1); K_e: depuration rate constant (d−1); Tb½: biological half-life (d); SE: standard error; R^2: determination coefficient.

		C_0 (ng g^{-1}) ± SE	C_{ss} (ng g^{-1}) ± SE	K_u (d^{-1}) ± SE	K_e (d^{-1}) ± SE	$T_{b1/2}$	R^2	Sy.x
Uptake	D1	10.05	33.57 ± 1.34	1.38 ± 0.36		–	0.98	1.88
	D2	10.05	356.4 ± 41.40	0.25 ± 0.07		2.79	0.94	36.70
Depuration	D1	–	–	–	–	–	–	–
	D2	315.2 ± 21.67	49.60 ± 16.86		0.57 ± 0.12	1.20	0.97	22.41

Table 2
Bioconcentration factors (log BCF15 d) and elimination factors (EF15 d) of *M. meretrix* tissues in the two contaminated treatments (D1 and D2) considering 15 d exposure. Results are expressed by mean ± standard error (n = 3 per sampling time).

	Uptake	Depuration
	Log BCF	EF (%)
Treatment D1	3.79 ± 0.03	67.14 ± 2.56
Treatment D2	3.86 ± 0.04	92.63 ± 3.95

4.2. 4,4′-DCBP uptake and depuration rates

Awareness of contamination and depuration processes in organisms, such as bivalves is an important issue to understand the possible biomagnification of contaminants through the food web, especially in the case of edible organisms such as the *M. meretrix*. This study focused on the kinetics of the metabolite 4,4′-DCBP rather than the parent compound, dicofol, providing new data in a topic where the information is still scarce. To our knowledge, there are no published data on 4,4′-DCBP kinetics with which it is possible to compare our results. Therefore, most of the comparison will be done using data from similar/related compounds (i.e. organic compounds, organochlorinated pesticides, DDT). Moreover, we also discuss the importance of using a very high concentration (10x D1) to mimic possible spills and understand the possible impact of extreme situations, which may occur in sporadic situations. For example, it has been reported that 119 spills incidents occurred from 1947 to 2011, which contained a total of 187 substances spilled. From these substances, the third largest group involved in marine accidental spills was pesticides, such as lindane or endosulfan (Cunha et al., 2015).

In this study, different kinetic patterns were observed between environmental (D1) and supra-environmental (D2) concentrations. For example, D1 treatment showed a baseline at initial time (plateau) (Fig. 2), where accumulation of the compound was not remarkable. After this initial phase, both treatments could be explained by pseudo-first order association kinetics. The initial absence of plateau in D2 may be due to the higher quantified concentrations (4,4′-DCBP was > LOQ), which were significantly different from SC, after 24 h exposure. In both cases, during uptake, the organisms assimilated a certain fraction of the compound until a steady state was reached. The steady state for D1 was reached faster than for D2, which was expected considering the k_u. Higher contaminant concentrations, like the ones spiked in D2 treatment (500 ng/L), may induce alterations in respiration rates and filtration capability (Bourdelin, F., 1996; Vijayavel, K., et al., 2007). The same behaviour in bivalves has been reported in other studies. For example, Cardoso et al. (2013) observed the same when *Cerastoderma edule* was exposed to different mercury concentrations while Gomez et al. (2012) when *Mytilus galloprovincialis* was exposed to different concentrations of tetrazepam.

Regarding the k_u, Richardson et al. (2005) estimated values of 9.66×10^3 and 3.82×10^4 in mussels exposed for 20 days to 100 ng/L of α-HCH and dieldrin, respectively. These values are much higher than the ones obtained in this work (1.38 and 0.35 for D1 and D2, respectively). Several studies have reported that, in a sediment-water system in which the direct source of contaminant is the dissolved phase, the tendency for accumulation of organic contaminants can be correlated with *n*-octanol/water partition coefficients (K_{ow}) of the compounds (Geyer et at. 1982; Mackay, 1982; Pruell et al., 1986). In such systems, organic compounds are bioaccumulated through passive diffusion across the gills, rather than ingestion. Dicofol, as well as 4,4′-DCBP, tends to bind to particulate matter (WHO/FAO, 1996) rather than the water column, therefore lower amounts will be available in the dissolved phase for passive diffusion. This could explain the difference between 4,4′-DCBP k_u rates and other OCPs like α-HCH and dieldrin, that tend to accumulate in the dissolved phase (Richardson et al., 2005).

The bioconcentration of organic compounds is often associated with the molecule lipophility (high K_{ow}) and the molecule aqueous solubility (low S_w), which are inversely related (Arnot and Gobas, 2006). For example, Katagi (2009) showed a strong positive correlation between log BCF and log K_{ow} in fish for pesticides developed in the most recent 10-years period. The more hydrophobic a pesticide is, the higher bioconcentration is observed with more distribution in the organs having higher lipid content (Katagi and Ose, 2014). In this study, the average log BCF values obtained for D1 and D2 treatment was 3.83, which is in the same range of values obtained by Richardson et al. (2005) for dieldrin (5.43), aldrin (3.92) and α-HCH (3.76). These values, and considering the low S_w and high K_{ow} (Table S2), indicate that the compound can concentrate more in the organism than in the surrounding water, and therefore biomagnification process may happen affecting the food web (Kanazawa, 1981).

Regarding the depuration period, this study revealed different kinetic patterns between treatments. We observed a fast recovery of the animals exposed to the lower concentration (D1) after 24 h (showing values < LOQ) while organisms exposed to higher concentration (D2) did not fully depurate over a period of 15 days. The same behaviour was observed by Richardson et al. (2005) in mussels depurated for 8 days, after being exposed to 100 ng/L of DDT, although k_e observed by this author was (0.015 d^{-1}) lower than in our study for 4,4′-DCBP (0.58 d^{-1}).

Our organisms also showed an elimination rate (k_e) 2.32x times higher than the uptake rate (k_u). Contrarily to these results, Uno et al. (1997) obtained higher k_u (338 d^{-1}) than k_e (0.054 d^{-1}) in clams exposed to 1700 ng/L of thiobencarb (30 mg/L, solubility) during 14 and 15 days, respectively. Studies previously mentioned (Richardson et al., 2005; Gomez et al., 2012) also showed the same trend as Uno et al., (1997). Kinetic studies focused on parent compounds and metabolites behaviour, may follow different patterns. For example, during uptake phase the animal is continuously exposed to the parent compound and it will start its accumulation in the organism. It is expected to get higher concentrations of the parent compound during this phase than the metabolite. However, during depuration, when no more parent compound is added, it will be expected to get higher metabolite concentrations due to metabolization or degradation of parent compound in the system. This hypothesis could explain the differences obtained between our results and other previous studies, where the parent compound instead of the metabolite was measured during uptake and depuration. Our study showed the importance of understanding the behaviour of the metabolites, since they can still be very active, and may present different kinetics pattern.

In addition, the efficiency of eliminating contaminants seems to be higher when there is more concentration in the system. The EF between treatments (Table 2) after 15 days of depuration indicated that organisms exposed to D2 were able to eliminate more 4,4′-DCBP than those exposed to D1. Another elimination route could be depuration by passive diffusion into surrounding water. However due to the hydrophobic character of the compound this way is less expected. In an open system, and depending on the affinity of the compound for the feces, the contaminants may then desorb and reenter the water column. In this case, 4,4′-DCBP levels in the water during depuration (data not shown) were similar to the Control (SC) ones, which could indicate that 4,4′-DCBP besides being accumulated by the organisms, could be adsorbed to the substrate (i.e. sand), to some remaining fecal material or to the glass of the aquarium, rather than to the water col-

umn. More studies may be needed in order to fully understand the metabolism process of 4,4′-DCBP in marine invertebrates.

Furthermore, 4,4′-DCBP levels obtained after 15 days of depuration (26.19 ng/g ww) in animals previously exposed to D2, presented concentrations 2.6-fold higher than the MRLs established by the European legislation (10 ng/g) for any kind of food for Human consumption (European Commission, 2019).

In summary, considering all the information mentioned above, we can highlight that although depuration of 4,4′-DCBP is happening in a more effective way than uptake ($k_e > k_u$), longer depuration may be needed to fully eliminate higher concentrations to reach levels that are safe for human consumption.

5. Conclusion

There is still a lack of data on the toxicity and effects of pesticides' metabolites on bivalves, whether individually or in mixture with their parent compounds.

In the present work, we studied the kinetics of the metabolite 4,4′-DCBP, considered as the main degradation product of dicofol. Our results showed that uptake of the contaminant was less effective than elimination, which could be associated with high metabolism of the compound by the organism. Therefore, quantification of metabolites could be a new alternative and a better approach to understand pesticide metabolism in bivalves, and its impact on the marine environment.

Moreover, these results raise to a certain extent issues of concern, since for both dicofol exposures, the organisms reached limit values accepted by the EU (i.e. 10.05 ng/L for D1) or did not have the ability to return to safe values (in case of D2) for food consumption after 15 days of depuration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.06.155.

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