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How can environmental conditions influence dicofol genotoxicity on the edible Asiatic clam, *Meretrix meretrix*?☆

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ABSTRACT

Genotoxic effects of dicofol on the edible clam *Meretrix meretrix* were investigated through a mesocosm experiment. Individuals of *M. meretrix*, were exposed to environmental concentration (D1 = 50 ng/L) and supra-environmental concentration (D2 = 500 ng/L) of dicofol for 15 days, followed by the same depuration period. DNA damage (i.e., strand breaks and alkali-labile sites) was evaluated at day 1, 7 and 15, during uptake and depuration, using Comet assay (alkaline version) and nuclear abnormalities (NAs) as genotoxicity biomarkers. The protective effects of dicofol against DNA damage induced by *ex vivo* hydrogen peroxide (H_2O_2) exposure were also assessed. Comet assay results revealed no significant DNA damages under dicofol exposure, indicating 1) apparent lack of genotoxicity of dicofol to the tested conditions and/or 2) resistance of the animals due to optimal adaptation to stress conditions. Moreover, *ex vivo* H_2O_2 exposure showed an increase in the DNA damage in all the treatments without significant differences between them. However, considering only the DNA damage induced by H_2O_2 during uptake phase, D1 animals had significantly lower DNA damage than those from other treatments, revealing higher protection against a second stressor. NAs data showed a decrease in the % of cells with polymorphic, kidney shape, notched or lobbed nucleus, along the experiment. The combination of these results supports the idea that the clams used in the experiment were probably collected from a stressful environment (in this case Pearl River Delta region) which could have triggered some degree of adaptation to those environmental conditions, explaining the lack of DNA damages and highlighting the importance of organisms' origin and the conditions that they were exposed during their lives.

1. Introduction

Dicofol (an acaricide that affects invertebrates and vertebrates nerve system) is an organochlorine pesticide (OCPs) that has been used in the crop industry to protect a variety of fruits and vegetables from mites (Thiel et al., 2011). This pesticide has been used since the late 1950s, however it is considered a worldwide environmental concern due to its capacity to biomagnify, similar to dichlorodiphenyltrichlorethane (DDT), which was extensively and predominantly used in Southeast Asia (between 2000 and 2012, Asia used around 22 Kt which is 77% of the worldwide total production) (Li et al., 2015), and known to cause high toxicity to animals, especially to aquatic life (Guo et al., 2008). Recent monitoring data demonstrate dicofol as adequately persistent to be transported from rivers to the open sea and to remote regions (UNED, 2016). As a consequence, the use of dicofol has been restricted or banned in many countries and usage has markedly decreased in the last decade (ca. 80% between 2000 and 2012) (UNED, 2016). Although banned in numerous countries, dicofol and its metabolites, mainly 4,4′-dichlorobenzophenone, can still be detected in Asian water bodies (Ivorra et al., 2019), biota (Wang et al., 2011) or sediments (Syed et al., 2013). Like other OCPs, dicofol can be toxic to the aquatic fauna causing genotoxic and endocrine disruptor effects, at least in aquatic vertebrates (Thibaut

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and Porte, 2004).

One of the strategies for pollutants monitoring in the aquatic environments, includes the use of bivalves, because of their feeding behavior (filter-feeders), geographical location, immobile lifestyle, high resilience and rapid and effective assimilation of toxic substances (Suarez et al., 2013). Moreover, considering their economic interest and their high consumption rate, especially in China (approx. 14 000 10^3 tons/-year; FAO, 2018), it is relevant to study dicofol toxicity in these marine invertebrates. In this work, the edible bivalve, *Meretrix meretrix* —also known as Vietnamese clam—was used as a model.

The ability of bivalves to bioaccumulate contaminants, and their use for environmental monitoring studies, have been widely studied (Siu et al., 2008; Ching et al., 2001; D'Costa et al., 2018). Previous work also showed how *M. meretrix* was able to accumulate 4,4′-dichlorobenzophenone (the main metabolite of dicofol) during and after 15 days exposed to dicofol (Ivorra et al., 2019); however, little is known about its genotoxicity in non-target organisms under realistic exposure scenarios. Therefore, the main aims of this work were: (1) to evaluate genotoxic effects of dicofol in the bivalve *M. meretrix* during exposure and depuration phase, and (2) to evaluate the protective or potentiation effects of the dicofol against DNA damage induced by H_2O_2 after *ex vivo* exposure. For that, two different approaches will be used: the comet assay (also called the single-cell gel electrophoresis assay) and nuclear abnormalities (NAs) evaluation. These techniques are routinely used as biomarkers for monitoring aquatic pollution by contaminants (D'Costa et al., 2018). The Comet assay is a rapid and sensitive technique that requires only a small number of cells to provide information regarding DNA damage and repair in individual cells (Lee & Scott, 2003). In the case of NAs—like segmented nucleus, binucleated cells, kidney shape, polymorphic cells and micronucleus— can be used to examine the exposure and effects of contaminants (Strunjak-Perovic et al., 2009; Carrola et al., 2014). This test is applied in environmental biomonitoring studies mainly due to its ability to detect chromosomal aberrations, structural and numerical, at specific stages in the cells' life cycle (Riva et al., 2007). While Comet assay (alkaline version) identifies single strand breaks and alkali-labile sites, NAs point out chromosomal damages that can also lead to potential germ cell mutations (Binelli et al., 2008). To summarize, some hypotheses have been raised in this study. Knowing that dicofol provoke DNA damage and mutagenic effects on humans and wildlife (Choi et al., 2004; Ahmad & Ahmad, 2017), and being the bivalves a sessile and filter-feeder organism (invertebrate), we expect (H1) that exposure to dicofol will induce genotoxic effects on the haemocytes of *M. meretrix*.

As mammalian cells have the capacity to recover from an initial genotoxic insult through the activation of their DNA repair mechanisms (Marques et al., 2014), similar response might be expected in bivalves after dicofol exposure (depuration phase) (H2).

Since previous exposure to a stress agent may influence the response to a second insult (Rocher et al., 2006), it is expected that previous exposure to dicofol can potentiate the DNA damage resultant from a second insult (H3.1) or give protection against it (H3.2).

2. Material and methods

2.1. Test organism/sample description

Bivalves (originally from Guangdong province) were acquired from a local market in Macao in 2017. Upon arrival in the laboratory, the clams (ca. 200) were immediately transferred in two 15 L containers and maintained in oxic conditions for an acclimation period of 4 days. During this period, animals were fed with a commercial mixture of spirulina and kelp (Kent Marine Microvert), with a daily dose of 600 μL, (1:10 dilution) and maintained under a photoperiod of 12:12 light/dark cycle. Temperature (1 °C/day) and salinity (2 ppt/day) were gradually adjusted until reaching 27 °C and 16–18 ppt.

2.2. Experimental design

The set up for this experiment included 36 sub-experiments (4 treatments*3 sampling times*3 replicates). The treatments used were: 1) C: control, with artificial seawater only (ASW), 2) SC: solvent control (0.1% methanol), 3) D1 = dicofol at environmental concentration (50 ng/L) based on concentrations quantified by Zheng et al. (2016), and 4) D2 = supra-environmental dicofol concentration (500 ng/L).

The total duration of the experiment was 30 days, divided into uptake phase where animals were exposed to dicofol during 15 days; and depuration phase where animals were exposed to ASW during the same period. At both phases, three sampling times (days) were considered (T1, T7 and T15). Diagram of the experimental design is shown in Fig. 1.

Distribution of the clams in the aquaria was done after the initial acclimation period, where same oxic conditions and feeding routine were kept. Each aquaria contained 1 kg sand (Xin Jing aquarium gravels) and 2.5 L of ASW. In order to ensure the stability of the system, clams were maintained in each aquaria during 24 h before starting the experiment. Distribution of the aquaria was done randomly into the water baths with controlled temperature and aeration.

Water was daily removed (by using a peristaltic water pump (BT100 M, Generic)) and replaced. In order to get homogenous concentrations in the system, dicofol's was spiked and previously mixed with an aliquot of ASW (250 mL) before adding to the aquarium. Due to dicofol's instability in aquatic environments (<1–4 days at pH 7–9), 4,4′-DCBP levels (dicofol's main metabolite) were quantified from water samples, regularly (right after spiking and 24 h later), as in Ivorra et al. (2019).

In order to avoid cross-contamination, individually glass covers were placed on top of the aquaria. Finally, 2 individuals of each replicate (total n = 6), were collected at each sampling time, and extraction of the haemolymph (ca. 150 μl per individual) from posterior adductor muscle with a hypodermic syringe (24 G, 0.55 μm pore size and 25 mm needle length) previously heparinized, was performed for further cell viability and genotoxicity evaluation (Comet assay and NAs). As an indicator of the health status of the bivalves (Hyötyläinen et al., 2002), condition index (CI) was also calculated as: (fresh weight/shell weight) x 100. Survival rate (%) of the animals was also checked along experiment. Temperature (daily), and pH and dissolved oxygen (DO) (weekly) were measured through the entire experiment using a multiprobe (YSI pro plus, USA).

2.3. Chemicals and reagents

Details regarding all the chemicals and reagents used in this work can be found in the Supplementary Material.

2.4. Cell isolation and sample viability

The collected haemolymph was transferred to a 1.5 mL tube, and placed on ice. Prior to the genotoxicity assays, cell count and cell viability were evaluated to ensure an optimum number of cells to perform the assay. The cell viability was done using the trypan blue exclusion test (0.1% in PBS) with 1:1 dilution. Cells were counted in a Neubauer chamber and only animals showing more than 80% viable cells were considered for genotoxicity assays.

2.5. Comet assay (in vivo procedure)

Comet assay technique followed the methodology described in Cruzeiro et al. (2019) with some modifications for the *M. meretrix* cells. The haemolymph (20 μL with about 2.0×10^4 cells) was mixed with 100 μL of low melting point agarose (0.5%). Each sample was divided in two slides pre-coated with 1% normal melting point agarose (60 μL), and covered immediately with coverslips. The rest of the procedure was done according to Cruzeiro et al. (2019); and can be found in the Supplementary Material section.

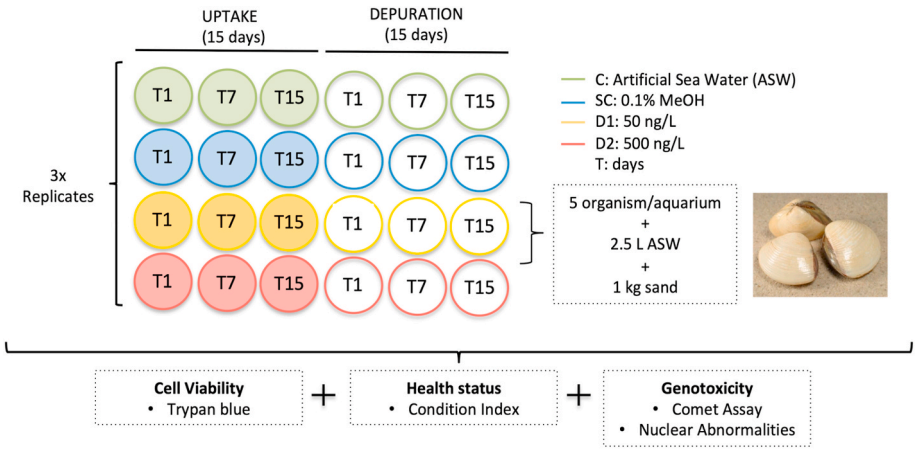

Fig. 1. Diagram of the experiment set-up, indicating the treatments (C: control; SC: solvent control; D1: dicofol 1; D2: dicofol 2) and sampling times (T1/T7/T15), in triplicates. Aquaria were randomly distributed inside water baths.

Processing software used to capture and score the images were Cell A (version 2.8), and the plugin Open Comet within the Image J software (version 1.51), respectively. Duplicated slides for each animal were included in the analysis. Per each individual, a total number of 100 comets were scored. To evaluate the DNA damage, the % DNA tail was measured. The average value per treatment was obtained considering the average value of each individual. Fig. 2 shows an example of no-tailed (A) and tailed (B) nucleoids. Data were organized by experiments, where exposure phases, treatments and times, were compared for each case. Base on the % of DNA in tail frequency of the distribution of nucleoids was also included and classified as: class 0 (0–5%); class 1 (5–20%); class 2 (20–40%); class 3 (40–75%); class 4 (>75%) (Azqueta et al. (2009)). All the results are expressed as average ± SE.

2.6. Comet assay (ex vivo procedure)

For the *ex vivo* exposure, similar protocol as the one described in 2.5 section was adopted. In this case, after 10 min at 4 °C, the coverslips were removed carefully and the slides were submerged in 40 mM of H_2O_2 for 5 min on ice. Then, slides were rinsed two times with PBS and immersed in lysis solution. The subsequent steps and data arrangement were the same as for the *in vivo* experiment.

Evaluation of H_2O_2-induced DNA damage, was done considering the basal DNA damage (% of DNA in tail without H_2O_2 exposure) and subtracting the DNA damage after H_2O_2 exposure. Data are expressed as average ± SE.

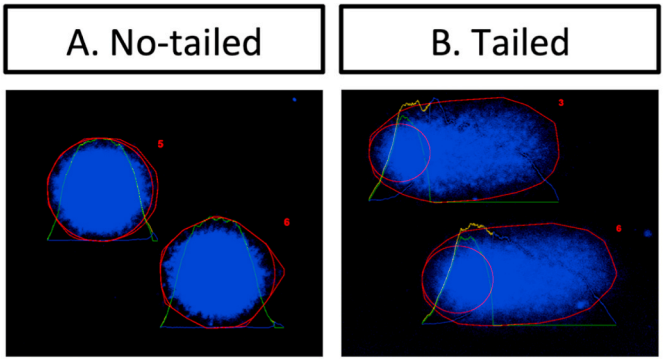

Fig. 2. No-tailed (A) and tailed (B) nucleoids from *M. meretrix* detected by Comet assay using Open Comet software analysis. Red color outlines the entire nucleoid, green profile indicates comet head; yellow profile indicates comet tail. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.7. Nuclear abnormalities procedure and analysis

Manual cytospin, followed by May-Grunwald-Giemsa stain was used for the identification of nuclear abnormalities, as described by Marcos et al. (2016). Firstly, 20 μl of the haemolymph suspension (ca. 2.0×10^4 cells) together with 30 μl of PBS were mixed carefully and smeared in the slide. Manual centrifugation (cytospin) was done for 3 min in order to obtain more homogenous distribution of the cells. After this step, slides were disassembled and oven dried for 30 min at 37 °C. Previous to the stain, slides were fixed in absolute methanol for 10 min. The stain protocol consisted of applying May-Grunwald eosine-methylene blue solution for 15 min, followed by Giemsa (5% in distilled water) for 30 min. Washing steps with distilled water were included after each stain. Finally, the slides were oven dried as indicated previously, and mounted with DPX medium (Sigma Aldrich, Germany) for further microscope observation. A total of 1000 haemocytes per animal were analyzed under a Leica DMB6000B light microscope (100 × objective). Intact haemocytes were classified in eight categories, as indicated in Fig. 3: Normal haemocytes (N); Segmented (SM) — symmetrical/asymmetrical "eight" shaped nuclei; Kidney Shaped (KS) — nuclei with a kidney shaped profile; Bilobated, lobed and nuclear buds (BLN) — presence of evagination or invagination in the nuclear envelope; Micronucleus (MN) — two nuclei clearly separated, and one of them very small; Binucleated (BN) — two nuclei clearly separated, and equal sizes, but clearly separated; Polymorphic (P) —with irregular nuclei and inconsistent pattern; and Nucleoplasmatic Bridge (NPB) — presence of a chromatin bridge between two nucleus. The mean frequencies of each category were estimated and expressed as a percentage.

2.8. Statistical analysis

All the obtained data was checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test). Data transformations were applied in order to fit the assumptions of normality. In order to check the health status of the organisms along the experiment, differences in condition index were assessed by 3-Way ANOVA, followed by Tukey's test. To determine differences in Comet assay (*in vivo* and *ex vivo*) and NAs results between treatments (C/SC/D1/D2), sampling times (T1/T7/T15), and phases (uptake/depuration), a 3-Way ANOVAs on ranks followed by Tukey's post-hoc test were applied to the different factors. Statistics of the classes was assessed by applying 2-Way ANOVA on ranks within each class (0/1/2/4) and for each independent phase (uptake/depuration), followed also by Tukey's post-hoc test. Statistics of the H_2O_2-induced DNA damage for each treatment was assessed by applying 2-Way ANOVA on ranks, followed by Tukey's post-hoc test. The significant differences found for all the

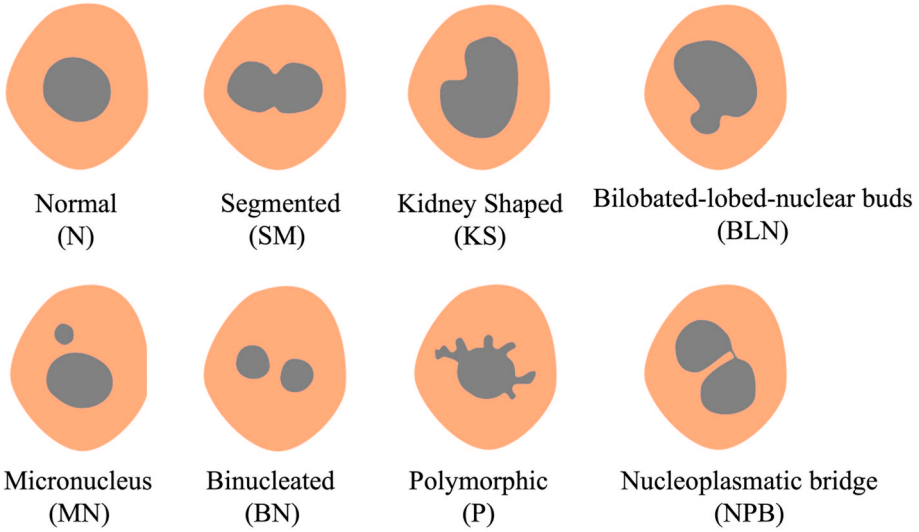

Fig. 3. Schematic representation of the different types of NAs in haemocytes from *M. meretrix*; image based on Strunjak-Perovic et al. (2009) and Carrola et al. (2014).

analysis, are represented by symbols or letters in the figures. Additionally, information related to the significant interactions can be found in SM file. The software Statistica version 7.00 was used for the analyses.

3. Results

3.1. Experimental design and maintenance

During the experiment, water temperature in the aquaria (26.92 ± 0.17 °C), pH (8.45 ± 0.14) and DO (106 ± 4.01%) were stable, indicating the successful maintenance conditions of the system. The survival rate did not vary among the treatments, presenting average values of 97.9 ± 3.9% of survival. Additionally, cell viability (assessed by trypan blue) was not affected by dicofol exposure, since all the values were above 80% for all the animals in all the treatments. Information and details related to the condition index (CI) can be found in the supplementary material (Figure S1). Overall, the CI declined significantly from uptake to depuration phase. Additionally, the CI of individuals exposed to dicofol was significantly higher than those from control.

3.2. Comet assay (in vivo)

DNA damages were estimated at different sampling times during uptake and depuration. The DNA damages (% tail DNA) induced *in vivo* by different concentrations of dicofol (50 ng/L and 500 ng/L) are shown in Fig. 4. *In vivo* results showed no significant differences for all of the factors individually, but a significant interaction between phase and time was found (3-Way ANOVA on ranks, $F_{(2,109)} = 5.7$, $p < 0.005$) (see Table-S1 in SM). Overall, a common pattern was observed, with a relevant increase of DNA damage in T7 of uptake relative to T1 of the same phase, and a relative decrease of DNA damage in T7 of depuration relative to T1 and T15 of the same phase.

Classes' classification (considering % DNA tail) as described in Materials and Method section was applied in order to evaluate the *in vivo* DNA damage distribution (Table 1). For all the treatments, the highest frequencies were observed in class 0 and 1. However, during uptake phase, in T7, distribution of these frequencies was different between D2 and the rest of the treatments. Animals exposed to D2 were mainly distributed in class 0 (62%) and class 4 (19%), while for the rest of the treatments (C, SC and D1) were between class 0 (65–69%) and class 1 (15–22%). During uptake, and looking to class 0, control presented significantly higher percentage of DNA damage than D1 (2-Way ANOVA on ranks, $F_{(3,\ 59)} = 3.45$, $p < 0.05$). Considering the sampling time, T7

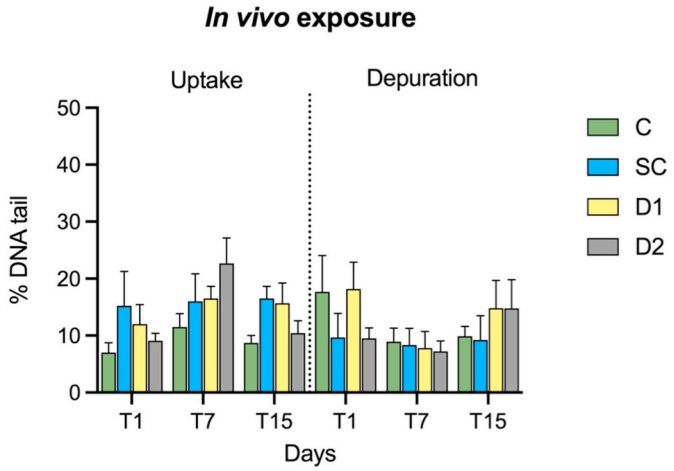

Fig. 4. *In vivo* results of DNA damage in haemocytes of *M. meretrix* during uptake and depuration phases. Results are expressed as average % DNA tail (±SE; n = 6 animals/treatment). C: control; SC: solvent control; D1: dicofol 50 ng/L; D2: dicofol 500 ng/L.

also had significant higher values than the other sampling times for data related to class 4 (2-Way ANOVA on ranks, $F_{(2,\ 59)} = 5.5$, $p < 0.01$) (Table 1). Depuration phase did not show any significant difference regarding time or treatment.

3.3. Protective effects against H_2O_2 (ex vivo)

Haemocytes (*ex vivo*) were exposed to H_2O_2, and single strand breaks were quantified by alkaline version of Comet assay (Fig. 5). The DNA damages, in Fig. 5A, were higher than in haemocytes without H_2O_2 exposure (Fig. 4). However, no significant effects of treatments or time were observed. Only a significant interaction between phase and time was found (3-Way ANOVA on ranks, $F_{(2,105)} = 9.2$, $p < 0.001$) (see Table-S2 in SM). Results obtained during uptake, indicated an increase in the frequency of nucleoids in class 1 to 4 for all the treatments, when compared with unexposed ones (Table 2). For example, classes 2 and 3 presented the highest increase during uptake (2.8- and 3-fold, respectively) and depuration (4.2- and 2.3-fold, respectively). In the uptake phase, no significant differences were observed between times, however, in class 0, significant lower values were observed in control when

Table 1
Average frequencies (%) of DNA damage distribution in each class (average ± SE, n = 6 animals/treatment) in haemocytes of *M. meretrix* exposed to different treatments C: control; SC: solvent control; D1: dicofol 50 ng/L; D2: dicofol 500 ng/L. For class 0, superscript letters indicate significant differences among treatments during uptake. For class 4, different symbols (in front of T1, T7 and T15) indicate significant differences among times, also during uptake.

			Class 0	Class 1	Class 2	Class 3	Class 4
Uptake	T1°	C	70.9 ± 6.6[a]	19.6 ± 3.3	3.1 ± 1.3	2.1 ± 1.0	4.2 ± 2.9
		SC	59.4 ± 9.6[a,b]	20.7 ± 4.2	5.9 ± 2.3	2.4 ± 0.7	11.6 ± 6.0
		D1	60.8 ± 11.3[b]	21.1 ± 6.6	8.3 ± 4.6	4.2 ± 1.6	5.6 ± 2.6
		D2	71.6 ± 4.9[a,b]	14.9 ± 3.9	2.9 ± 0.8	2.9 ± 0.9	7.5 ± 1.3
	T7Δ	C	65.4 ± 3.5[a]	22.4 ± 1.8	5.2 ± 1.0	1.6 ± 0.9	6.3 ± 1.9
		SC	68.6 ± 7.7[a,b]	14.9 ± 2.8	3.1 ± 1.2	2.1 ± 1.1	11.3 ± 4.8
		D1	64.6 ± 3.7[b]	15.1 ± 2.9	5.0 ± 0.9	3.5 ± 0.8	11.8 ± 2.5
		D2	61.9 ± 5.3[a,b]	10.4 ± 1.9	4.2 ± 0.6	3.7 ± 1.2	18.8 ± 4.9
	T15°	C	70.3 ± 6.4[a]	22.1 ± 4.4	1.8 ± 0.8	2.7 ± 1.1	3.0 ± 0.5
		SC	61.5 ± 5.5[a,b]	24.4 ± 4.1	6.3 ± 1.7	2.8 ± 0.5	4.9 ± 1.5
		D1	60.6 ± 4.7[b]	20.5 ± 4.9	5.7 ± 1.2	3.4 ± 0.7	9.8 ± 3.8
		D2	68.7 ± 4.9[a,b]	17.8 ± 2.9	3.9 ± 1.8	4.5 ± 1.2	5.0 ± 1.7
Depuration	T1	C	60.6 ± 8.3	18.9 ± 3.1	4.7 ± 0.7	4.5 ± 1.5	11.4 ± 6.5
		SC	77.3 ± 8.7	11.9 ± 3.1	2.6 ± 1.9	4.2 ± 2.5	3.9 ± 1.9
		D1	59.2 ± 3.9	21.5 ± 1.7	2.8 ± 0.7	2.8 ± 1.2	13.6 ± 5.9
		D2	79.9 ± 5.3	9.9 ± 2.6	2.1 ± 1.3	1.0 ± 0.7	7.0 ± 2.2
	T7	C	76.7 ± 6.4	13.5 ± 3.8	1.9 ± 0.9	2.6 ± 1.1	5.2 ± 2.3
		SC	77.4 ± 7.5	14.3 ± 4.4	2.1 ± 0.9	2.3 ± 0.9	4.0 ± 1.9
		D1	81.7 ± 5.5	9.4 ± 2.1	3.3 ± 1.3	1.7 ± 0.9	3.9 ± 2.2
		D2	73.9 ± 5.1	18.5 ± 3.3	2.1 ± 0.6	2.1 ± 0.9	3.3 ± 1.3
	T15	C	71.2 ± 6.3	16.5 ± 3.8	2.3 ± 0.7	3.5 ± 1.1	6.6 ± 1.9
		SC	77.9 ± 8.2	13.5 ± 3.6	0.9 ± 0.4	2.8 ± 1.5	4.9 ± 3.3
		D1	72.8 ± 9.2	8.5 ± 1.6	3.6 ± 0.9	7.1 ± 2.7	10.2 ± 4.4
		D2	69.4 ± 7.2	11.6 ± 2.1	4.8 ± 1.4	3.9 ± 1.8	10.4 ± 3.9

Table 2
Average frequencies (%) of DNA damage distribution in each class (average ± SE, n = 6 animals/treatment) in haemocytes of *M. meretrix* after *ex vivo* exposure to H_2O_2. C: control; SC: solvent control; D1: dicofol 50 ng/L; D2: dicofol 500 ng/L. For class 0, during uptake, superscript letters indicate significant differences among treatments. For classes 0, 2, 3 and 4, during depuration, different symbols (in front of T1, T7 and T15) indicate significant differences among times.

			Class 0	Class 1	Class 2	Class 3	Class 4
Uptake	T1	C	31.6 ± 4.5[a]	36.9 ± 4.2	11.3 ± 1.6	8.4 ± 3.4	11.8 ± 3.4
		SC	44.1 ± 6.7[b]	24.9 ± 2.2	9.3 ± 2.8	9.8 ± 2.9	12.0 ± 5.3
		D1	45.1 ± 9.4 [b]	24.9 ± 3.4	11.0 ± 5.0	8.1 ± 3.0	10.9 ± 2.1
		D2	47.6 ± 5.8 [b]	25.9 ± 2.6	10.7 ± 1.5	8.1 ± 1.7	7.7 ± 1.8
	T7	C	29.8 ± 6.1[a]	28.6 ± 4.7	12.2 ± 2.0	12.9 ± 6.5	16.6 ± 2.7
		SC	41.9 ± 8.8[b]	26.8 ± 4.8	9.3 ± 2.4	5.1 ± 1.5	16.9 ± 3.7
		D1	37.8 ± 6.0[b]	35.4 ± 4.3	10.6 ± 2.4	3.4 ± 0.6	12.8 ± 3.3
		D2	43.1 ± 3.4[b]	26.9 ± 2.6	7.8 ± 1.5	5.0 ± 1.9	17.2 ± 3.1
	T15	C	17.6 ± 4.9[a]	35.4 ± 4.1	16.9 ± 3.6	13.7 ± 2.9	16.4 ± 4.6
		SC	47.0 ± 11.8[b]	31.0 ± 5.0	8.0 ± 3.0	6.3 ± 2.4	7.6 ± 2.2
		D1	53.8 ± 7.8[b]	23.1 ± 3.8	8.0 ± 2.1	8.0 ± 1.3	7.0 ± 2.0
		D2	37.1 ± 8.7[b]	30.1 ± 3.6	10.7 ± 2.9	10.0 ± 3.3	12.1 ± 3.6
Depuration	T1°	C	34.0 ± 4.8	29.1 ± 1.3	15.6 ± 1.6	10.3 ± 2.7	11.0 ± 1.7
		SC	23.2 ± 7.3	21.6 ± 3.0	11.1 ± 0.5	14.7 ± 3.2	29.3 ± 7.2
		D1	15.6 ± 2.7	48.5 ± 3.4	13.4 ± 1.2	9.0 ± 3.4	13.5 ± 3.2
		D2	37.9 ± 7.7	34.2 ± 4.8	11.6 ± 2.0	3.5 ± 2.0	12.8 ± 2.7
	T7Δ	C	51.8 ± 9.8	30.4 ± 3.6	6.6 ± 2.8	4.3 ± 1.8	6.8 ± 2.9
		SC	54.3 ± 12.1	28.7 ± 5.2	6.3 ± 2.5	3.5 ± 1.8	7.1 ± 4.1
		D1	72.9 ± 7.7	18.2 ± 3.9	3.7 ± 1.9	1.5 ± 1.1	3.6 ± 1.8
		D2	54.0 ± 7.4	29.7 ± 3.9	7.2 ± 2.1	4.0 ± 1.7	5.0 ± 1.4
	T15°	C	34.8 ± 8.4	33.7 ± 5.1	8.8 ± 2.1	7.9 ± 3.6	14.8 ± 5.9
		SC	29.1 ± 6.0	38.4 ± 3.0	11.6 ± 2.9	8.8 ± 3.3	12.1 ± 3.0
		D1	46.0 ± 6.5	26.9 ± 1.7	7.6 ± 1.4	7.1 ± 2.5	12.4 ± 3.3
		D2	25.0 ± 6.9	29.2 ± 6.7	15.7 ± 2.6	8.9 ± 2.6	21.2 ± 6.4

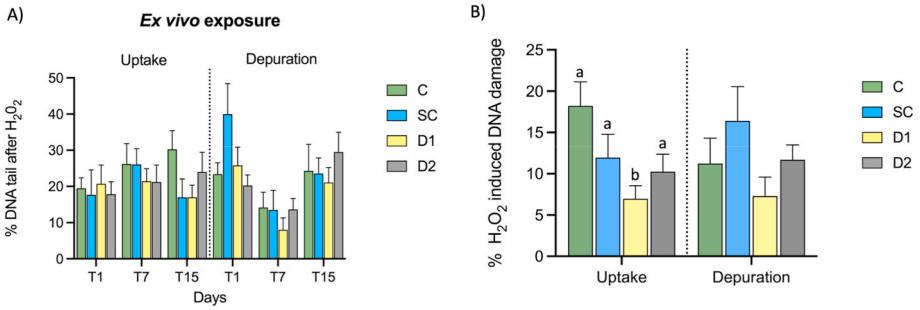

Fig. 5. DNA damage in haemocytes of *M. meretrix* after *ex vivo* exposure to H_2O_2 during uptake and depuration phase. Results are expressed as average (±SE; n = 6 animals per treatment). A) % DNA in tail after H_2O_2 exposure; B) % DNA damage induced by H_2O_2 (DNA damage after H_2O_2 exposure - basal DNA damage). C: control; SC: solvent control; D1: dicofol 50 ng/L; D2: dicofol 500 ng/L.

compared to the rest of the treatments (2-Way ANOVA on ranks, $F_{(3,57)} = 4.38$, $p < 0.01$; Table 2).

Contrarily, depuration results presented significant differences between sampling times, with significantly higher frequency of nucleoids in class 0, at T7 (2-Way ANOVA on ranks, $F_{(49,2)} = 11.4$, $p < 0.0001$) than T1 and T15; and significantly lower in class 2 (2-Way ANOVA on ranks, $F_{(49,2)} = 8.7$, $p < 0.001$), class 3 (2-Way ANOVA on ranks, $F_{(49,2)} = 6.6$, $p < 0.01$), and class 4 (2-Way ANOVA on ranks, $F_{(49,2)} = 10.4$, $p < 0.001$). Only class 1, during depuration presented a significant interaction between treatments and sampling times (2-Way ANOVA on ranks, $F_{(49,6)} = 3.4$, $p < 0.01$) (see Table-S3 in SM).

Concerning the % of DNA damage induced by the H_2O_2 (Fig. 5B), there were no differences between uptake and depuration. However in the uptake, considering all the times, the organisms exposed to D1 presented a significantly lower damage (a reduction of 61% in relation to control) (2-way ANOVA on ranks, $F_{(3,99)} = 3.02$, $p < 0.05$) caused by H_2O_2 than the other treatments (see statistical table in Fig. 5B). In the depuration phase, this reduction was not statistically significant.

3.4. Nuclear abnormalities

The results of NAs (i.e., segmented, kidney, polymorphic and BLN) are presented in Figs. 6 and 7. Morphological NAs, such as MN, binucleated and bridge shape were barely observed (<0.1%) in this study, therefore were not included for further discussion. Differences were studied within each category and between phases. Regarding the percentage of total NAs (Fig. 6), significant differences were observed between uptake and depuration (3-Way ANOVA on ranks, $F_{(1,106)} = 15.3$, $p < 0.05$), showing a significant decline in the total NAs along the experiment. In addition, significant interactions between treatment and time (3-Way ANOVA on ranks, $F_{(6,106)} = 2.3$, $p < 0.05$) and time and phase (3-Way ANOVA on ranks, $F_{(2,106)} = 3.5$, $p < 0.05$) were also observed (see Table-S4 in SM).

For the polymorphic type, there was a significant decline in the percentage of cells with this type of NAs from uptake to depuration phase (3-Way ANOVA on ranks, $F_{(1,\ 106)} = 33.2$, $p < 0.0001$) and between sampling times from T1 to T15 (3-Way ANOVA on ranks, $F_{(2,\ 106)} = 5.0$, $p < 0.01$; Fig. 7).

For kidney type, there was a significant decline in the percentage of cells from uptake to depuration phase (3-Way ANOVA on ranks, $F_{(1,\ 106)} = 6.07$, $p < 0.05$; Fig. 7).

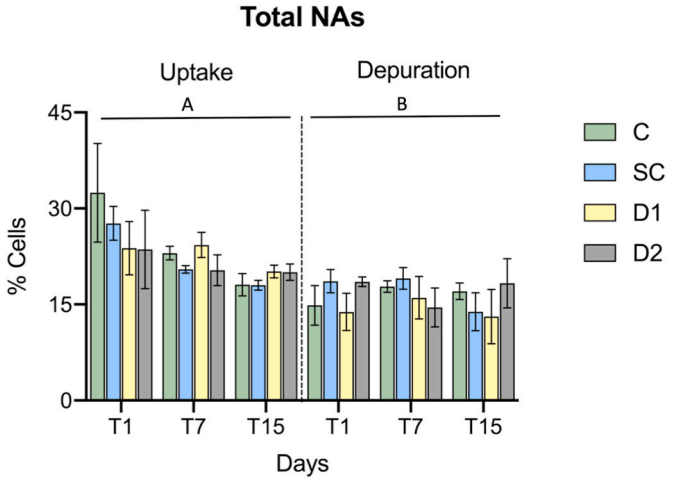

Fig. 6. Total nuclear abnormalities (NAs) in haemocytes cells from *M. meretrix* during uptake (exposed to dicofol) and depuration phase. Results are expressed as average % Cells (±SE; n = 6 animals per treatment). Sampling times are expressed in days (T1/T7/T15) for each phase. C: control; SC: solvent control; D1: dicofol 50 ng/L; D2: dicofol 500 ng/L. Different uppercase letters represented in the graph indicate significant differences between phases.

For BLN, significant differences were observed between uptake and depuration (indicated in Fig. 6). In addition, significant interactions were observed between treatment and time (3-Way ANOVA on ranks, $F_{(6,\ 106)} = 2.2$, $p < 0.05$) and time and phase (3-Way ANOVA on ranks, $F_{(2,\ 106)} = 12.01$, $p < 0.0001$; Fig. 7) (see Table-S5 in SM).

4. Discussion

4.1. Comet assay (in vivo)

One of the novelties of this work is the study of the genotoxic effects of dicofol, as well as its potential effects against H_2O_2-induced DNA damage on haemocytes of *M. meretrix*. Haemocytes are cells of the open vascular system that have been used routinely for monitoring cytogenetic damage (Pavlica et al., 2001), since they play an important physiological role in detoxification of xenobiotics in invertebrates (Calisi et al., 2008). Previous studies have already demonstrated that dicofol can be highly toxic for aquatic organisms (Singh et al., 2016; Grisolia, 2002). Despite the lack of information regarding dicofol genotoxicity in invertebrates, there is some literature with aquatic vertebrates that demonstrates its genotoxicity. For example, Grisolia (2002), has shown that dicofol induced significant MN frequency in the fish *Tilapia rendalli* when exposed to three different concentrations (50, 100 and 200 mg/L). However, the concentrations used in this study were much higher than those used in our study, which can explain the positive results. Another study from Ahmad and Ahmad (2017) reinforced the genotoxic effects of dicofol in humans. This work strongly suggests that dicofol induces oxidative stress in erythrocytes through generation of reactive oxygen species (ROS). Despite there is no much information in the literature regarding the mode of action of dicofol, and considering that dicofol presents a similar molecular structure as DDT (MCS Tanimoto coefficient of 0.95 over 1), we can assume a similar response by the cell/target animal. Previous studies with DDT demonstrated that it can act as an effective agent, inducing oxidative stress by overproduction of reactive oxygen species (ROS) that damage DNA (Jin et al., 2014; Tebourbi et al., 2011). So, we can assume a similar response regarding dicofol.

The results of this study demonstrated that, apparently, dicofol did not cause DNA damage detected by alkaline version of comet assay (i.e., strand breaks and alkali-labile sites), despite the accumulation of its main metabolite (4,4′-dichlorobenzophenone) in the bivalves throughout the exposure period (Ivorra et al., 2019), which goes against H1. A possible explanation for the inexistence of DNA damage can be related to the dicofol concentrations used in this mesocosm experiment.

Another explanation for the lack of genotoxicity observed, can be related to the origin of studied organisms. The results obtained suggest that *M. meretrix* individuals were, probably collected from a disturbed area, with some pre-exposure to stressful environmental conditions (i.e. pollution, abiotic factors), which could have given some resistance and adaptation through life to the exposure to a second stressor (Rocher et al., 2006). This last hypothesis is in accordance with the multixenobiotic resistance (MXR) concept. MXR consist in the ability of xenobiotic expulsion, by for example the expression of a membrane permeability glycoprotein that confers the ability to lower the intracellular concentration of the xenobiotic and to transport toxic substances out of the cell. In accordance with this hypothesis, some environmental xenobiotics, mainly hydrophobic pesticides, have been reported to interact with the mussel MXR-protein (Galgani et al., 1995) and differential expression levels of the MXR-protein have been found in mussels living in polluted and unpolluted waters (Minier et al., 1993).

Animals used in this work, were acquired from a local market supposedly from the Pearl River Delta region (Guangdong province), an area with serious issues regarding environmental water pollution (Wang and Hao, 2020). Authors, such as Grung et al. (2015), through an extensive review work, considered the Pearl River basin as one of the areas with highest elevated concentrations of DDTs in water (>250 ng/L). So, *M. meretrix* may have developed throughout their lives,

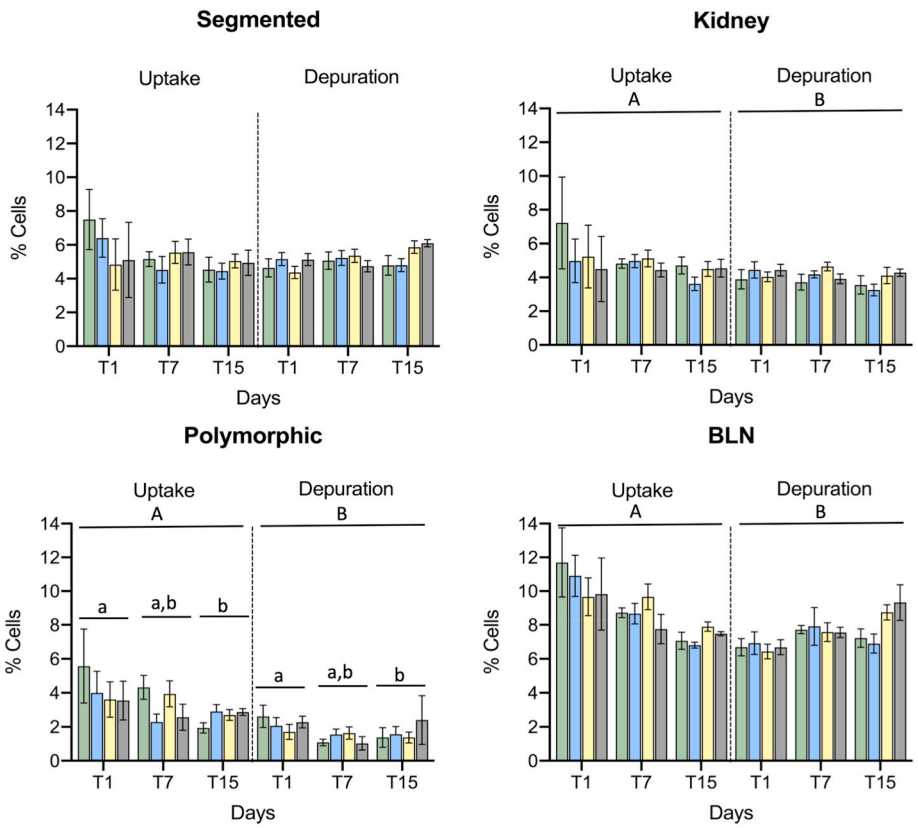

Fig. 7. Specific nuclear abnormalities in haemocytes cells from *M. meretrix* during uptake (exposed to dicofol) and depuration phase. Results are expressed as average % Cells (±SE; n = 6 animals per treatment). Sampling times are expressed in days (T1/T7/T15) for each phase. C: control; SC: solvent control; D1: dicofol 50 ng/L; D2: dicofol 500 ng/L. Different letters represented in the graph indicate significant differences between phases (uppercase) or times (lowercase).

mechanisms of adaptation to disturbed conditions, for instance, through the activation of anti-oxidant defense and/or DNA repair ability. It is important to consider that DNA damage is the result of two opposite processes: damage induction and DNA repair. As some authors already suggest, the augmented antioxidant defenses in marine organisms exposed to organic pollutants or trace metals may play a role in the ability of the organism for protection. Rocher et al. (2006), clearly suggested an activation of cellular defense mechanisms in bivalve species chronically exposed to waterborne contaminants. Activation of those cellular defenses is thought to protect DNA and other cellular macromolecules against oxidation and adduct formation. In case that DNA damage induction cannot be avoided, DNA repair is activated to maintain the genomic stability (Eastman and Barry, 1992). The lack of damage observed in our study (for both concentrations) could be due to a successful activation of the repair system. Some authors suggested that a "threshold" has to be reached before any DNA repair process can be maximized (Ching et al. (2001), Siu et al. (2003, 2004)), and this is generally happening at higher concentrations. For example, Black et al. (1996) observed significant DNA strand breaks in the freshwater mussel (*Anodonta grandis*) exposed to lower concentrations of lead (50 μg/L) but not at higher ones (500 and 5000 μg/L), over 4 weeks experiment. Also, Siu et al. (2003) and Ching et al. (2001) reported the same tendency when mussels (*Perna viridis*) were exposed to different concentrations of bezo[a]pyrene (B[a]P) during 12 and 24 days, respectively. In the present study, no DNA strand breaks were observed for both dicofol concentrations, which means that none or some DNA repair activation occurred.

Moreover, since no DNA strand breaks were induced during the uptake phase, we could not evaluate their capacity to recover through activation of the DNA repair mechanisms during depuration phase, as we firstly hypothesized (H2).

4.2. Protective effects against H_2O_2 (ex vivo)

Comet assay results revealed an increase of DNA damage after H_2O_2 exposure compared to basal damage. This patter was observed for all the treatments, without significant differences between them. However, considering that no differences between times were observed, the results regarding the percentage of DNA damage induced by the H_2O_2 were partially in agreement with H3.1 since during uptake phase, organisms pre-exposed to D1 treatment had a higher protection against a second stressor than those from D2. These results also corroborate with the theory that clams came from a disturbed system (i.e., stressful environment) in which the organisms were probably exposed, during their lives, to certain levels of contamination and a posterior exposure to a genotoxic agent did not cause expressive DNA damages as initially expected. Therefore, results obtained herein indicated that the organisms used could present resistance to *in vivo* exposure to dicofol and *ex vivo* exposure to H_2O_2 due to previous adaptation to stressful conditions, which is in accordance with H3.2.

4.3. Nuclear abnormalities

Concerning the percentage of nuclear abnormalities, results were also against the H1, indicating that exposure to dicofol is not inducing genotoxic effects on the heamocytes of *M. meretrix*. Once again, these results support the idea of adaptive mechanisms of the organisms coming from stressful environments, since apparently an improvement on the percentage of total NAs was observed along the experiment. For all the treatments, total NAs in the organisms during the first 24 h (T1-uptake) were significantly higher than for the other sampling times. As it was previously mentioned, the natural habitat of these organisms should have been a more stressful environment than the experimental

conditions that they were exposed to, explaining this significant decline with time. Results obtained by Rabei et al. (2018), also support the idea that mollusks (*Donax trunculus*) coming from contaminated sites have improved their health status when transplanted to less stressful conditions.

Herein, we also reported each type of nuclear abnormality individually that may potentially provide a more refined approach. Results regarding individual alterations also followed the same pattern, supporting the idea of a better health status of the animals along the experiment. Despite the vestigial occurrence of MN found in our study, other abnormalities such as polymorphic, kidney, BLN or segmented cells seemed to be more sensitive biomarkers to assess genotoxicity. The different frequencies of these abnormalities are related to specific genotoxic events associated to the different mechanisms of action of the carcinogenic/mutagenic agents (Bolognesi et al., 2006). In this work for example, nuclear abnormalities related to cell division such as MN, binucleated cells or nuclear bridge, were less obvious, while biomarkers related to nuclear evaginations or buds (indicating gene amplification, cellular necrosis or/and cellular apoptosis; Fenech et al., 1999) were more frequent.

5. Conclusions

In this study, dicofol concentrations did not induce genotoxic effects in haemocyte cells from *M. meretrix,* as assessed by comet assay and NAs. However, variations on the level of DNA damage were observed during the experimental period as well as a significant decline of NAs. The lack of genotoxicity of dicofol may be due to: 1) dicofol concentrations that were not enough to trigger DNA damage and/or 2) the adaptation of the bivalves throughout their lives to certain stressful environments. This preconditioning could result in the activation of certain mechanisms of the antioxidant defense system and/or DNA repair system conferring organisms' adaptation, fact that was also supported by the *ex vivo* exposure to H_2O_2 results. This work also revealed different kinds of NAs (i.e., polymorphic, segmented, BLN and kidney) associated to different mechanisms of action of mutagenic agents.

Evidence found in this study may indicate that the environmental conditions that animals were exposed to during their life-time are quite relevant to the results obtained. However, since little is known about DNA damage/repair system and antioxidant defenses in bivalves, complementary studies should be done to better understand the results obtained herein.

Author statement

Lucia Ivorra: Investigation, Data curation, Formal analysis, Writing-Original draft preparation **Catarina Cruzeiro**: Conceptualization, Methodology, Formal analysis,Writing- Reviewing and Editing, **Alice Ramos**: Methodology, Writing- Reviewing and Editing **Patricia G. Cardoso**: Conceptualization, Methodology, Writing- Reviewing and Editing, Supervision **Karen Tagulao**: Conceptualization, Investigation, Writing- Reviewing and Editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.118467.

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