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Evaluation of toxicity of *Deepwater Horizon* slick oil on spat of the oyster *Crassostrea virginica*

Vignier Julien ^{1,2,*}, Rolton Anne ^{2,3}, Soudant Philippe ³, Chu Fu-Lin E. ⁴, Robert Rene ⁵, Volety Aswani K. ^{2,6}

¹ Cawthron Inst, 98 Halifax St East, Nelson 7010, New Zealand.

² Florida Gulf Coast Univ, Dept Marine & Ecol Sci, Coll Arts & Sci, Ft Myers, FL 33965 USA.

³ IUEM UBO, Lab Sci Environm Marin, UMR LEMAR 6539, Technopole Brest Iroise, F-29280 Plouzane, France.

⁴ VIMS, Dept Aquat Hlth Sci, Coll William & Mary, Gloucester Point, VA 23062 USA.

⁵ IFREMER, Unite Littoral, Ctr Bretagne ZI Pointe Diable, CS 10070, F-29280 Plouzane, France.

⁶ Univ North Carolina Wilmington, Bear Hall 146,601 S Coll Rd, Wilmington, NC 28403 USA.

* Corresponding author : Julien Vignier, email address : julien.vignier@cawthron.org.nz

Abstract :

The 2010 explosion of the Deepwater Horizon (DWH) oil rig generated the largest marine oil spill in US history with millions of barrels of crude oil released in the Gulf of Mexico (GoM). The eastern oyster, *Crassostrea virginica*, is an ecologically and economically important species in the northern GoM. Due to its biological characteristics (sessile, filter feeding), juvenile oysters may have been affected. This study investigated the effects of surface-collected DWH oil prepared as high-energy water-accommodated fraction (HEWAF) on the survival of 2-month-old oyster spat, and evaluated the potential impacts of HEWAF on particle clearance rate and spat tissue. Exposure of oysters to a range of oil/HEWAF (0-7-66-147-908-3450 $\mu\text{g tPAH50}$ (sum of 50 polycyclic aromatic hydrocarbons) L⁻¹) resulted in non-dose-dependent mortalities and reduced clearance rates of algal food (*Tisochrysis lutea*). A morphometric study of the digestive tubules (DGTs) indicated a dose-dependent response to oil exposure on lumen dilation, on epithelium thinning of the DGT, and a significant change in DGT synchrony (LOEC = 66 $\mu\text{g tPAH50 L}^{-1}$). This finding suggests that structural changes occurred in the digestive gland of exposed oysters most likely due to an oil-related stress. In addition, histological observations showed that tissues in contact with HEWAF (gills, palp, connective tissue, digestive gland) were adversely impacted at $\geq 7 \mu\text{g tPAH50 L}^{-1}$, and exhibited pathological symptoms typical of an inflammatory response (e.g., hemocyte diapedesis and infiltration, syncytia, epithelium sloughing).

Keywords : Oyster spat, *Deepwater Horizon* oil, Sublethal, Tissue, Inflammatory response, Histopathology

23 1. Introduction

1
2 24 On April 20th 2010, the explosion at the *Deepwater Horizon* (DWH) oil drilling rig located 50 miles
3
4 25 southeast of the Louisiana Coast in Mississippi Canyon Block 252 (28°55'12''N, 88°23'14''W), led
5
6
7 26 to the largest marine oil spill in United States history (Carriger & Barron, 2011; National
8
9
10 27 Commission, 2010). Until the well was capped and the flow finally stopped, 87 days after the DWH
11
12 28 oil rig sank, several million barrels of light Louisiana crude oil were released into the Gulf of Mexico
13
14 29 (GoM) (U.S District Court, 2014; 2015). The escaping oil created a deep sea “cloud” or “plume”, and
15
16
17 30 a surface slick covering up to 112,000 km² of the Gulf waters (Boesch, 2014; ERMA, 2015)]. The
18
19 31 coastal extent of the spill was from Louisiana to Florida, with at least 2,113 km of shoreline visibly
20
21
22 32 oiled (Nixon et al., 2015).

23
24 33 Petroleum hydrocarbons or crude oil constituents are one of the most common contaminants released
25
26
27 34 into the marine environment: some of them are of particular concern due to their high chemical
28
29 35 stability, low solubility, low degradation, and lipophilic nature. Although oil spills represent a small
30
31
32 36 fraction of the total crude oil discharge into the sea, they have acute and long term impacts on marine
33
34 37 ecosystems, including effects from physical damage (physical contamination and smothering) and
35
36
37 38 toxicity from chemical compounds (NRC, 2003). Polycyclic aromatic hydrocarbons (PAHs) are
38
39 39 considered to be the most acutely toxic components of crude oil (Neff, 1985; Neff & Stubblefield,
40
41 40 1995). Given their lipophilic nature, these contaminants can bind to particulate organic matter,
42
43
44 41 sediment and microalgae, thus entering the food web (Graham et al., 2010; Lee et al., 1978).
45
46 42 Sedentary, benthic filter-feeding organisms such as oysters have an enormous filtration capacity and
47
48
49 43 are particularly effective in taking up hydrocarbons *via* filtration (Newell, 2004). Following ingestion
50
51 44 of hydrocarbons, oysters may accumulate high concentrations within their tissues and may be
52
53
54 45 susceptible to the negative effects of these contaminants. For this reason, bivalve mollusks, such as
55
56 46 oysters and mussels, are employed on a world-wide scale as sentinel organisms to monitor
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47 concentrations of contaminants in estuarine and coastal environments (Farrington et al., 1983;
48 O'Connor & Lauenstein, 2006).

49 The Eastern oyster (*Crassostrea virginica*) is one of the most commercially important shellfish
50 species in the GoM, representing about \$74 million in landings value in coastal regions of the
51 northern Gulf for 2012 (NMFS, 2012). In addition to its economic value, it is also an ecologically
52 vital species. Oyster reefs provide food, shelter and habitat for many fish and shellfish species,
53 improve water quality, stabilize bottom areas, and influence water circulation patterns within
54 estuaries (Coen et al., 2007; Newell, 2004; Wells, 1961). Although the location of the spill was in
55 deep water, currents carried the oil to shallow coastal waters and estuaries, which are spawning and
56 nursery grounds for many marine species, including oysters. In the North of the GoM, two peaks in
57 settlement of *C. virginica* occur in early and late summer (Supan, 1983). Given the time of the DWH
58 spill, newly settled spat and juveniles of *C. virginica* are thus likely to have been exposed to DWH
59 crude oil and associated PAHs. The physiological and ecological effects of environmental stress are
60 numerous. While the ultimate endpoint of stress is mortality, sub-lethal effects may interfere with
61 normal metabolic processes of the animals, thus increasing expenditure of energy reserves at the
62 expense of growth, survival and ultimately reproduction and recruitment (Capuzzo, 1996).

63 Given their role of sentinel in coastal environment, bivalve species have the capacity to survive in
64 highly polluted waters, despite the fact that pollutant levels often overload the normal physiological
65 mechanism of biotransformation or detoxification present in the cell (Gilewicz et al., 1984; Moore,
66 1985). Exposure to some petroleum hydrocarbons can cause alterations in the condition of certain
67 molluscs, by reducing the feeding efficiency of the animals (Axiak & George, 1987; Barszcz et al.,
68 1977; Galtsoff et al., 1935). As a result, variations in clearance rates of oil-exposed oysters may
69 influence the degree of assimilation and exposure of specific tissues, and, subsequent damage. After
70 long-term and sub-lethal exposure to petroleum hydrocarbons, vacuolization and atrophy of the
71 digestive cells leading to a thinning of the digestive epithelium and subsequently to an increase in
72 tubule lumen size have been reported for molluscs (Cajaraville et al., 1989; Lowe et al., 1981;

73 Widdows et al., 1982). In addition, a loss of the normal synchrony of the digestive cells of mussels
74 exposed long-term to oil has been found, to a point where almost all the tubules exhibited a similar
75 appearance (i.e. reconstituting stage) (Lowe et al., 1981). To avoid some of the subjectivity
76 associated with descriptive histology, quantitative techniques, such as stereology and morphometric
77 analysis, have been employed to measure alterations in cells following contaminant exposure
78 (Cajaraville et al., 1989; Lowe et al., 1981). Accordingly, in the present study, morphometric
79 analyses were employed to quantify alterations on the digestive tubules of oil-exposed *C. virginica*.
80 Histopathological changes are powerful indicators of prior exposure to environmental stressors,
81 especially xenobiotics, and histopathology is often seen as the easiest method for assessing both
82 short- and long-term toxic effects in the field (Kim et al., 2006). The long-term effects of oil
83 exposure in oysters collected from the field were studied after the *Amoco Cadiz* spill and included
84 necrosis, inflammation, and lesions of the digestive tract epithelium, gonads, connective tissue, and
85 gills (Berthou et al., 1987; Neff & Haensly, 1982). After an exposure to petroleum hydrocarbons,
86 degeneration of epithelial cells in the foot and alimentary canal, activation of mucus secretory cells
87 and inflammatory responses in the gills of the clam *Venus verrucosa* were observed (Axiak et al.,
88 1988). These studies suggest that after exposure to oil, an inflammatory response will likely occur,
89 mainly located in the gills and the digestive system of exposed bivalves. Studies have also shown
90 evidence that contaminant-stimulated “reactive oxygen species” (ROS) production and resulting
91 oxidative damage may be a mechanism of toxicity in aquatic organisms exposed to pollution (Di
92 Giulio et al., 1989; Livingstone et al., 1989; 2001).

93 The objective of the present study was to determine the physiological (e.g. clearance rates), tissue
94 (e.g. digestive tubules staging, tubule lumen sizes, inflammatory responses) and biochemical (e.g.
95 lipid peroxidation) responses of *C. virginica* spat exposed to increasing concentrations of high-
96 energy water accommodated fraction (HEWAF) of oil.

98 2. Material and methods

99 2.1 Preparation of exposure solutions

100 Surface oil, also referred to as “Slick A”, was collected on July 29th 2010, from the hold of barge
101 number CTC02404, which was receiving surface slick oil from various skimmer vessels near the
102 *Macondo* well, and was delivered under chain of custody (sample CTC02404-02). Slick A incurred
103 significant natural weathering at sea before collection. HEWAF exposure solutions were prepared
104 following a standardized procedure extensively detailed in Vignier et al. (2015). Briefly, two-liters of
105 UV-sterilized and 0.1 µm-filtered seawater (FSW) were added to a stainless-steel blender pitcher
106 (Waring™ CB15 commercial food blender) and 4 g of Slick A were added using a gastight syringe
107 (1:500 dilutions of oil). After blending for 30 s at low speed, the solution was transferred to a 2-L
108 aspirator bottle and left to settle for at least one hour to allow separation of the solution from residual
109 floating oil. The stock solution (2 g oil L⁻¹) was obtained by carefully draining the bottom layer of
110 the mixture from the aspirator bottle and used for PAH analysis and test dilutions with FSW. Four
111 liters of stock solution were prepared every other day with fresh oil. The solution was not filtered,
112 and thus contained whole particulate oil in addition to dissolved PAHs.

113 2.2 Water quality

114 Throughout the exposure period, temperature and dissolved oxygen (DO) were monitored daily by
115 means of a Pro ODO optic probe (YSI™), while salinity and pH were measured daily using a
116 refractometer (Pentair Aquatic Eco-systems Inc.™) and a “Pinpoint” pH monitor (American Marine
117 Inc.™) respectively. Total ammonia was measured in each treatment, and analyzed using a SEAL
118 Analytical Auto-Analyzer 3. All water quality parameters were measured daily, and from both
119 discarded and fresh exposure solutions. Each exposure chamber was aerated to maintain oxygen
120 levels above 5 mg L⁻¹ and light photoperiod was maintained at 12h: 12h using fluorescent lamps.

121 2.3 Experimental design

122 2.3.1 Test organisms

1
2 123 Two month-old single seed oyster spat (mean shell length \approx 10-15 mm; mean individual dry weight \approx
3
4 124 0.5 mg) from the Auburn University shellfish laboratory (Dauphin Island, AL) were used during
5
6 125 spring 2013. After reception, oyster spat were gradually acclimated at the experimental hatchery in a
7
8
9 126 flow-through tank for 2 weeks. Using a sump and a submersible pump, seawater was semi-
10
11 127 recirculated and salinity was gently increased over several days, from its origin (i.e. 20 PSU \pm 2) to
12
13
14 128 the local conditions (ambient temperature of 23°C \pm 1 and salinity of 30 PSU \pm 2). Oyster were fed
15
16 129 *ad libitum* with Instant Algae/Shellfish Diet[®] (Reed Mariculture Inc. [™]) for optimal growth.
17
18
19
20 130

23 131 2.3.2 Experiment 1: HEWAF exposure and clearance rate measurements

26 132 Clearance rate (or filtration) measurements were carried out in 600 mL glass beakers for 24 h using
27
28
29 133 the unicellular microalgae *Tisochrysis lutea* (CCMP 1324). Gentle central aeration by means of a
30
31 134 glass pipette was provided at 100 mL min⁻¹ to each beaker to homogenize exposure solutions. Ten
32
33
34 135 spat per beaker were exposed, in triplicate, to increasing concentrations of HEWAF ranging from
35
36 136 100 to 2000 mg oil L⁻¹. Total PAH content was not quantified in exposure solutions used in the 24-h
37
38
39 137 filtration experiment. As a result, exposure treatments will be referred to as nominal concentrations
40
41 138 of HEWAF and expressed in mg oil L⁻¹. At the start of the exposure, spat were fed *ad libitum* with 1
42
43
44 139 billion cells (equivalent to 100 mL) of *T-lutea* per beaker. After thorough mixing, residual algal
45
46 140 cells were assessed through the exposure (T₀ and T₂₄) by taking a 1.5 mL aliquot from each replicate
47
48
49 141 and then fixing the sample by addition of 100 μ L of Glutaraldehyde. After thorough agitation, three
50
51 142 10 μ L subsamples were taken from the fixed aliquots and counted under a microscope using a
52
53
54 143 hemocytometer. Algal cells counts were then averaged for each sampling point and 24 h clearance
55
56 144 rates were determined for each HEWAF condition. To compare different treatments, clearance rates
57
58
59
60
61
62
63
64
65

145 (CR) were normalized per gram of whole dry tissue weight of oyster, and determined using the
146 following equation, adapted from Coughlan (1969):

$$147 \quad CR = (V/(n \times t)) \times \ln (C_{i}/C_{f})/\bar{\omega}$$

148 where “CR” is the clearance rate in L h⁻¹ g⁻¹; “V” is the volume of each container in L (i.e. 0.5L); “n”
149 is the number of spat; “t” is the time of experiment in h; “C_i” and “C_f” are the initial and final counts
150 of *T. lutea* cells at t_i or t_f; “ $\bar{\omega}$ ” is the mean individual dry weight of spat in g ($\bar{\omega}$ =0.0005g).

151 We assumed that pumping rate of animal was constant, particle retention was 100 % efficient and the
152 algal concentration was homogenous at all times thanks to aeration.

153 2.3.3 Experiment 2: Acute exposure to HEWAF for 10 days

154 2.3.3.1 Mortality measurement

155 Single oyster spat were haphazardly distributed at a density of 15 per beaker, in triplicate (5
156 replicates for the control). Gentle central aeration by means of a glass pipette was supplied to each
157 beaker at 100 mL min⁻¹. Spat were exposed for 10 days to increasing concentrations of HEWAF (0 –
158 10 – 50 – 100 – 500 – 2000 mg oil L⁻¹ corresponding to 0 – 7 – 66 – 147 – 904 – 3450 μ g tPAH50 L⁻¹
159 ¹). This range of doses tested and exposure duration were chosen according to partial mortality
160 results observed during preliminary range finding tests. Exposure was performed in 600 mL glass
161 beakers filled up with 500 mL of solution, under static-renewal conditions, with the exposure media
162 renewed every other day (i.e. new fresh stock of HEWAF was prepared every other day). Spat from
163 each beaker were fed daily with 2 mL of Instant Algae[®]. Every other day, spat were collected on a 2-
164 mm stainless steel sieve, and observed using a dissecting microscope for examination of dead spat,
165 measured by their failure to close their valves in seawater. Beakers were cleaned using deionized
166 water and Kim-Wipes[®], and refilled with new exposure media.

167 2.3.3.2 Histopathological study

168 *a) Histopathological observation*

1
2 169 At the start of the experiment and following acclimation, histopathological condition of 26 individual
3
4 170 oyster spat was examined (Control Day 0; results presented in Table 2). At the end of the 10 day
5
6 171 exposure to HEWAF, 15 spat (excluding dead) from each concentration were randomly selected for
7
8 172 histological examination. The number of individuals sampled represented a third of the population.
9
10 173 Oyster spat were then fixed in a solution of Davidson's fixative for 48 h at 4°C. After rinsing
11
12 174 thoroughly with tap water, animals were placed in individual histological cassettes in a solution of 10
13
14 175 % EDTA (pH = 7.5) for decalcification (Howard et al., 2004). Solution of EDTA was changed every
15
16 176 other day during the week of decalcification process. After decalcification and thorough rinsing, spat
17
18 177 were transferred to cold 70% ethanol solution. Following dehydration through an ascending ethanol
19
20 178 series, tissues were dealcoholized with xylene using a tissue processor (Shandon Citadel 1000), and
21
22 179 embedded in paraffin wax. Sections (5 µm) were cut on a rotary microtome (Microm HM325),
23
24 180 stained with Harris' hematoxylin and eosin (Winstead, 1995), and examined with light microscopy.
25
26 181 A four-level semi-quantitative scale, from 0 to 3, was established to assess the intensity of each
27
28 182 histopathological condition observed in the gastro-intestinal system (i.e. hemocyte diapedesis,
29
30 183 hemocyte infiltration, mucus infiltration, and hyperplasia of mucus secretory cells). 0 = no
31
32 184 occurrence of the pathological condition was examined in the tissue area; 1= light (i.e. covering less
33
34 185 than 5% of the tissue); 2 = moderate (i.e. covering about 10% of the tissue); and 3 = severe (i.e.
35
36 186 covering about 25% of the tissue).

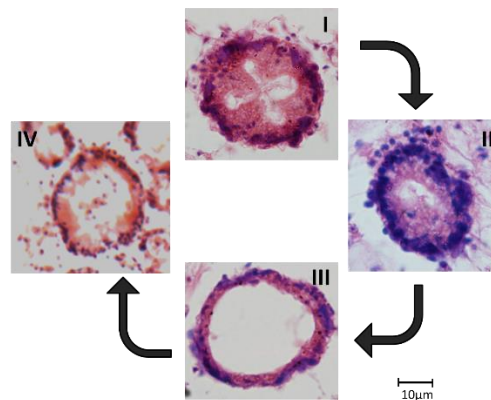
187 *b) Morphometric analysis of the digestive tubules*

188 Using an Olympus IX73 inverted microscope equipped with an Olympus DP73 camera and the
189 CellSens image analysis software, digestive tubules were measured at random from histological
190 sections of 6 surviving animals haphazardly selected in each treatment. Morphometric analysis
191 followed a protocol adapted and modified from Cajaraville et al. (1989) and Winstead (1995).
192 Histological cross sections of the digestive gland were divided into 4 fields of observation per

193 animal. Eight randomly selected tubules from each field (32 total tubules per spat) were measured.
194 Two sets of measurements, internal (luminal) surface and total tubule surface, for each tubule were
195 determined using the CellSens image analysis software, and tubule luminal ratio (luminal
196 surface/total surface) was calculated.

197 *c) Grading of digestive tubules*

198 The phasic activity of the digestive gland was assessed by means of a subjective grading of the same
199 tubules selected previously for morphometric measurements. Thus, at least 30 tubules per individual
200 and a minimum of 180 per experimental group (6 oysters) were therefore categorized. Four tubule
201 types were distinguished as described by Cajaraville (1989) (Fig. 1): holding/absorbing (combined as
202 stage I), disintegrating (stage II), reconstituting (stage III), and a fourth tubule type, not related to the
203 digestion process, and named “necrotic” (stage IV). The percentage occurrence of the 4 different
204 tubule types with respect to the total number measured was then calculated.



205
206 **Figure 1:** Subjective grading of digestive tubules into the 4 different stages observed. I: Absorbing /
207 Holding; II: Disintegrating; III: Reconstituting; IV: Necrotic (sloughing).

208 *2.3.3.3 Biochemical analysis (lipid peroxidation assay)*

209 Surviving oysters, which were not processed for histological analyses, were used for lipid
210 peroxidation assay. Lipid peroxides derived from polyunsaturated fatty acids are unstable and
211 decompose to other complex compounds of which malondialdehyde (MDA), a marker for oxidative

212 stress, is the most abundant by-product. MDA was measured by a spectrophotometric assay using a
213 commercial BIOXYTECH[®] MDA-586 kit (Oxis Research). The method is based on the reaction of a
214 chromogenic agent, N methyl-2-phenylindole with MDA at 45°C. A minimum of 100 mg wet weight
215 of oyster tissue was homogenized by sonication for 15 min in 990 µL of Phosphate Buffer Saline
216 (PBS, pH = 7.4) and 10 µL of Butylated Hydrotoluene (BHT) to avoid intra-assay oxidation. The
217 homogenate was then centrifuged at 10,000 g for 10 min, and the supernatant (200 µL) was added to
218 a mixture of probucol in methanol, N-methyl-2-phenylindole, and hydrochloric acid as indicated in
219 the MDA-586 kit. The reaction was conducted at 45°C for 60 min. After centrifugation at 10,000 g
220 for 10 min, the clear supernatant was transferred into a microtitration plate and the absorbance was
221 measured at 586 nm. A standard curve was established with a solution of tetramethoxypropane
222 (TMP) hydrolyzed during the acid incubation step generating MDA. Results were expressed in µmol
223 of MDA per g of wet mass of oyster tissue.

2.4 Analytical chemistry

225 During experiment 2, samples of every preparation of HEWAF (including FSW control, stock
226 solution and dilutions) were collected at the start and before the first exposure solution renewal, and
227 shipped (4°C) to ALS Environment (Kelso, WA, USA) for chemical analyses. After that and
228 throughout the 10-day exposure, only stock solutions of oil prepared every 2 days with fresh oil and
229 FSW control were sent for chemical analyses. Polycyclic aromatic hydrocarbons (PAHs), including
230 alkyl homologues, were determined by gas chromatography with low resolution mass spectrometry
231 using selected ion monitoring (GC/MS-SIM) and a sum of 50 different PAHs were quantified (Table
232 1). The sum concentrations of these 50 PAH analytes are hereafter referred to as tPAH50. The
233 analytical procedure was based on EPA Method 8270D with the GC and MS operating conditions
234 optimized for separation and sensitivity of the targeted analytes (US EPA, 1986). Additional details
235 regarding the methods used (e.g. standards used, QC criteria for surrogate recovery, internal
236 standards, spiked blanks) can be found in the analytical QAPP provided by the analytical laboratory

237 and applied to all samples analyzed for the Deepwater Horizon Natural Resource Damage
238 Assessment (DWH NRDA): [https://pub-dwhdatadiver.orr.noaa.gov/dwh-ar-documents/945/DWH-
239 AR0101767.pdf](https://pub-dwhdatadiver.orr.noaa.gov/dwh-ar-documents/945/DWH-AR0101767.pdf)

240 2.5 Statistical analyses

241 All percentage data (mortality, tubule types) were arcsine-square root transformed to improve
242 normality prior to statistical analyses. Effects of oil on mortality, tubule stages, tubule luminal ratio
243 and lipid peroxidation were analyzed by one-way analysis of variance (ANOVA), after checking for
244 normality (Shapiro-Wilk test) and homogeneity of variances between treatments (Levene's test).
245 Whenever significant differences among groups were found ($p < 0.05$), a Tukey post-hoc test for
246 multiple comparisons was performed. Non-parametric Kruskal-Wallis one-way ANOVA on ranks
247 and Dunnett's T3 post-hoc test were completed whenever normality and homogeneity of variance
248 requirements were not met after transformation of the data.

249 For histopathological results, a non-parametric Mann-Whitney/U-test ($p = 0.1$) was used to assess the
250 effect of oil treatment on the prevalence of various pathological conditions compared to the control at
251 day 10. Additionally, the total number of pathologies observed per individual as well as gastro-
252 intestinal pathologies, scored by intensity, were analyzed using the Mann-Whitney/U-Test ($p = 0.1$)
253 and compared between treatments. All analyses were performed using SPSS® 22.0 statistical package
254 (IBM Corp., NY).

256 3. Results and Discussion

257 3.1 Water quality and analytical chemistry of oil solutions

258 Temperature and salinity for all experiments were $25.4^{\circ}\text{C} \pm 1.0$ and $25.4 \text{ PSU} \pm 0.8$, respectively. DO
259 and pH averaged $6.3 \text{ mg L}^{-1} \pm 0.4$ and 8.09 ± 0.2 respectively, and total ammonia concentration

260 remained below deleterious levels ($\text{NH}_3 = 0.0147 \text{ mg L}^{-1} \pm 0.005$). Two faulty aeration lines in 2
 1 261 replicates within the control treatment of experiment 2 caused levels of DO to drop below 2 mg L^{-1}
 2
 3 262 on day 9, resulting in a mass mortality; as a result, only 3 replicates for the control condition were
 4
 5 263 included. Seawater used for control treatments contained background concentrations of PAHs
 6
 7 264 ($\text{tPAH}_{50} = 0.03 \text{ } \mu\text{g L}^{-1} \pm 0.01$) considered negligible for the present study. Nominal concentrations
 8
 9 265 tested with corresponding sum concentration of 50 PAHs quantified by GC/MS-SIM are presented in
 10
 11 266 Table 1, whereas PAH profile of the stock solutions of HEWAF throughout the 10-day exposure is
 12
 13 267 shown in the Supplementary File.

19 268 **Table 1:** Range of nominal test dilutions in percentage of stock of HEWAF (2 g L^{-1}), nominal
 20 269 concentrations in mg oil L^{-1} , and corresponding measured tPAH_{50} (i.e. sum of 50 PAHs)
 21 270 concentrations, in $\mu\text{g L}^{-1}$, of the initial exposure solutions. Measured tPAH_{50} were only quantified in
 22 271 the stock solutions at renewal throughout the 10-day exposure, i.e. at Day 4, Day 6, and Day 8.

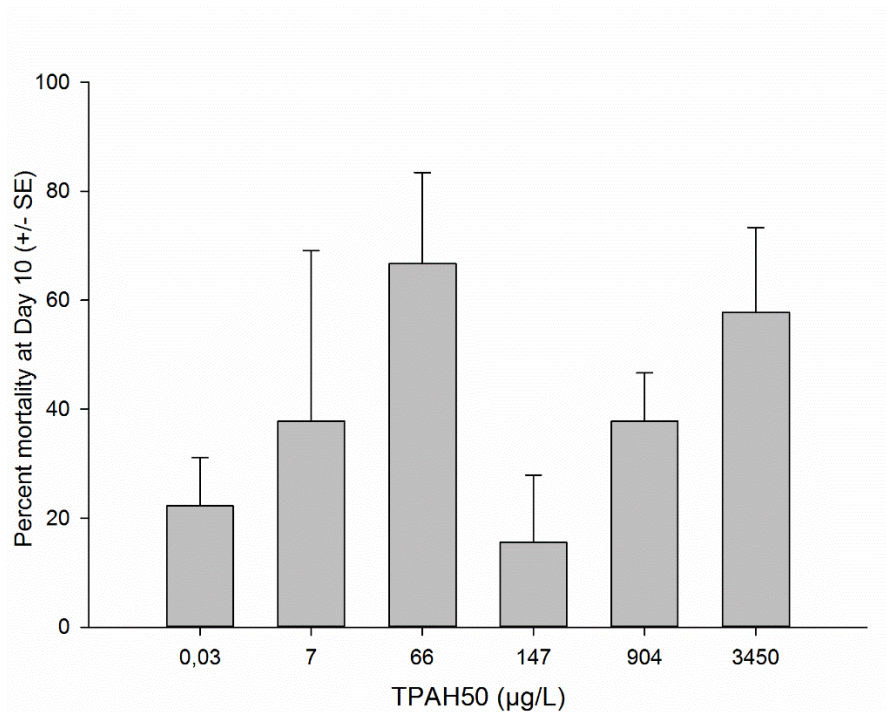
	% Stock	Nominal (mg oil L^{-1})	Day 0 ($\mu\text{g tPAH}_{50} \text{ L}^{-1}$)	Day 4 ($\mu\text{g tPAH}_{50} \text{ L}^{-1}$)	Day 6 ($\mu\text{g tPAH}_{50} \text{ L}^{-1}$)	Day 8 ($\mu\text{g tPAH}_{50} \text{ L}^{-1}$)
	100	2000	3450	3588	3710	3383
	25	500	904	-	-	-
HEWAF	5	100	147	-	-	-
Slick A	2.5	50	66	-	-	-
	0.5	10	7	-	-	-
	0	0	0.03	-	-	-

37 272 HEWAF = high-energy water accommodated fraction; tPAH_{50} = sum concentration of 50 polycyclic aromatic
 38 273 hydrocarbons analytes quantified by GC-MS/SIM by ALS (Kelso, WA).

40 274
 41
 42 275 Bi- and tri-aromatic compounds were the major contributor to the PAH content of the stock solutions
 43
 44 276 of HEWAF used throughout the spat exposure. Furthermore, alkylated PAHs such as C1-C4
 45
 46 277 naphthalene, fluorene or phenanthrene were more abundant than parent PAHs, a result in accordance
 47
 48 278 with our previous toxicity tests (Vignier et al., 2015; 2016) and other studies (Achten & Andersson,
 49
 50 279 2015; Neff & Anderson, 1981). These alkylated derivatives were shown to have increased toxicity to
 51
 52 280 invertebrates as a result of increased lipophilicity (Hawthorne et al., 2006).

281 3.2 Effects of HEWAF on mortality

282 Oyster spat ($\approx 10\text{-}15$ mm) were exposed for 10 days to concentrations of HEWAF ranging from 7 to
283 3450 $\mu\text{g tPAH50 L}^{-1}$. Non-parametric statistical tests showed no significant differences in mortality
284 between control and exposed treatments (Kruskal-Wallis: $p=0.280$) (Fig. 2). Overall, only partial
285 mortalities were encompassed by the broad range of HEWAF concentrations tested, and no clear
286 dose-dependent mortality response in exposed oyster spat was observed. Highest mortalities were
287 observed at a relatively low concentration of oil ($66.7\% \pm 16.8$ at $66 \mu\text{g tPAH50 L}^{-1}$) and at the
288 highest concentration ($57.8\% \pm 15.5$ at $3450 \mu\text{g tPAH50 L}^{-1}$) compared to control ($22.2\% \pm 8.9$) (Fig.
289 2).



291 **Figure 2:** Mortality of spat following 10 days of exposure to increasing concentrations of HEWAF,
292 expressed in $\mu\text{g tPAH50 L}^{-1}$ (initial oil loading rate of 2 g L^{-1}). Data are presented as mean
293 percentages ($n=3$) \pm standard errors (SE). Seawater control treatment corresponds to $0.03 \mu\text{g tPAH50}$
294 L^{-1} . TPAH50 = sum concentration of 50 polycyclic aromatic hydrocarbons analytes quantified.

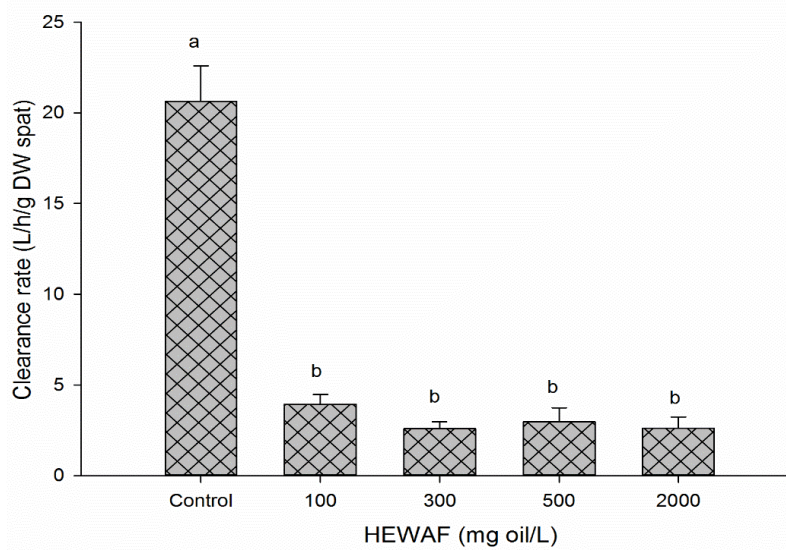
295
296 This is in accordance with our preliminary spat exposures (data not shown) and our previous study
297 exposing veliger stage of oyster larvae to HEWAF in which we found a non-dependent dose-

298 response (Vignier et al., 2016). This lack of a clear dose-response relationship is noteworthy. A
299 recent study by Forth et al. (2017) determined how different WAF preparation methods and
300 concentrations influenced the chemical composition and concentration of PAHs in the dissolved and
301 particulate phases over time. In HEWAF solutions prepared with the same starting oil-to-water ratio
302 (i.e. 2 g oil L⁻¹), the composition and concentration of the dissolved and particulate fractions varied
303 substantially across a range of dilutions (same range used in the present toxicity testing) (Forth et al.,
304 2017). The authors also found that in the stock solutions of HEWAF (containing oil droplets), the
305 dissolved phase was a small fraction of the total PAH concentration whilst the dissolved phase
306 became the dominant fraction when diluted to lower concentrations. Previous studies have
307 demonstrated that most toxicity of oil to aquatic organisms can be imputed to the PAH derived from
308 its dissolved fraction rather than its particulate form (Barron et al, 1999; 2003; Carls et al., 2008;
309 Hansen et al., 2012). Consequently, the dynamic nature of HEWAF preparations (i.e. proportion of
310 the dissolved and particulate phases) may explain the accrued lethal effects observed on oyster spat
311 at low doses of oil tested in the present work.

312 In addition, spat in the seawater control suffered over 20 % mortality. This may have been caused by
313 a stress due to change of salinity conditions between the hatchery where the spat came from (20
314 PSU) and the salinity conditions at the laboratory (26-30 PSU). Ultimately, if we consider solely the
315 mortality data obtained in the current study, it must be concluded that oyster spat were relatively
316 tolerant to DWH oil (LC50 value exceeded the range of concentrations tested, i.e. LC50 > 3450 µg
317 tPAH50 L⁻¹). However, the sub-lethal impacts of mechanically dispersed DWH oil on filtration,
318 histopathological features and lipid peroxidation of exposed spat were further investigated to
319 determine any long-term effects on the population dynamics of oysters and on the whole estuarine
320 ecosystem.

321
322
323 **3.3 Effects of HEWAF on clearance rates**

324 Clearance rates of spat fed *Tisochrysis lutea* were significantly impacted at all concentrations of
 1 325 HEWAF tested for 24h ($F_{4, 10} = 59.08$, $p < 0.001$; Fig. 3). Each treatment reduced oyster feeding
 2
 3 326 activity by at least 5-fold compared to the control ($F_{4, 10} = 59.08$, $p < 0.05$). In response to an
 4
 5 327 environmental stress such as oil pollution, filter-feeding oysters can reduce their clearance rates by
 6
 7 328 closing their valves, as a defense mechanism (Akberali & Trueman, 1985). Concentrations of oil \geq
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 9 329 100 mg L^{-1} (equivalent to $147 \mu\text{g tPAH50 L}^{-1}$) caused a significant reduction in the clearance rate of
 10
 11 330 oyster spat regardless of the concentration tested (Fig. 3).



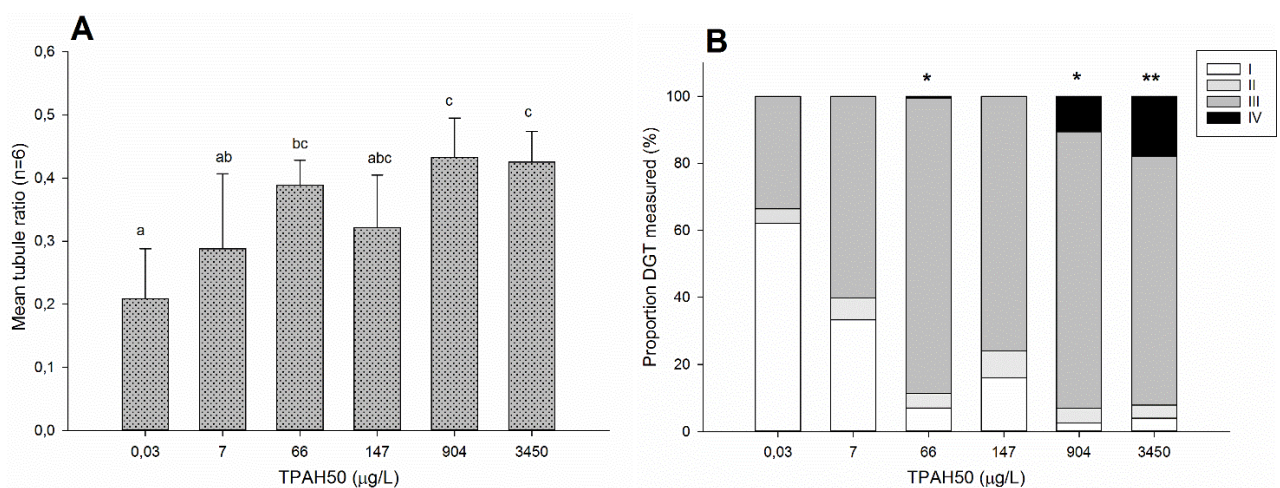
331
 332 **Figure 3:** Clearance rates of *Tisochrysis lutea* by oyster spat exposed for 24 h to nominal concentrations of
 333 HEWAF (in mg oil L^{-1}), expressed in $\text{L}^{-1} \text{ h}^{-1} \text{ g}^{-1}$ DW of oyster tissue. Data are presented as mean percentages
 334 ($n=3$) \pm standard errors (SE). Different letters denote statistical differences compared to control (ANOVA;
 335 $p=0.05$). HEWAF = high-energy water accommodated fraction; DW = Dry weight.

336
 337 This inhibition of clearance rates, similar across all treatments, suggests valve closure as a potential
 338 avoidance response. Other studies have also reported a decline in filtration rates of bivalves in
 339 response to hydrocarbons (Barszcz et al., 1977; Galtsoff et al., 1935; Widdows et al., 1982). Several
 340 reasons could explain this reduced feeding activity, the most obvious being a mechanical action of oil
 341 droplets by coating of the gills, thus disabling the normal efficiency of the ctenidia to sort and absorb
 342 food particles (Galtsoff et al., 1935). Exposure of the gill tissue of hard clams to toxic algae exerted

343 an irritation, causing a cessation of ciliary activity (Gainey & Shumway, 1991). In the same way,
 1 344 oil/PAHs could have induced a cessation of the ciliary motion, inhibiting feeding rates. In the current
 2
 3 345 study, instances of erosion of gill filaments were observed in some individuals, indicative of an
 4
 5 346 apparent irritation and toxicity by contact. Our results (i.e. high concentrations of 2 and 3 ring
 6
 7 347 alkylated PAHs; see supplementary file) could also be explained by another study which suggested
 8
 9 348 that bi- and tri-aromatic hydrocarbons could induce a narcotizing effect on the ciliary feeding
 10
 11 349 mechanisms of *Mytilus edulis*, reducing clearance rates (Donkin and Widdows, 1990).

350 3.4 Effects of HEWAF on digestive morphometry

351 Measurements of the digestive tubule (DGT) lumen ratio in tissue sections of both control and
 352 experimental oysters indicated that exposure to increasing concentrations of slick oil prepared as
 353 HEWAF induced a significant increase of the luminal tubule ratio compared to control ($F_{5, 30} = 7.817$,
 354 $p < 0.001$; Fig. 4A). The number of DGT with dilated lumens (Type III, Fig. 1) increased in a dose-
 355 dependent manner, with concentrations of oil/HEWAF (e.g. 66, 904 or 3450 $\mu\text{g tPAH50 L}^{-1}$) having
 356 a significant effect ($F_{5, 30} = 7.817$, $p \leq 0.01$; Fig. 4A).



357
 358 **Figure 4:** (A) Mean tubule luminal ratio (\pm SD) from 6 oysters exposed for 10 days to increasing
 359 concentrations of HEWAF, expressed in $\mu\text{g tPAH50 L}^{-1}$. Different letters denote a statistical difference
 360 (ANOVA: $p = 0.05$). (B) Proportion (in percent) of the different digestive tubule (DGT) types observed in
 361 histological sections of spat ($n = 6$), exposed for 10 days to HEWAF ($\mu\text{g tPAH50 L}^{-1}$). Scoring method

362 (2.3.3.2c) as follow: stage I: absorption; stage II: disintegration; stage III: reconstitution; stage IV: necrotic.
363 Asterisks denote a statistical difference compared to control (ANOVA: $p=0.05$).

364 Furthermore, compared to control oysters, 10 days of exposure to oil induced significant changes in
365 phasic activity between the different stages of tubules observed among treatments ($F_{5, 30} = 11.221$,
366 $p<0.001$; Fig. 4B). Concentrations of HEWAF, i.e. 66, 904, and 3450 $\mu\text{g tPAH50 L}^{-1}$, significantly
367 impacted the proportion of tubule types ($F_{5, 30} = 11.221$, $p\leq 0.05$; Fig. 4B) in a dose-dependant
368 manner with a significant shift observed from stage I to III (Fig. 4B). Overall, histological sections of
369 oil-exposed oysters showed that digestive diverticula were typical in appearance to that seen during
370 starvation of mollusks (Morton, 1977). Symptoms such as severe atrophy of the DGT, with increased
371 dilation of the lumina and a loss of epithelial cell height (stage III) were noted, but more markedly
372 than during starvation observed by the above cited study. This result suggests that crude oil/PAHs
373 might disrupt the normal food uptake by exposed oysters, hence inducing a nutritional stress. This is
374 in agreement with other studies, which showed that environmental stress can cause or mimic
375 starvation (Barszcz et al., 1977; Winstead, 1995; Wikfors & Smolowitz, 1995).
376 Oil and PAH exposures were previously reported to lead to atrophy of the digestive diverticula and
377 severe changes in the structure of the cells comprising the epithelium in the digestive gland of
378 molluscs (Cajaraville et al., 1992; Lowe et al., 1981; Luna-Acosta et al., 2017). As described by
379 Moore (1986) and Lowe (1981), PAHs could affect lysosomal activity, by altering their structure
380 (size, number, membrane permeability) and function, leading to damage of digestive cells, thus
381 impacting intracellular digestion.

383 **3.5 Effects of HEWAF on tissue condition**

384 A variety of pathological conditions were observed after 10 days of exposure to mechanically
385 dispersed oil (HEWAF). Our observations focused on connective tissue (Fig. 5A), gills, labial palp
386 (Fig. 5C), and mantle which were in direct contact with the oil solutions. In addition, organs of the
387 digestive system, such as stomach (Fig. 6), intestine, digestive ducts and tubules (Fig. 7) were

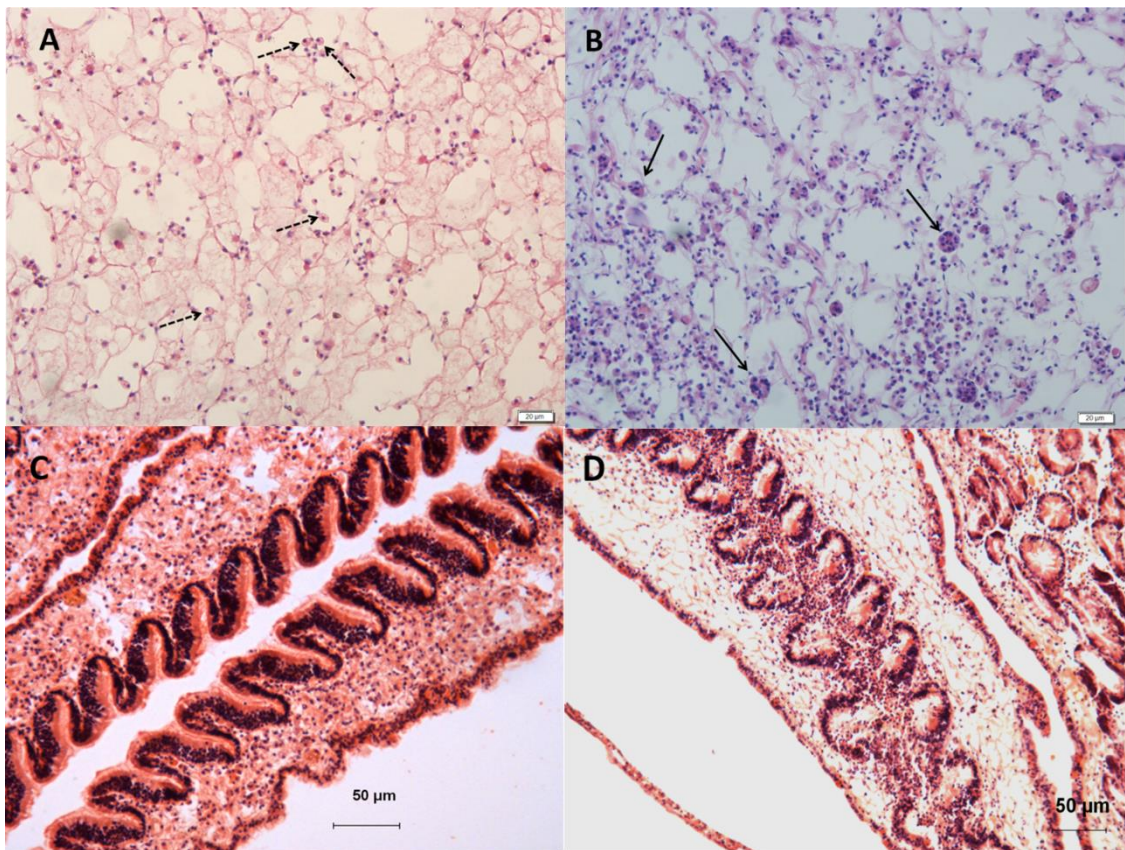
388 specifically studied. Conditions such as; erosion of gill filaments, hemocyte infiltration in the water
389 tubules of the gills or in the labial palp, high instances of mucus secretory cells in the digestive
390 tubules and digestive ducts epithelia (i.e. mucus diapedesis), or parasitic infestation (*Tylocephalum*)
391 were commonly observed in the oysters after 10 days, but distributed in all experimental treatments,
392 exposed or not to oil. The histopathological condition of non-exposed spat at the start of the exposure
393 (i.e. control Day 0; Table 2) suggests that a severe change in salinity from the original to the
394 experimental condition during acclimation, as well as a potential poor health condition of the spat to
395 begin with may have caused the observed high mortality in the non-exposed, control oysters after 10
396 days.

397 Nonetheless, oil-exposed spat differed from the control group in a number of aspects. In the stomach
398 for instance, little food or digested material was found, particularly in those exposed to 66 μg
399 tPAH50 L^{-1} and higher. This finding is in line with our observations of oyster spat upon exposure,
400 i.e. filtration reduction and occurrence of atrophy in the digestive diverticula.

401 One of the main histological features observed in exposed animals was the significant presence of
402 syncytia in the connective tissue, in very high numbers compared to control (Fig. 5A & B) (U-test:
403 $p < 0.05$, Table 2). In the gills, the presence of syncytia was consistently observed in all oil treatments
404 except the 66 μg tPAH50 L^{-1} dose, reaching up to 38.5% of the oysters at 7 μg tPAH50 L^{-1} as
405 compared to 5.3% in control (Table 2). Syncytia are defined as a multinucleate mass of protoplasm
406 produced by the fusion of several cells (Dorland, 2011). Syncytia can be normal cells in animal
407 biology (e.g. some type of fungi), but hemocytes are mononuclear cells and their merging together to
408 form syncytia is a serious pathology, indicative of a severe inflammatory response. For instance,
409 syncytia can form when cells are infected by certain types of viruses such as HIV, causing changes in
410 immunology and surface antigens of the cells and ultimately apoptosis (Huerta et al., 2009).
411 Formation of polykaryons cells (giant cells, syncytia) has been recorded in vertebrates in response to
412 inflammation (Lewis & Lewis, 1926; Mariano & Spector, 1974) but more rarely reported in
413 invertebrates (Anderson, 1987). The presence of syncytia may impair the immune response of

414 affected organisms, and the relationship between syncytia formation and oil-exposure requires
1 415 further research.

2
3 416 In the labial palp epithelium, hemocytic diapedesis was observed more often in oil-exposed spat (Fig.
4
5 417 5D) than in non-exposed spat (Fig. 5C), particularly at the highest doses (100% of the oysters) (U-
6
7 418 test: $p \leq 0.05$, Table 2). Combined with hemocyte infiltration in the water tubules, these features
8
9 419 indicate a direct contact between soluble PAHs and/or oil droplets with the labial palp via filtration.



44 421 **Figure 5:** (A) healthy connective tissue with normal hemocytes and presence of phagocytosing
45 422 hemocytes (dashed arrows), observed in non-exposed group after 10 days; (B) connective tissue
46 423 observed in oil-exposed spat (at 10 mg oil L⁻¹ treatment equivalent to 7 μg tPAH50 L⁻¹) after 10 days
47 424 showed high instance of syncytia (arrows); (C) Normal labial palp observed after 10 days in control
48 425 oyster; (D) abnormal labial palp exhibiting major hemocyte infiltration (100 mg L⁻¹ treatment
49 426 equivalent to 147 μg tPAH50 L⁻¹).

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56 427 Acute exposure to oil prepared as HEWAF negatively affected the digestive system of oyster spat. In
57
58 428 addition to DGT atrophy mentioned previously, histological examination of the digestive diverticula

429 and the gastro-intestinal tract revealed a major inflammatory response upon oil-exposure. After 10
1 430 days, the occurrence of severe pathological conditions such as ulcers (Fig. 6B) and epithelial
2
3 431 sloughing (Fig. 6D) were found in a greater number of oysters at 66 and 147 $\mu\text{g tPAH50 L}^{-1}$
4
5 432 exposure concentrations compared to controls (U-test, $p \leq 0.1$, Table 2). Hemocyte proliferation in the
6
7 433 connective tissue surrounding the digestive diverticula, as well as severe hemocyte diapedesis (Fig.
8
9 434 6C) and infiltration into the lumen of the alimentary tract (Fig. 6D) occurred significantly more in
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11
12
13 435 oil-exposed oysters than in non-exposed oysters (U-test, $p \leq 0.1$, Table 2).

14
15 436 A recent study from Luna-Acosta (2017) also found that the digestive gland of oysters exposed to
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17 437 mechanically dispersed oil showed hemocyte infiltrations. Significant migration of hemocytes by
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19
20 438 diapedesis from the vascular system into the stomach and the intestine is part of a defense
21
22 439 mechanism in bivalves (Feng, 1966; Galimany et al., 2008). In the presence of oil, oyster hemocytes
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24
25 440 can migrate to engulf and encapsulate foreign material (Sami et al., 1992). Enzyme activities can be
26
27 441 activated in hemocytes of *C. gigas* exposed to crude oil, and may play an important role in protection
28
29
30 442 against xenobiotics (Luna-Acosta et al., 2011). Thus, as an attempt to eliminate oil, hemocytes may
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32 443 migrate from the connective tissue through the epithelia of the stomach/intestine by diapedesis,
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34
35 444 carrying the compounds to the alimentary canal (intestine) for elimination by excretion in the feces.
36
37 445 This process was confirmed in our study by observation of actively phagocytosing hemocytes in the
38
39
40 446 connective tissue (Fig. 5A & 6C) and apoptotic hemocytes in the lumen of the stomach/intestine
41
42 447 (Fig. 6D). Such a detoxification pathway has been described in mussels challenged with
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45 448 environmental stressors or toxic algae; this process was associated with an increase of tissue lipid
46
47 449 peroxidation due to an oxidative stress (Alves de Almeida et al., 2007; Galimany et al., 2008).

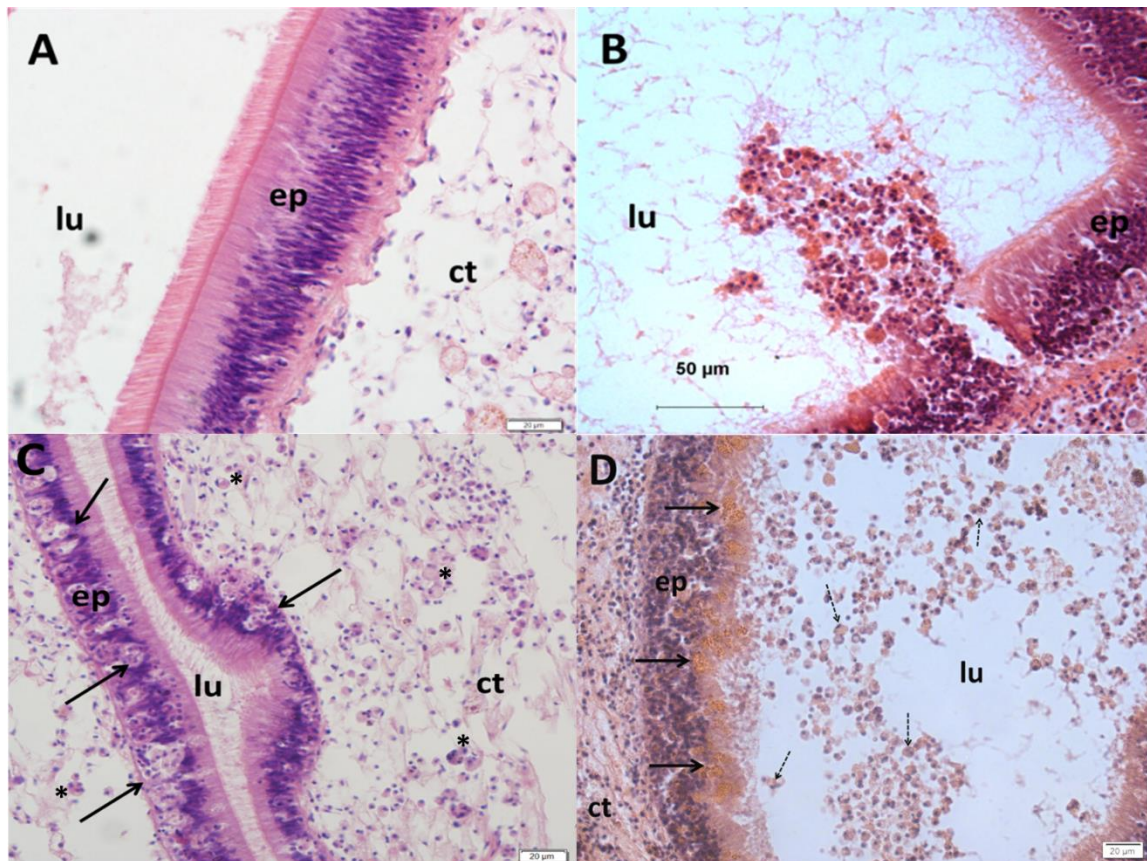


Figure 6: Section of the stomach of experimental oyster after 10 days of exposure to HEWAF. (A) Normal epithelium in non-exposed oyster; (B) example of ulcer and consequent hemorrhaging of hemolymph in the stomach lumen observed in oysters exposed to $66 \mu\text{g tPAH50 L}^{-1}$ treatment; (C) heavy hemocyte diapedesis (arrows) from the connective tissue into the lumen of the stomach ($7 \mu\text{g tPAH50 L}^{-1}$). Asterisks note the presence of phagocytosing hemocytes as well as syncytia; (D) severe hemocyte infiltration coupled with epithelium sloughing and diapedesis in the stomach ($147 \mu\text{g tPAH50 L}^{-1}$); dashed arrows show apoptotic hemocytes; arrows show oil accumulated in epithelia. ct = connective tissue; lu = lumen; ep = epithelium.

An excess of mucus in the alimentary canal and a high prevalence of hypertrophic mucus secretory cells in the epithelia (referred as mucus diapedesis) of the stomach/intestine were also consistently observed in exposed spat (U-test: $p \leq 0.05$, Table 2, Fig. 6D). Although mucus secretory cells are a normal cell type in epithelia of the digestive gland, their increased number and abnormal size in oil-exposed oyster may indicate hyperplasia, which could be a severe pathology directly imputable to contact with oil droplets/dissolved PAHs (Domingos et al., 2007).

467 **Table 2:** Histological parameters recorded in *Crassostrea virginica* tissues at day 0 and after 10 days
 468 of exposure to DWH oil (HEWAF), expressed in $\mu\text{g tPAH50 L}^{-1}$. Data are presented in percent of
 469 occurrence per treatment. Mann-Whitney/U-test compares treatments with control Day10: statistical
 470 difference at $p=0.1$.

Histological features	Measured HEWAF ($\mu\text{g tPAH50 L}^{-1}$)						
	Control Day 0	Control Day 10	7	66	147	908	3450
GILLS							
<i>Syncytia</i>	0	5.3	38.5**	0	15.8	18.2	9.1
<i>Hemocyte infiltration</i>	0	0	15.4#	12.5	5.3	9.1	9.1
<i>Erosion of filament</i>	8.6	0	0	12.5	0	0	9.1
PALP							
<i>Diapedesis</i>	5.2*	46.2	75	66.7	57.1	100*	100*
<i>Hemocyte infiltration</i>	0	7.7	50*	33.3	14.3	57.1*	28.6
CONNECTIVE TISSUE							
<i>Syncytia</i>	11.5	31.8	92.3***	75*	84.2***	92.3***	84.6**
STOMACH/INTESTINE							
<i>Epithelium sloughing</i>	0	5.3	11.1	33.3#	26.7#	15.4	0
<i>Ulcer</i>	0	0	0	33.3**	20*	7.7	0
<i>Hemocyte diapedesis</i>	40*	79	100	100	100#	100#	84.6
<i>Hyperplasia mucus cells</i>	25	21.1	78**	66.7*	60**	76.9**	69.2**
<i>Hemocyte infiltration</i>	15*	57.9	100*	83.3	93.3#	92.3*	76.9
<i>Mucus infiltration</i>	10	31.6	88.9**	50	46.7	53.9	61.5#
DIGESTIVE DIVERTICULA							
<i>Epithelium sloughing</i>	0	31.8	100***	37.5	89.5***	76.9**	66.7*
<i>Digestive cells in lumen</i>	7.7*	36.4	100***	50	94.7***	84.6**	66.7#
<i>Hemocyte diapedesis</i>	0	13.6	53.9**	50*	47.4*	61.5**	8.3
<i>Hyperplasia mucus cells</i>	7.7	9.1	30.8	12.5	15.8	30.8#	33.3#
<i>Oil in lumen®</i>	0	0	0	0	5.3	7.7	8.3

471 Data were categorized as 0 or 1, according to the absence (0) or presence (1) of the observed feature; Mann-
472 Whitney/U-tests were performed: # $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, @ Three oysters had this
473 feature, all in the highest doses tested ($> 147 \mu\text{g tPAH50 L}^{-1}$) see Fig. 7D.

474
475 Compared to non-exposed oysters (Fig. 7A), the digestive diverticula of oil-exposed oysters showed
476 a predominance of dilated lumina of the DGT (Fig. 7B). In addition, migration of hemocytes by
477 diapedesis into the lumen of the digestive ducts (DD), epithelium sloughing and presence of
478 digestive cells in the lumina of the DD and DGT, were observed in most of the exposure groups (U-
479 test, $p < 0.05$, Table 2) (Fig. 7C). More specifically, a prevalence (10 to 18%) of necrotic tubules was
480 noted at the highest HEWAF concentrations tested (904 and 3450 $\mu\text{g tPAH50 L}^{-1}$), with cellular
481 debris and digestive cells, most often in apoptosis, sloughed into the lumen of tubules and ducts (Fig.
482 7C). It is evident from these observations that a degeneration of the digestive cells lining the DGT
483 epithelia and subsequent sloughing into the lumen was related to exposure to hydrocarbons.
484 Likewise, epithelium sloughing was described in oysters collected from the field following the
485 *Amoco Cadiz* spill (Neff & Haensly, 1982) and with chronic experimental exposures (Barszcz et al.,
486 1977).

487 Instead of partially digested food material ready to be taken in by digestive cells for intracellular
488 digestion, abnormal excretion product (i.e. mucus) and possibly oil were observed in the lumen of the
489 DD and DGT from three spat exposed to the highest doses of HEWAF (Fig. 7D, Table 2). In light of
490 these observations, it is possible that oysters directly ingested oil as droplets, carried it to the stomach
491 *via* the labial palps, and transferred it to the lumen of DGT by the ciliated epithelia of the DD. As
492 part of the DWH natural resource damage assessment, Forth et al. (2017) characterized the oil
493 droplets size distribution in HEWAF dilutions used in our toxicity testing. Their results revealed that
494 the initial mean diameters of the HEWAF droplets were 8 μm and decreased to 5 μm after 48h, a size
495 range of particle well within the food spectrum available to filter-feeding oyster.

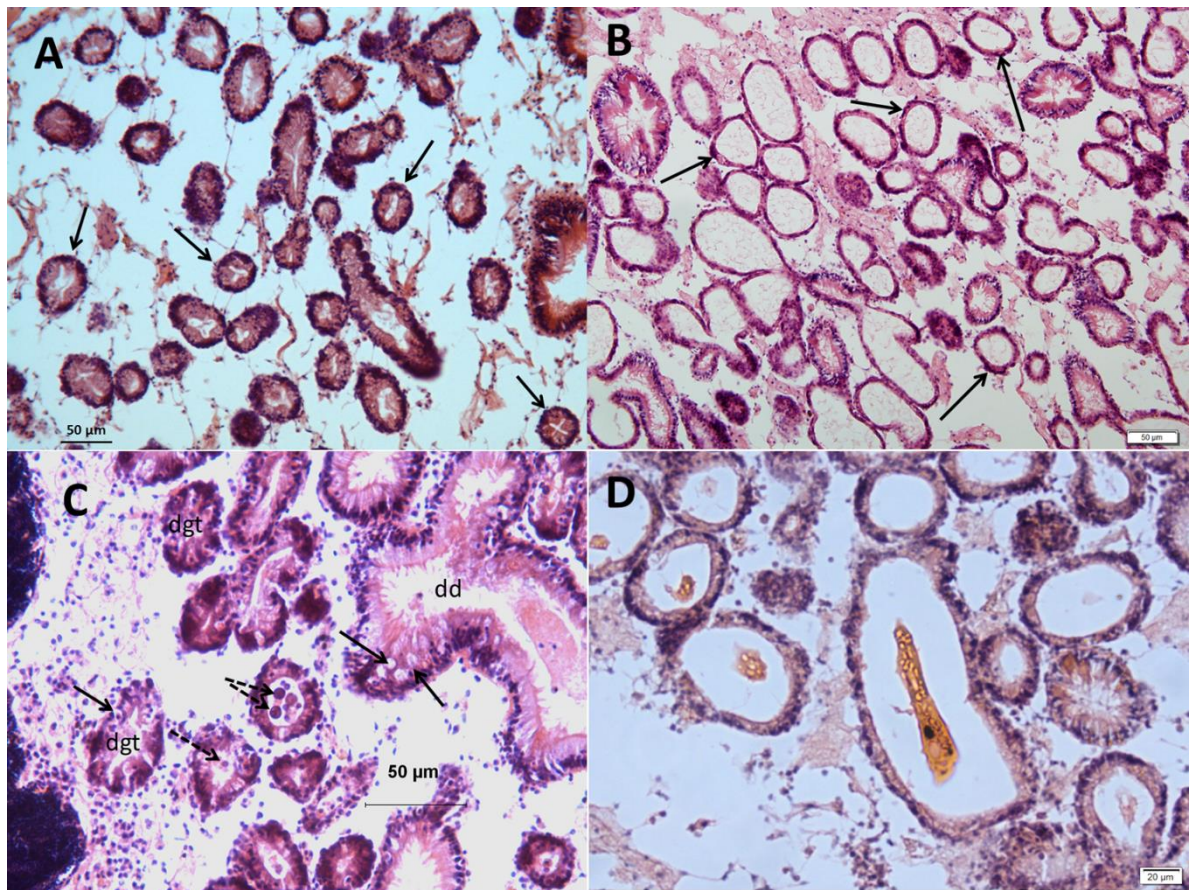
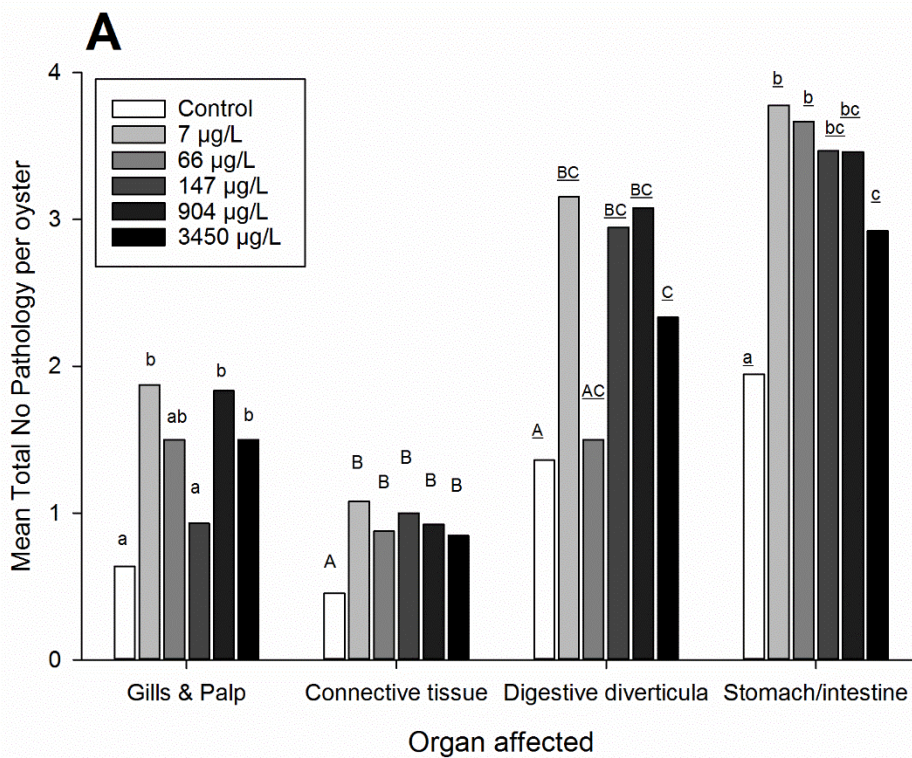


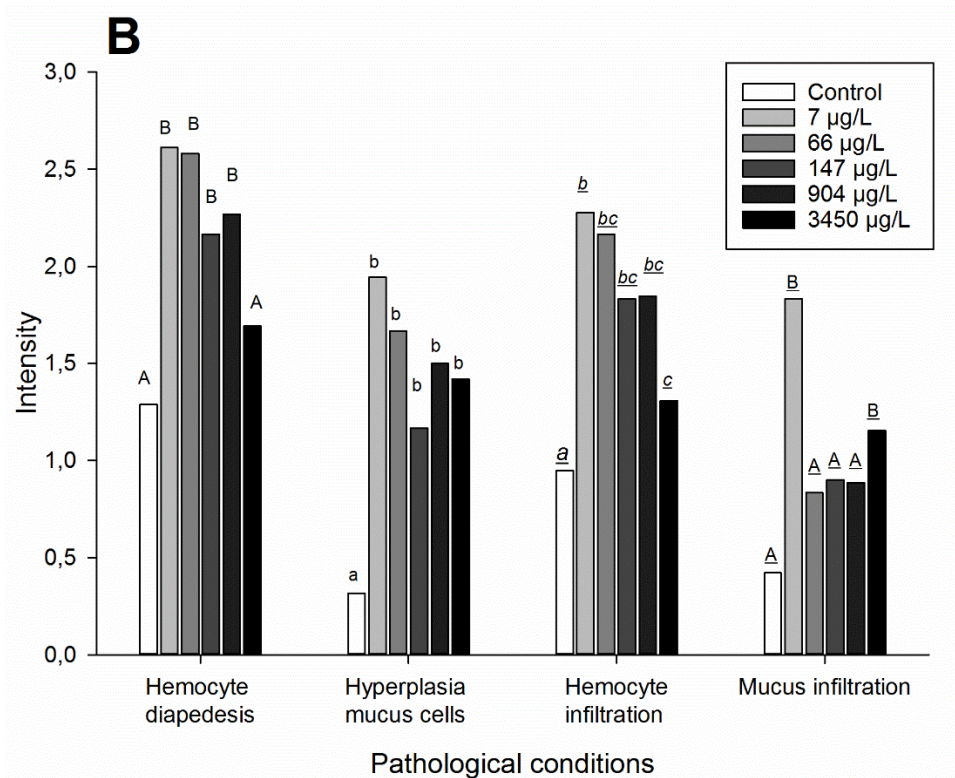
Figure 7: Histological preparations of *Crassostrea virginica* cross-sections (A) show predominantly normal (Type I) tubules (arrows); (B) arrows show severe metaplastic changes (Type III), with dilation of the lumina and loss of epithelial cell height; (C) section of digestive diverticulum (100 mg oil L⁻¹ treatment equivalent to 147 μg tPAH50 L⁻¹) showing necrotic stage of DGT (Type IV) with sloughing of digestive cells (dashed arrow) in the lumen of digestive tubules as well as hemocyte diapedesis (arrows) in epithelia of tubules and digestive ducts; (D) example of tubules and ducts with presence of oil in lumen observed in the 2000 mg L⁻¹ treatment (corresponding to 3450 μg tPAH50 L⁻¹). dgt = digestive tubules; dd = digestive ducts.

Figure 8A summarizes the mean number of pathological conditions observed per individual for each concentration of HEWAF tested. For most concentrations of HEWAF tested, an increase in the number of pathologies was observed in every organ studied in comparison to control oysters. Acute exposure to oil also affected the digestive system, where most histopathological conditions were reported, particularly in the gastro-intestinal system (Fig. 8A). A focus on the stomach and intestine revealed a significant increase in the intensity of pathologies compared to control (Fig. 8B). Surprisingly, concentration of 7 μg tPAH50 L⁻¹ seemed to induce more intense pathologies compared

512 to the highest dose of HEWAF tested (i.e. 3450 $\mu\text{g tPAH50 L}^{-1}$); for example, hemocyte diapedesis
 1 513 and hemocyte infiltration were significantly more intense at 7 $\mu\text{g tPAH50 L}^{-1}$ than at 3450 $\mu\text{g tPAH50 L}^{-1}$
 2
 3 514 tPAH50 L^{-1} (U-test, $p \leq 0.05$, Fig. 8B). Altogether, the gastro-intestinal system appeared to be the
 4
 5 515 most impacted tissues with a significant increase of pathology intensity as compared to control.
 6
 7
 8 516 It may be that the chemical characteristics of low concentrations of HEWAF and particularly the
 9
 10 517 proportion of dissolved to particulate (i.e. droplets associated) PAHs - which increases with
 11
 12 518 decreasing total concentration through time (Forth et al., 2017) - could have contributed to these
 13
 14 519 augmented sub-lethal effects. Filtration results, which indicated no difference of clearance between
 15
 16 520 oil treatments, and the variable mortalities observed could support this hypothesis. Further research
 17
 18 521 should be conducted on the respective cause of toxicity from dissolved and particulate oil/droplets.



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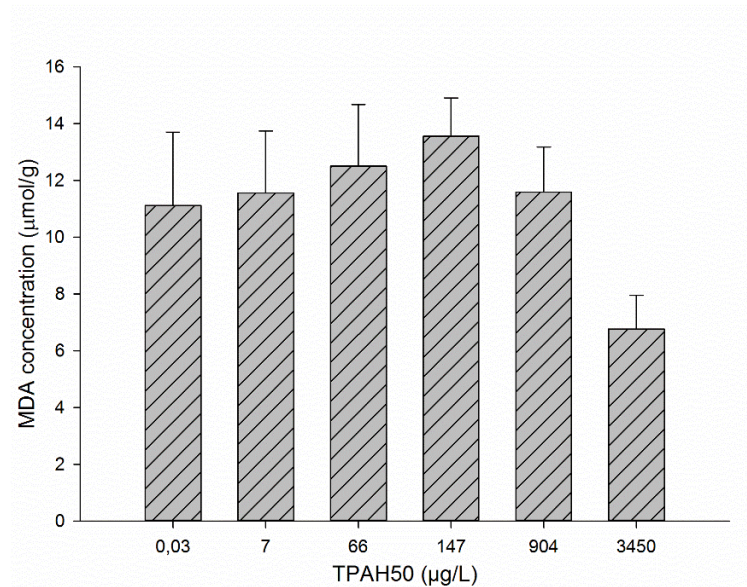


523
524 **Figure 8:** (A) Organ distribution of total pathological conditions observed per oyster ($n \geq 9$) following 10
525 days of exposure to increasing concentrations of HEWAF, expressed in $\mu\text{g tPAH50 L}^{-1}$. (B) Occurrence of
526 various pathological conditions observed in the gastro-intestinal system (stomach/intestine) of oyster spat ($n \geq$
527 9) after 10 days of exposure to HEWAF, expressed in $\mu\text{g tPAH50 L}^{-1}$. Occurrence of pathological conditions
528 are categorized by intensity following a semi-quantitative scale (see details in 2.3.3.2.a); 0: absence, 1: light,
529 2: moderate, 3: severe. Different letters within each organ/pathological condition denote statistical difference
530 at $p \leq 0.1$ (Mann-Whitney/U-test). Lettering differed between categories because separate analyses were
531 performed for each organ/condition.

532 533 3.5 Effects of HEWAF on lipid peroxidation

534 For oysters exposed to increasing HEWAF concentrations, a trend of increased malondialdehyde
535 (MDA, i.e. a marker for oxidative stress) level from 0 to 147 $\mu\text{g tPAH50 L}^{-1}$ and a trend of decreased
536 MDA level from 147 $\mu\text{g tPAH50 L}^{-1}$ to 3450 $\mu\text{g tPAH50 L}^{-1}$ (Fig. 9) were observed in the tissue.
537 Furthermore, concentrations of the lipid peroxidation byproduct were relatively high in the tissue
538 sampled from the 147 $\mu\text{g tPAH50 L}^{-1}$ group (13.6 $\mu\text{mol g}^{-1}$). However, none of these results show
539 any statistical significance ($F_{5, 15} = 0.946$, $p = 0.480$) due to the high individual variability. Chronic,

540 long-term exposure of bivalves to crude oil can induce an oxidative stress and impair membrane
541 integrity, associated with lipid peroxidation (Di Giulio et al., 1989; Downs et al., 2002; Livingstone
542 et al., 1989; 2001). Perhaps the duration of exposure tested in our study was too short to result in a
543 significant oxidative stress measurable using the MDA biomarker.



544 **Figure 9:** Mean malondialdehyde (MDA) concentration (\pm SE), expressed in $\mu\text{mol g}^{-1}$ of oyster tissue, in spat
545 exposed for 10 days to increasing doses of oil prepared as HEWAF, expressed in $\mu\text{g tPAH50 L}^{-1}$.

547 4. Conclusions

548 This study revealed lethal and sublethal effects of DWH oil on an ecologically important organism of
549 the Gulf of Mexico, the oyster *Crassostrea virginica*. Relatively high concentrations of DWH oil
550 (HEWAF) were necessary to elicit a lethal response ($> 3450 \mu\text{g tPAH50 L}^{-1}$ or $> 2 \text{ g oil L}^{-1}$) in oyster
551 spat. If we consider the mortality data in conjunction with the histopathological changes observed on
552 surviving spat however, exposure to relatively low doses of PAH ($< 66 \mu\text{g tPAH50 L}^{-1}$) caused
553 severe alterations in the physiology and metabolism of these animals. The present study also
554 demonstrated that an inflammatory response occurred, with various degrees of severity depending of
555 HEWAF doses, mostly located around the alimentary canal and the connective tissue. **Stress**
556 **associated with shipping, acclimation and holding oysters in static-renewal conditions during**

557 exposure could have contributed to the apparent poor condition of oysters in the control (both day 0
558 and day 10) and the unexpectedly elevated mortality in the control at the end of the exposure (>
559 20%). However, the differences between controls and experimental treatments showed significant
560 difference suggesting that oil had an impact.

561 Oysters are the principal benthic filter-feeders in the northern region of the Gulf of Mexico and
562 appear to be, from the present study, sensitive to crude oil. Although these laboratory results showed
563 significant sub-lethal effects in oysters exposed to DWH oil, these results cannot be extrapolated
564 directly to the environment or to the natural oyster population. The lowest effective dose of PAHs
565 determined in the current study (i.e., 7 $\mu\text{g tPAH50 L}^{-1}$ for tissue damage) was greater than most
566 tPAH50 concentrations measured in coastal-water samples during and after the DWH spill (e.g., 0.17
567 $\mu\text{g tPAH33 L}^{-1}$; Allan et al., 2012). It must be noted however that in the natural environment, oil can
568 potentially interact with other abiotic factors (e.g. temperature, salinity, UV irradiance, xenobiotics)
569 which may exacerbate the toxicity of its compounds to oysters (Pelletier et al., 1997; Powers et al.,
570 2015). Further research is warranted to elucidate the effects of multiple stressors in combination with
571 petroleum hydrocarbons on the physiology of oyster spat. Finally, information collected in the
572 present study can be used in the selection of meaningful endpoints in addition to lethality for
573 assessing oil spill effects.

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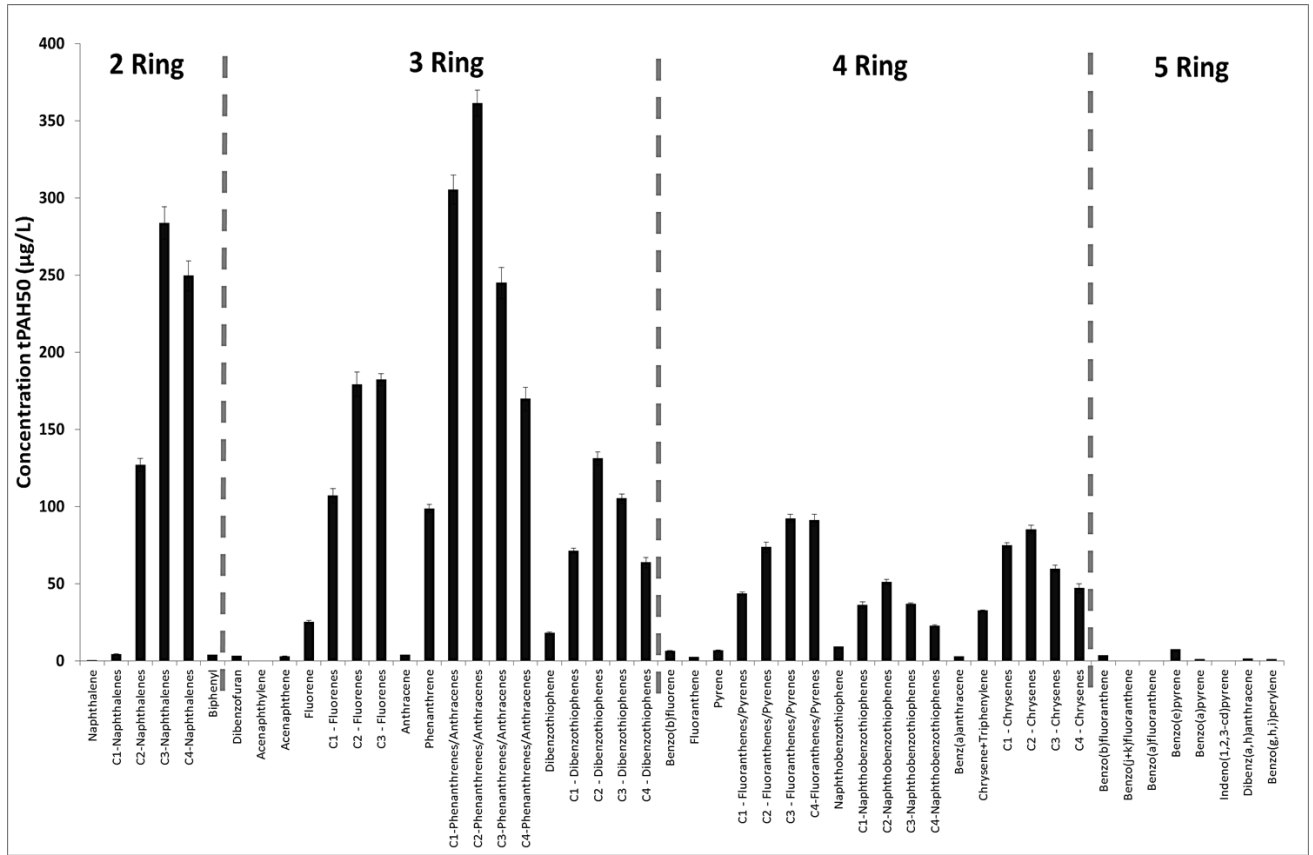
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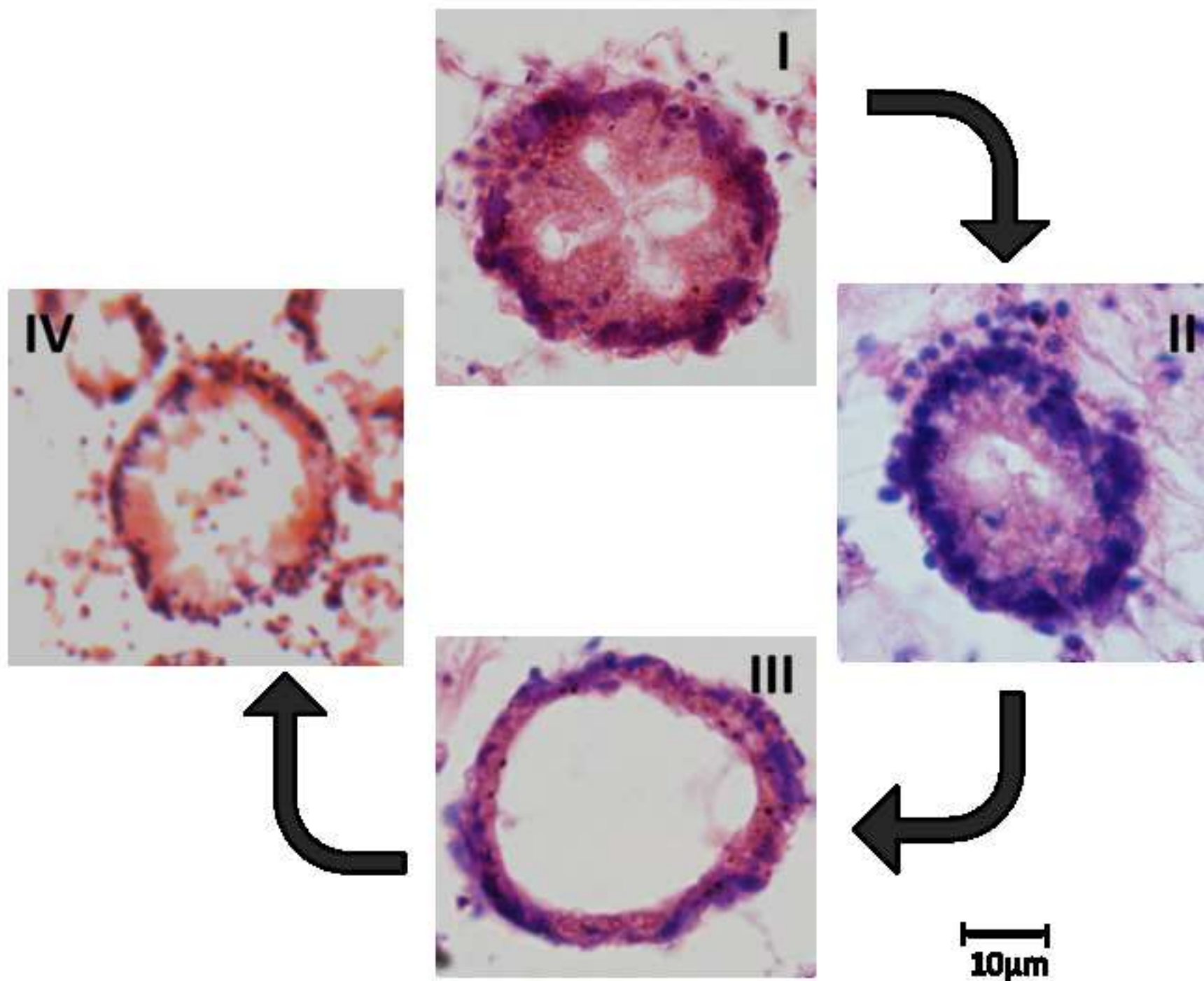
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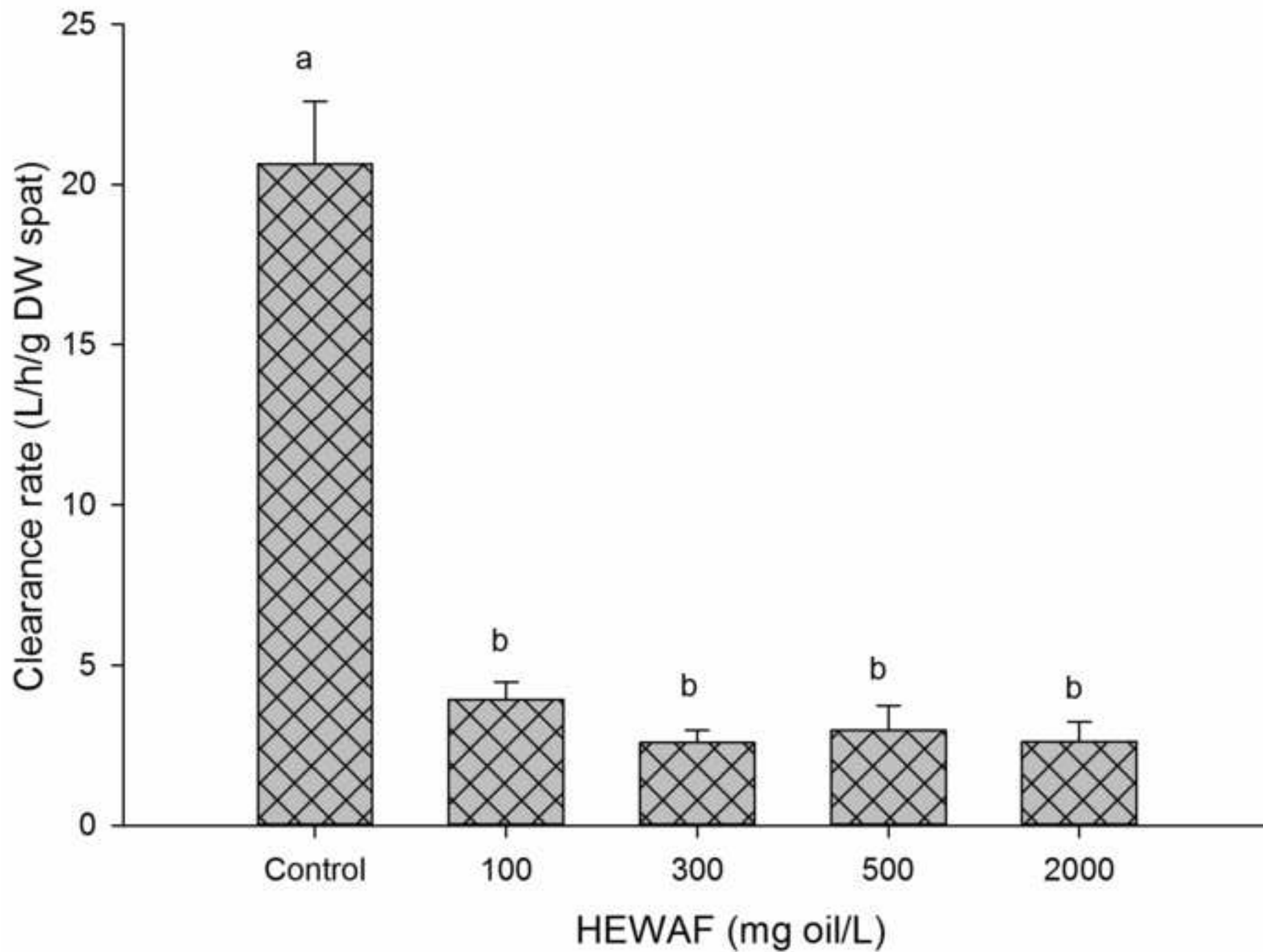
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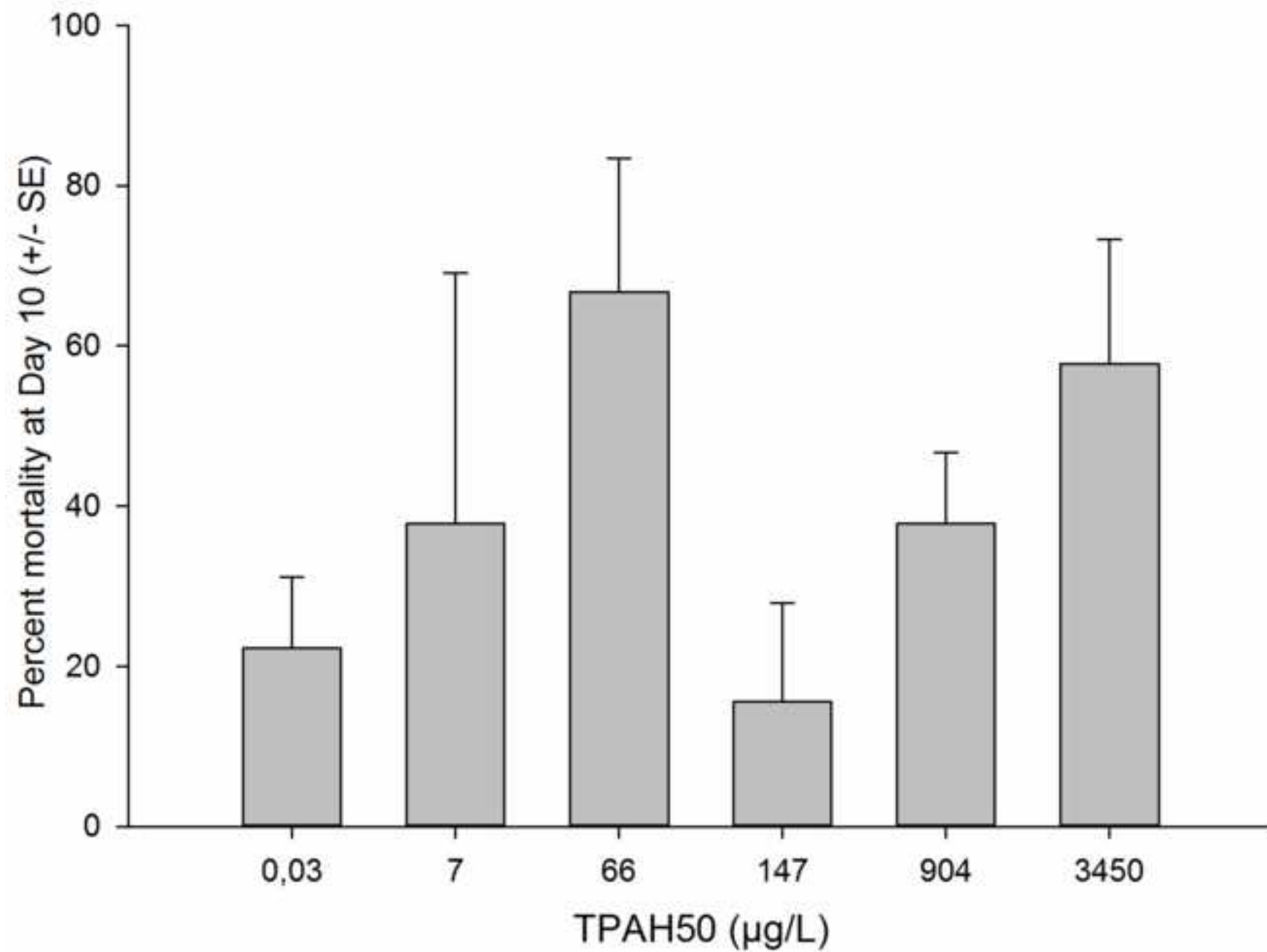


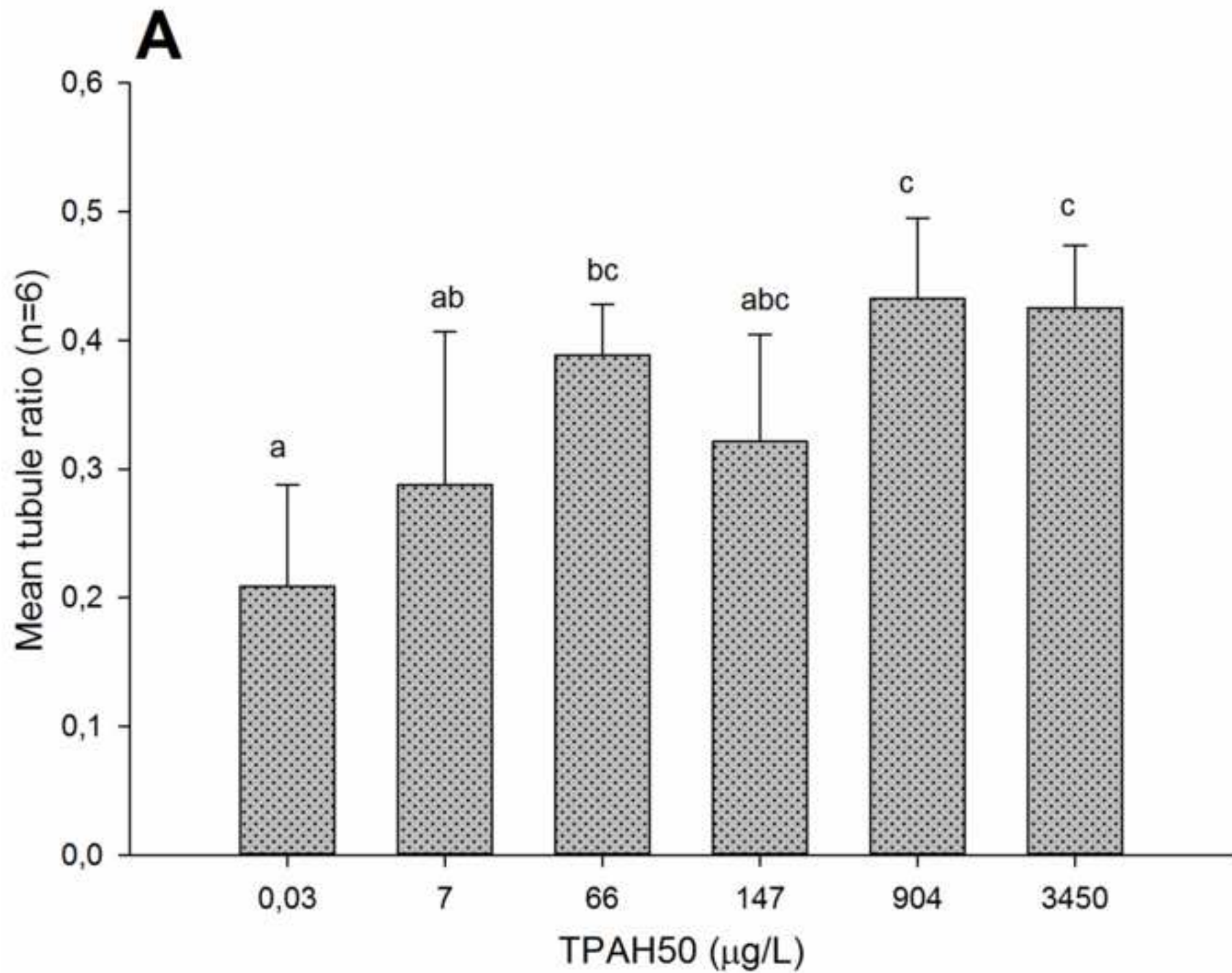
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 828 **Supplementary file:** Initial concentrations, expressed in $\mu\text{g L}^{-1}$, for the 50 PAH analytes (parent and alkyl
 829 homologs) measured in the fresh stock solutions of HEWAF (oil loading rate of 2 g L^{-1}) throughout the 10
 830 day-exposure of oyster spat. Generalized subclasses are divided by dotted lines and noted on the figure

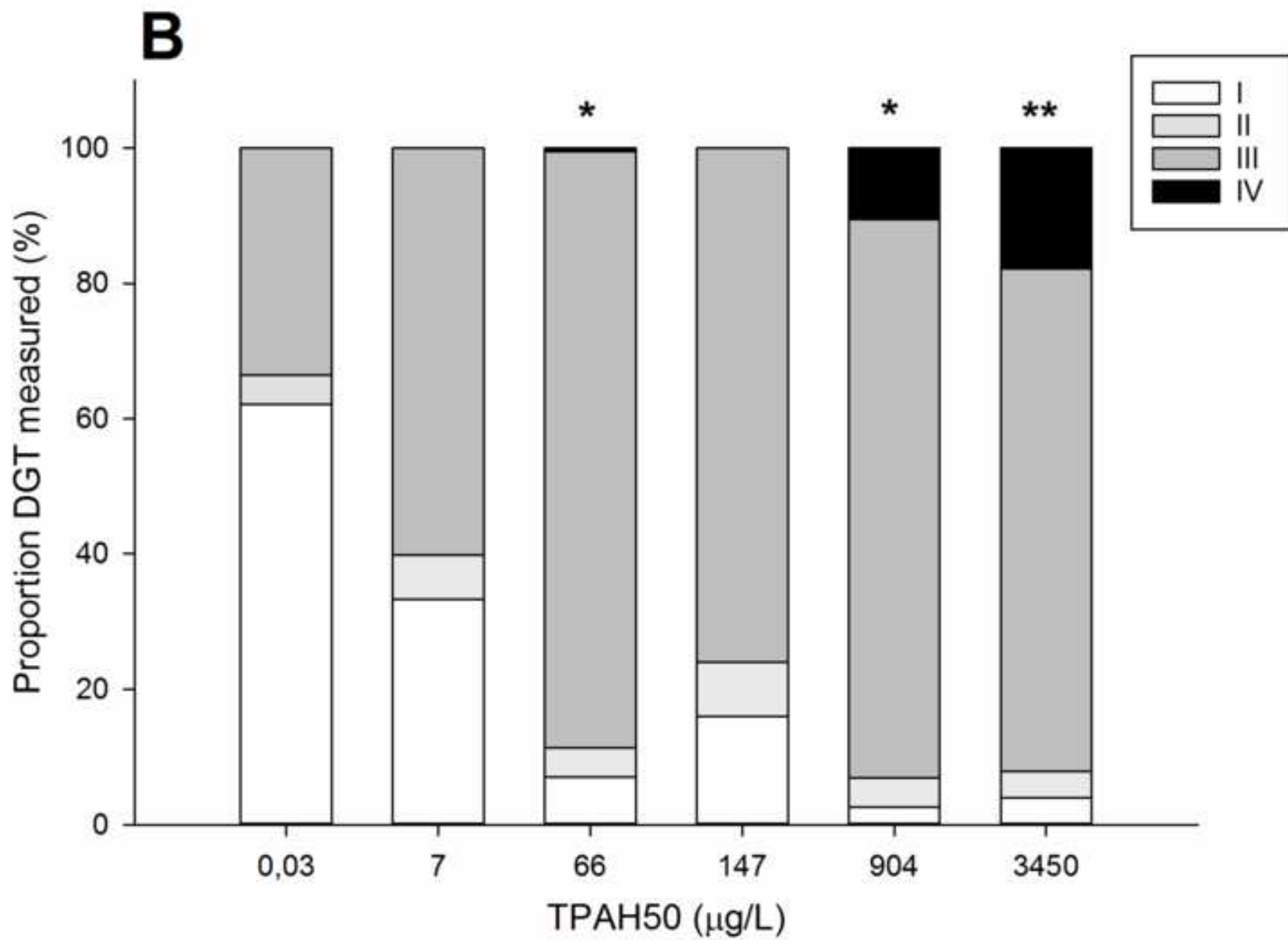
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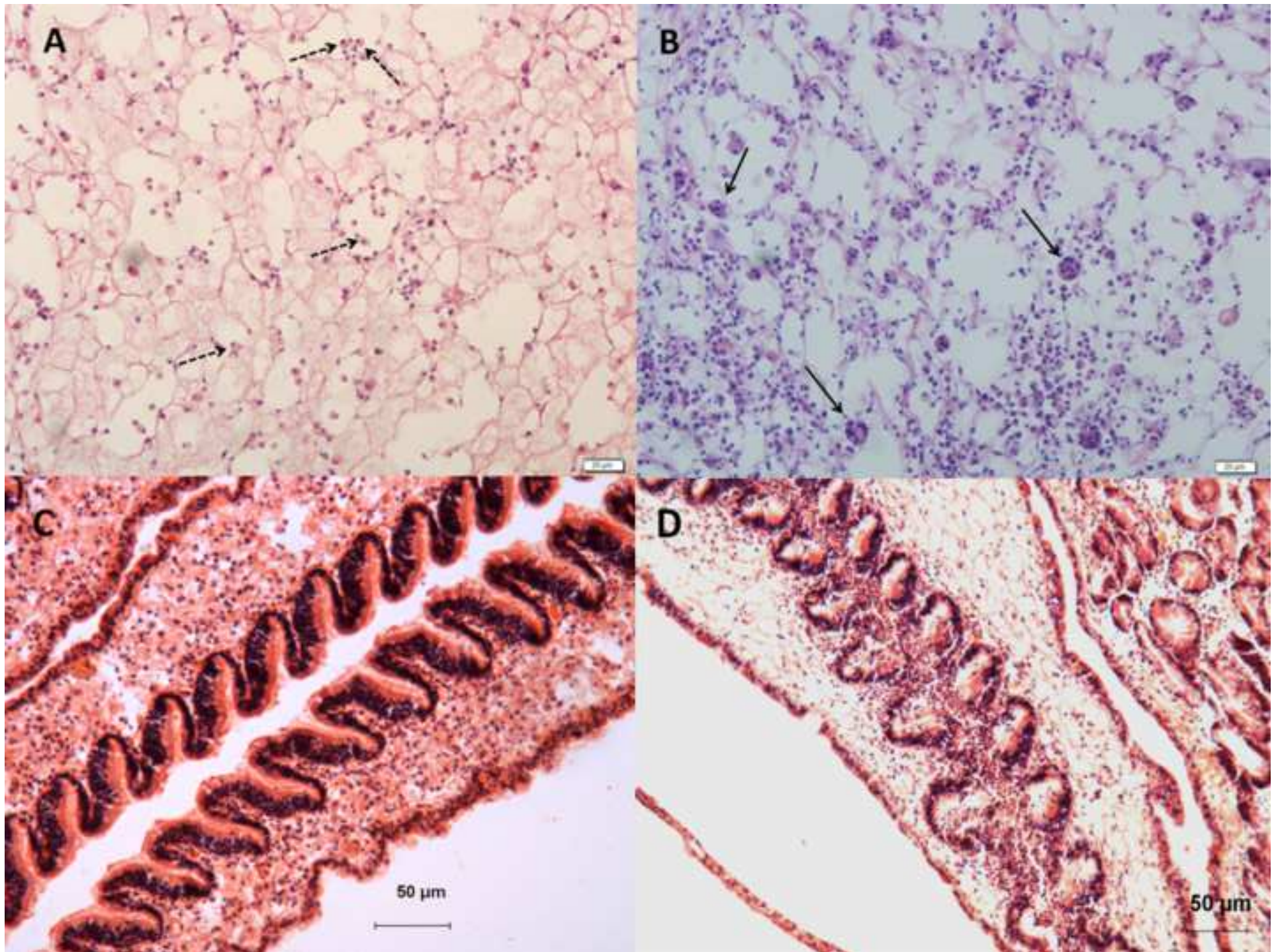


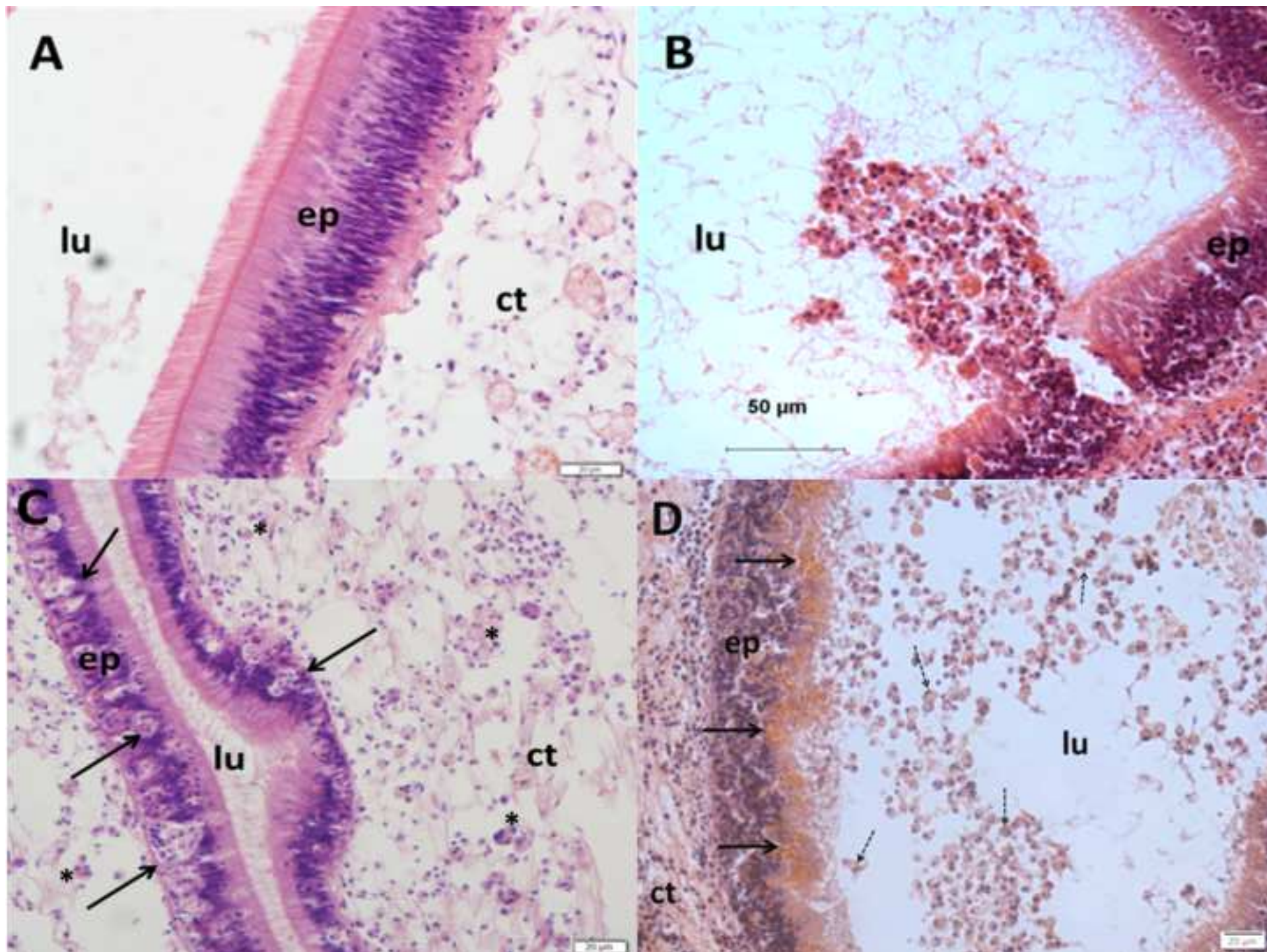


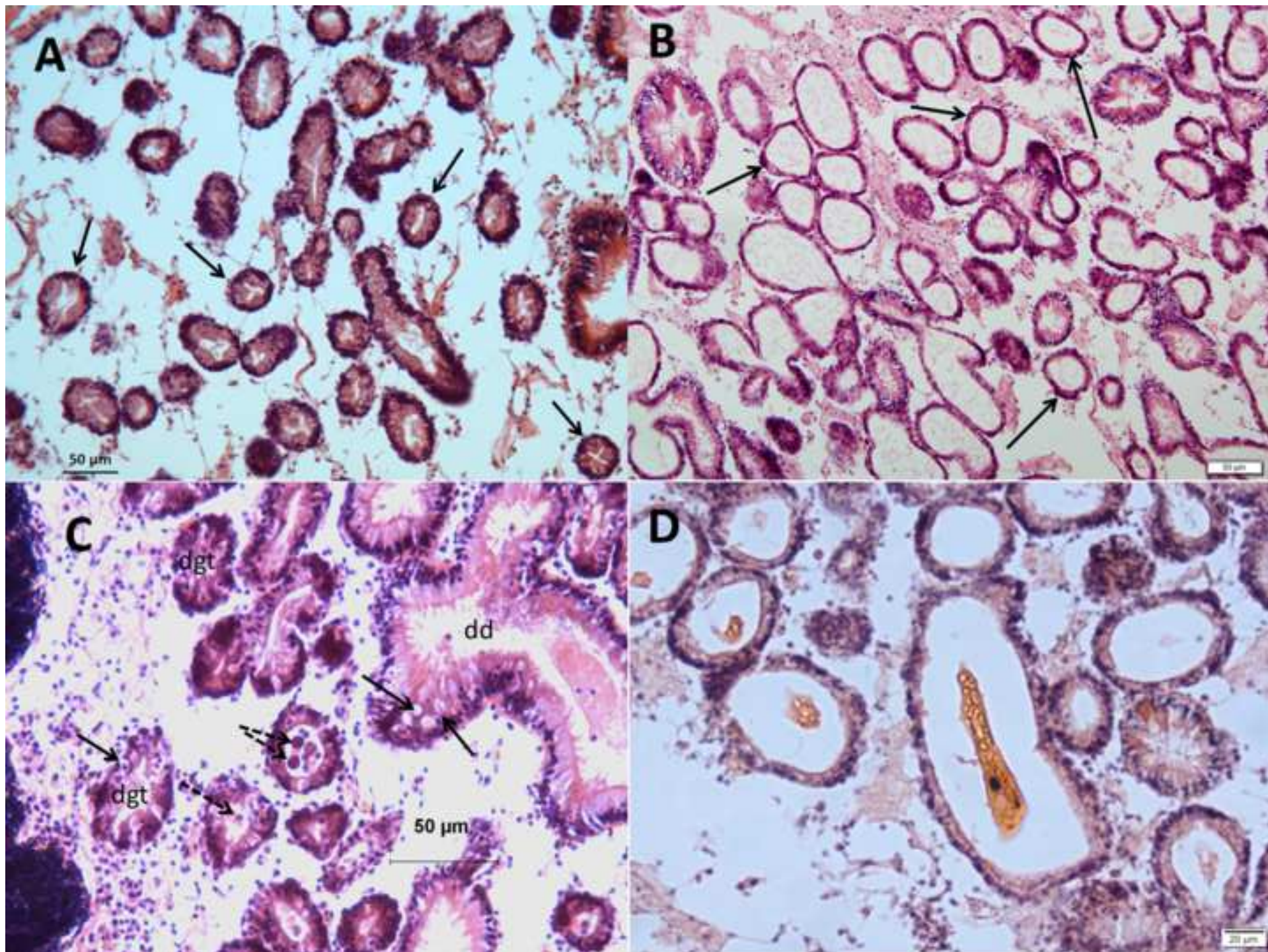


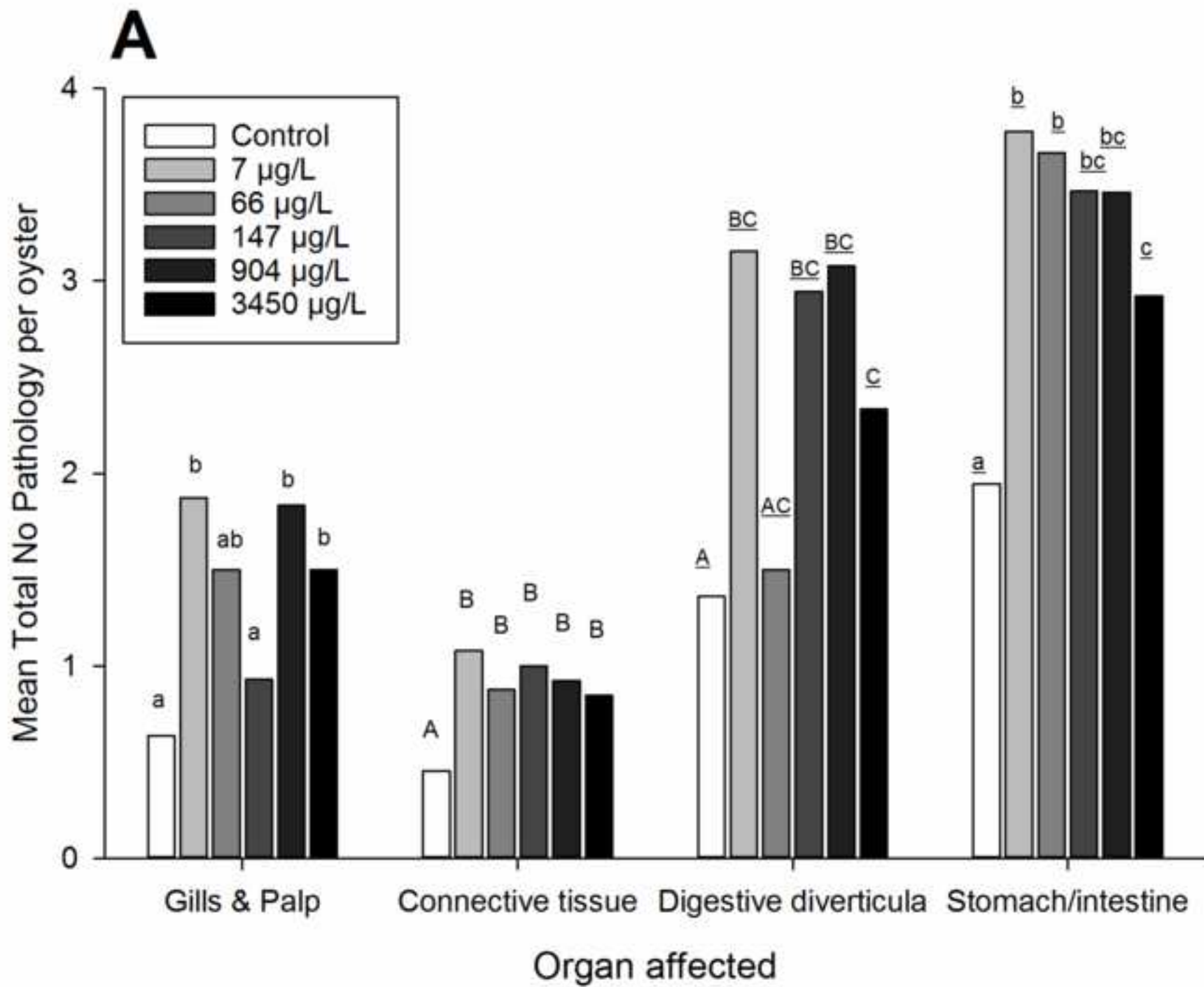


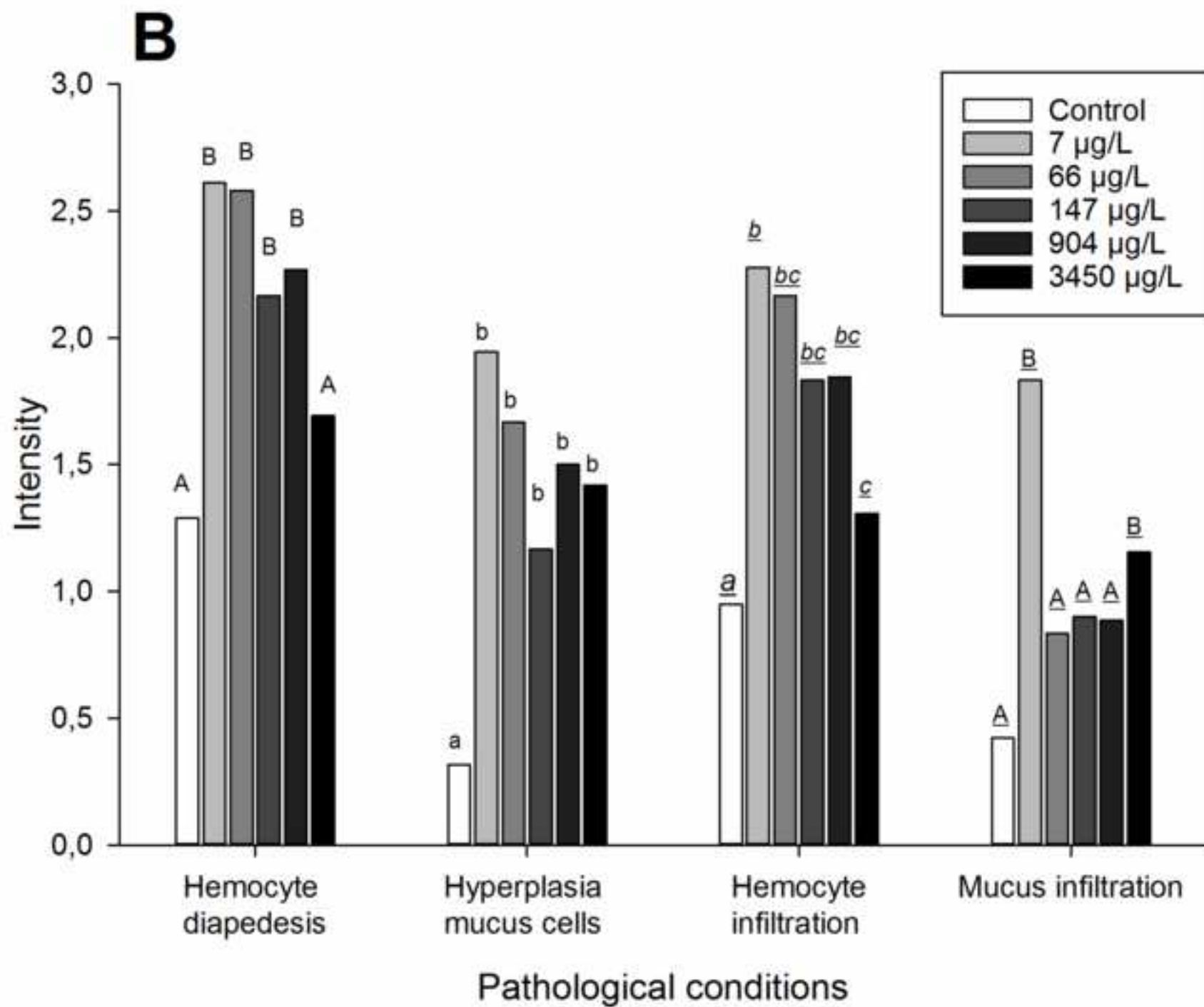


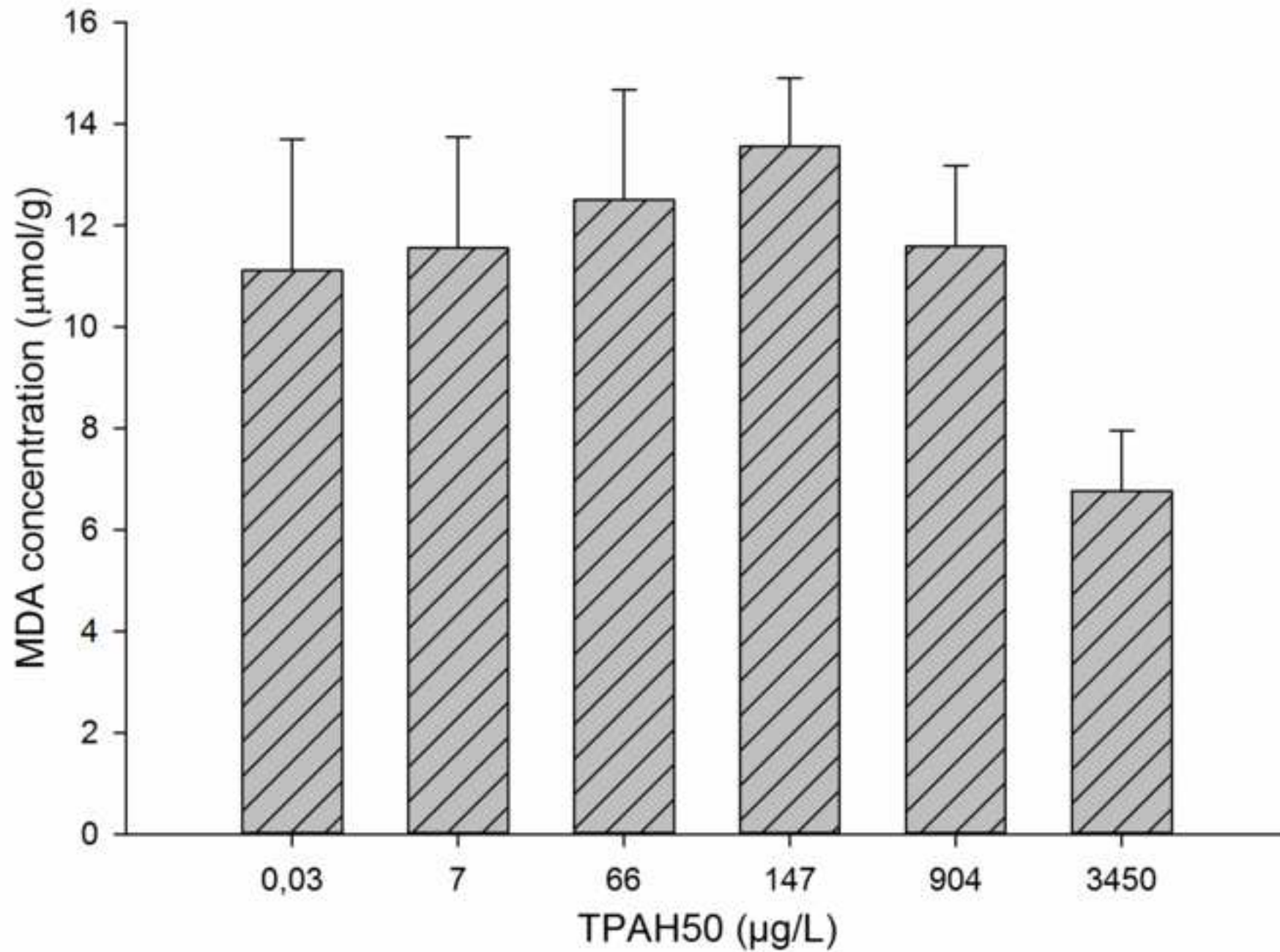














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Supplementary Material

Supplementary File PAH profile stocks.tif

