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Asiatic clam *Corbicula fluminea* exhibits distinguishable behavioural responses to crude oil under semi-natural multiple stress conditions.

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1. INTRODUCTION

Behavioural ecotoxicology is an emerging approach that can be used in the evaluation of ecological risk by serving as a bridge between laboratory and field studies (Pyle and Ford, 2017). Behaviour as a result of environment factors and physiological, cellular or biochemical processes (Amiard-Triquet, 2009; Saaristo et al., 2018) is considered as a particularly relevant and sensible marker for assessing environmental quality and the consequences of pollutants for organisms (Gerhardt, 2007). For example, Melvin and Wilson (2013) conducted a meta-analysis of the literature and showed that although behavioural studies are generally carried out for shorter periods than developmental or reproductive studies, they are often significantly more sensitive to a wide range of compounds and lower concentrations. Within this framework, the development of innovative approaches in relation to behaviour is fundamental for improving the monitoring of human activities on the environment. This is especially true for aquatic ecosystems, which are faced with strong anthropogenic pressures and where understanding the consequences of these pressures is a tremendous challenge (Borja, 2014). The investigation of *in situ* technologies for monitoring environmental aquatic status has shown that there are several methods and tools of interest, such as the use of biosensor behaviours proposed decades ago (Danovaro et al., 2016; Queirós et al. 2016). However, ensuring the ecological validity of the behavioural approach is a complex challenge (Parker, 2016). In the present work, the behavioural responses of Asiatic clams, *Corbicula fluminea*, were studied by HFNI Valvometry (High-frequency, noninvasive Valvometry; Andrade et al., 2016) in mono and multistress contexts by using freshwater outdoor artificial streams that were representative of a natural environment.

Regarding bivalve mollusks, many studies in laboratories, semi-natural environments or *in situ* have reported the potential of studying their behaviour to reflect environmental changes (e.g., Garcia-March et al., 2016; Guo and Feng, 2018; Hartmann et al., 2016; Miller and Dowd, 2017). They are ecologically relevant for behavioural biomonitoring by remote control because they have an exoskeleton composed of two hard shells to glue light electrodes and record behavioural responses without disturbing them, they are sedentary, sessile, abundant and available all year round and they are filter feeders which allows to overcome the need to feed them. However, few reports have focused on crude oil detection (Dragsund et al. 2013; Kramer et al., 1989; Redmond et al., 2017). Using the mussel *Mytilus edulis* under laboratory conditions and changes of its valve activity, Kramer et al. (1989) demonstrated the practical feasibility of detecting dispersed crude oil at a concentration of $<$ 6000 $\mu g \cdot L^{-1}$. More recently, Dragsund et al. (2013) reported additional information for crude oil concentrations $\geq$ 180 $\mu g \cdot L^{-1}$ in the context of leak detection in the natural environment. Finally, Redmond et al. (2017) showed a decrease in the valve gap of the marine mussel *Mytilus edulis* exposed for 4 days to North Sea crude oil under laboratory conditions at nominal concentrations of 60 and 250 $\mu g \cdot L^{-1}$.

In the current work, a main goal was to study the distinguishability and reproducibility of behavioural responses of *C. fluminea* to crude oil in a multistress context. In artificial streams fed by the Gave de Pau river (S.W. France) and subjected to natural variations, clams were exposed for 10 days to crude oil alone or to crude oil plus a metallic trace element (barium), noise pollution (cargo ship noise) or turbidity pulses. The rationale behind these choices is (i) few data are available regarding the effects of barium despite its significant presence in produced waters (Neff et al., 1987; 2011), (ii) underwater noise pollution is inherent to industrial activities, and bivalve mollusks are sensitive to noise, including that of cargo ships (Charifi et al., 2017; 2018) and continuous noise (Shi et al., 2019), and finally (iii) turbidity episodes are part of the background changes that routinely occur in many rivers and estuaries. We show that key aspects of the behavioural response to oil are visually and statistically discriminating. They were not confounded by the presence of the other disruptors tested, alone or in combination with crude oil, under semi-natural conditions. In addition, the analysis of PAH in different tissues allowed us to characterize the contamination status and its relationship with behavioural changes.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

The experiment was carried out from October to December 2016. Samples of Asiatic clams, *Corbicula fluminea*, (height, 24-28 mm) were taken from Parentis – Biscarrosse Lake, France (44°22'6"N, 1°11'3"W). They were maintained in 600 L tanks supplied with freshwater in an open circuit at the Arcachon Marine Station, France. The clams (n = 128) were equipped with HFNI electrodes (Andrade et al., 2016) one week before being transferred to the Pilot Rivers facilities of TOTAL, Lacq, France (Bassères and Tramier, 2001; Sourisseau et al., 2008; for an overall view of the experimental site see Fig 1 in Cailleau et al., 2019). We worked in 8 parallel artificial streams equipped as shown in Fig. 1A, B (length, 40 m; width, 0.5 m; depth, 0.5 m) and supplied by an open circuit with freshwater from the Gave de Pau River. Streams were exposed to natural variations; the water was not filtered or treated, and an upstream nursery constituted a reservoir of living organisms, promoting natural colonization (Bassères et al., 2004). For the specific requirements of the experiment, a water depth of 0.25 m in each stream was defined, and 2 quartz and silica sand zones *A* and *B* (length, 1 m; depth, 0.1 m) were created to allow natural burrowing of the clams. Measured from the injection site, zone *A* was at 19-20 m and zone *B* at 29-30 m (Fig. 1B). The water velocity in all streams was similar, at 3 $cm \cdot sec^{-1}$ (6 $cm \cdot sec^{-1}$ in zone *A* and *B*), and the residence time for a drifter was ≈ 22 min.

2.2. Experimental protocol

The experiment was divided into 4 periods with t_0 being the beginning of the exposure period. Period 1 was a 16-day acclimation period to the artificial streams (26th October – 9th November; t_{-26} – t_{-12}). Period 2, from 11th November (t_{-10}) to 20th November (t_{-1}), was the reference period used for comparison with the exposure period. Period 3 (21st November – 1st December), t_0 – t_{10}, was the exposure period. The exposure period was started simultaneously in all streams on t_0 at 03:15 PM (GMT+1; 21st November). It ended at t_{10} on 1st December at 03:15 PM (GMT+1). Period 4, from 2nd to 11th December (t_{11} – t_{20}), was a 10-day post-exposure period.

In each artificial stream, a group of 16 clams was equipped for the study of behaviour in all *A* zones (Fig. 1B). The area was covered with a wire mesh to prevent bird predation (Figs. $1A_2$ and $1A_3$). A second set of groups (n = 20) was placed in cages burrowed in the *B* zones for tissue sampling (Fig. 1B). The spacing between the *A* and *B* zones made it possible to sample in *B* without disturbing clams in *A*. Stream C was the control stream and was subjected to natural variations only. In stream O (oil), clams were exposed to oil only; in stream Ba (barium), to barium only; in stream N (noise), to noise pollution only; and in stream T (turbidity), to turbidity pulses only. In stream O+Ba, clams were exposed to oil plus barium; in stream O+N, to oil plus noise pollution and in stream O+T, to oil plus turbidity pulses.

The crude oil was a light oil from the North Sea. The density was 0.77 (at 15 °C), and the dynamic viscosity was 1.016 mPa·s (at 15 °C). SARA analysis indicated that the residue fraction (representing 28.2 % of the crude oil, compared to 71.8 % for the distillate fraction) contained 84 % saturated hydrocarbons, 15.5 % aromatic hydrocarbons and 0.5 % polar compounds. Crude oil stored under inert nitrogen was continuously injected (6.5 $mL \cdot h^{-1}$) into the streams by a piston pump (Prominent) and mechanically dispersed in the water as microdroplets through a high-pressure pump and a shearing valve (Netzsch, Nemo; Figs. $1A_1$ and 1B). Barium ($BaCl_2$, $(H_2O)_2$; Sigma-Aldrich; CAS Number: 10326-27-9) stored under inert nitrogen was continuously injected (40 $mL \cdot h^{-1}$) by a piston pump (Prominent). Turbidity pulses were carried out with green clay (Les argiles du soleil; CAS number: 1318-93-0) that was previously homogenized (Joffe Agitateurs) with the Gave de Pau water in 200 L tanks and injected into the streams (76 $L \cdot h^{-1}$) with a peristaltic pump (Cole-Parmer; Masterflex L/S) from 02:00 to 05:00 PM (GMT+1) on days t_0, t_1, t_2, t_3, t_4, t_7, t_8 and t_9. Noise pollution was achieved using 2 underwater loudspeakers (US-0130; Randson; France) positioned on either side of the two areas where clams were present and an amplifier (AM60A; RONDSON; France), Fig. 1B. A playlist was created (Cool Edit; version 2.0; Syntrillium Software Corporation; USA) using a 16 min cargo ship noise previously recorded in the port of Santander, Spain (see Charifi et al., 2018 for more

details). A 3-day sound pattern was created (5 to 8 cargo ship noises per day) and repeated during the experiment.

2.3. Follow-up of exposure parameters

2.3.1. Analysis of crude oil contamination

Oil injection rates were measured on days t_1, t_3, t_4, t_7, t_8 and t_9 to maintain a nominal concentration of 400 $\mu g \cdot L^{-1}$. The measured oil injection rates were 6.6 ± 0.43 $mL \cdot h^{-1}$ in stream O, 6.7 ± 0.07 $mL \cdot h^{-1}$ in stream O+Ba, 6.8 ± 0.26 $mL \cdot h^{-1}$ in stream O+N and 6.0 ± 0.39 $mL \cdot h^{-1}$ in stream O+T. The measured rates, and therefore the quantities of oil injected, were not different between the 4 contaminated streams (Tab. 1). In-stream measurements of total petroleum hydrocarbon (TPH) were performed on days t_1, t_3, and t_{10}. Water was sampled 35 m downstream of the injection point (Fig. 1B) in the centre of the stream and in the centre of water column in 1 liter glass bottles. It was stabilized with Methanol and Nitric acid. The TPH were extracted with 40 ml of hexane and concentrated under nitrogen flux to be analyzed by GC-FID with Agilent 7890 B GC system (equipped with a 15 m Agilent CP7491 column) and integration of total peak area between C10 and C40. The mean measured concentration was 167 ± 28 $\mu g \cdot L^{-1}$.

Tab.1. Inter-comparison p-values of the injection rates measured in the 4 streams involved in the oil contamination.

	O	O+Ba	O+N
O+Ba	1	-	-
O+N	1	1	-
O+T	1	0.5	0.24

2.3.2. Analysis of barium contamination

The water was analysed for barium (Ba) concentration on days t_0, t_1, t_3 and t_{10} in streams C, Ba and O+Ba. The samples were collected in previously cleaned polypropylene tubes (72 h in 5 % regal water and rinsing with ultrapure water). For all samples, 9 mL of water was sampled 35 m downstream of the Ba injection, in the centre of the stream and in the centre of water column (Fig. 1B). Samples were immediately filtered with a 0.2 µm syringe filter (PVDF 33 mm sterile; DDD), acidified with 1 mL of nitric acid (HNO_3^- 65%; Carlo Erba Reagents) and stored in the dark at 4 °C. Analyses were performed by inductively coupled plasma optical spectrometry (ICP OES 700 Series; Agilent). Concentrations of all analytical blanks were below the detection limit of Ba (0.03 $\mu g \cdot L^{-1}$); 14.2 ± 0.06 $\mu g \cdot L^{-1}$ was the mean geochemical background noise for samples taken before contamination in all

streams (n = 3). During contamination, the Ba concentration was 99.7 ± 5.6 µg·L^{-1} (n = 3) in the Ba stream and 90.4 ± 4.7 µg·L^{-1} (n = 3) in the O+Ba stream (not significantly different, p = 0.456). In contrast, these levels were significantly higher than that in the C stream (12.6 ± 0.55 µg·L^{-1}; n = 3; p = 0.051 between C and Ba streams, and p = 0.202 between C and O+Ba streams).

2.3.3. Analysis of turbidity pulses

The turbidity was analyzed manually with a portable turbidimeter (Hack; 2100Q*is*). Samples were collected 35 m downstream of the injection point, in the centre of the stream and water column (Fig. 1B). During pulses, the turbidity was 268 ± 20 NTU (n = 8) in the T stream and 263 ± 21 NTU (n = 8) in the O+T stream (not significantly different, p = 0.8732). The turbidity during pulses was significantly different from the natural turbidity measured in the C stream (43 ± 26 NTU; n = 8; p = 0.0020 between C and T streams, and p = 0.0023 between C and O+T streams).

2.3.4. Analysis of noise pollution

The background noise was measured using a broadband hydrophone with an internal buffer amplifier (H2a-XLR; sensitivity, -180 dB re 1 V·µPa^{-1}; useful range, 10 Hz to 100 kHz; Aquarian) and an Edirol recorder (H4n Handy; Zoom Corporation; Japan) that was previously calibrated (see Charifi et al., 2018 for details). Recordings were taken at the water–sediment interface in the centre of the stream, and therefore between the 2 loudspeakers (Fig. 1B). The clams were at a distance of between 0.2 and 1 m from the centre of the 2 loudspeakers. At 0.2 m, the maximum sound pressure level, SPL, was 161 ± 3 dBrms re 1 µPa (n = 3). At 0.6 m, it was 150 ± 1 dBrms re 1 µPa (n = 2), and at 1 m, 142 dBrms re 1 µPa (n = 1). The average background noise was 88 ± 1 dBrms re 1 µPa (n = 6).

2.4. Analysis of clam behaviour

2.4.1. HFNI Valvometry

The clam behaviour (i.e., valve activity) was studied by HFNI valvometry (Andrade et al. 2016; Tran et al., 2003). For this, 2 lightweight electromagnets were positioned face-to-face on each valve (Fig. $1A_3$). The voltage variation produced by the electromagnetic current between two electromagnets is governed by Maxwell's law. The frequency of data acquisition (time, bivalve number, voltage) in a group of 16 bivalves was 10 Hz, or every 1.6 sec per bivalve. The data were recorded by an acquisition card and were automatically transmitted daily to a processing unit located at the Arcachon Marine Station, France. The data were then automatically and daily processed with R (R Core Team, 2016) and published online on the professional pages of the MolluSCAN *eye* website

(https://molluscan-eye.epoc.u-bordeaux.fr/). All behavioural analyses were performed by remote control in Arcachon, France.

2.4.2. Behavioural parameters

Valve-opening amplitude (VOA). Each valve gap value was associated with a valve-opening-amplitude, expressed as a percentage. The valve amplitude values of 0 % and 100 % were defined as the minimum and maximum values of each bivalve over the previous 6 days. Therefore, the valve amplitude was calculated for each bivalve as the ratio of the valve gap (mm) subtracted from the minimum value (mm) and from the maximum value (mm) subtracted from the minimum value (mm). This parameter was then determined for each group of bivalves, averaging the individual values each hour to give the percentage of hourly valve-opening amplitude for the group.

Valve-closure duration (VCD). The mean percentage of the hourly valve-closure duration in the group was based on the closing duration of each bivalve, each hour (the bivalve was considered closed at less than 5 % of maximum valve opening). Thus, if the bivalve was closed for one hour, the percentage of valve-closure duration was 100 %. By contrast, if the bivalve remained open for one hour, the percentage of valve-closure duration was 0 %. This parameter was then determined for each group of bivalves, averaging the individual values each hour to give the percentage of hourly valve-closure duration for the group.

Valve agitation index (VAI). Valve agitation was obtained by measuring the distance travelled by the electrodes glued on the valves. Every 1.6 sec the distance travelled was measured, in absolute value, by subtracting the value of the valve gap from the previous value (mm) and then an hourly sum was realized for each bivalve. Lastly, this parameter was determined for each group of bivalves by averaging individual values each hour to give the hourly valve agitation. To weight this parameter, the hourly valve agitation of each group of bivalves was divided by the percentage of hourly valve-opening amplitude of the same group of bivalves—this was the hourly valve agitation index.

2.4.3. Response time

The first valve closure reaction or, more rarely, the beginning of a brief series of openings and closings followed by a continuous valve closure reaction were considered to be the first behavioural response of clams to the presence of disruptors in the water (Tran et al., 2003). A response percentage, based on the use of binary variables, was then established for the group of bivalves from the start of the exposure period (t_0; 03:15 PM; GMT+1) thanks to the individual behavioural analysis of each bivalve at different integration times (10; 20; 30; 60; 60; 12; 300; 480; 600; 720 min). A logistic

regression model was then used to estimate the response percentage of bivalves over time. The following logistic function was applied:

$$f(x) = (exp(\beta 0 + \beta 1 \cdot x)) / (1 + exp(\beta 0 + \beta 1 \cdot x))$$

$\beta 0$ and $\beta 1$ are unknown regression parameters. Once these parameters are known, the response times necessary for 10 to 90 % of the bivalves to react can be determined.

2.5. PAH analysis in clam tissues

A total of 21 PAHs (naphthalene, benzothiophene, biphenyl, acenaphthylene, acenaphthene, fluorene, dibenzothiophene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene and benzo(g,h,i)perylene), among which are 16 listed as priority pollutants by the US EPA (United States Environmental Protection Agency; underlined in the list) were analyzed in the gills, foot and adductor muscles of 5 randomly sampled clams per artificial stream at t_{10}. The mass of analyzed tissue (mean ± standard deviation) was 46 ± 8 mg wet weight (w.w.) for muscle, 67 ± 11 mg w.w. for foot and 53 ± 12 mg w.w. for gills. The analyses were performed by stir bar sorptive extraction-thermal desorption-gas chromatography-tandem mass spectrometry (SBSE-GC-MS/MS) as described in Lacroix et al. (2014). Briefly, each tissue was digested by saponification and analytes were extracted for 2 hours at 700 rpm using polydimethylsiloxane stir bars (Twister 20 mm x 0.5 mm, Gerstel). The bars were subsequently analyzed using a gas chromatography system (Agilent 7890A) coupled to an Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies) and equipped with a thermal desorption unit (TDU) combined with a cooled injection system (Gerstel). Thermodesorption and GC-MS/MS conditions were as previously described (Lacroix et al. 2014). Analytes were quantified relative to deuterated compounds using a calibration curve ranging from 0.01 ng to 10 ng per bar. Two compounds, benzo(b)fluoranthene and benzo(k)fluoranthene, were quantified as a sum named benzo(b+k)fluoranthene due to poor resolution. The limits of quantification (LOQ) were calculated by the calibration curve method (Shrivastava et al., 2011), and the limit of detection (LOD) was estimated by dividing the LOQ by 3.

2.6. Statistics

The results are reported as means ± 1 SE and box plots of mean hourly values. After checking for assumptions of normality and homoscedasticity of error term, comparisons between variables were investigated using the non-parametric Kruskal-Wallis test. For all pairwise comparisons of

254 independent samples, Dunn's or Conover's tests with Holm adjustment (PMCMR package; Pohlert,
255 2014) were considered. For all pairwise comparisons of paired samples, the pairwise Wilcoxon test
256 was used with Holm adjustment. For all statistical results, a probability of $p < 0.05$ was considered to
257 be significant. The data were computed and analyzed using R software (R Core Team, 2016).
258
259 **3. RESULTS**
260
261 **3.1. Individual clam behaviours**
262
263 No mortality was recorded during the exposure and post-exposure periods. During the reference
264 period, 1 clam died in stream T, and the recordings of 3 clams were lost due to technical problems (2
265 clams in stream O+Ba and 1 clam in stream O+N).
266
267 A visual study of the individual behaviours of each clam preceded the analysis of the group behaviour.
268 Figure 1B1 shows a 16-day record, including the 10-day exposure period of a clam exposed to crude
269 oil (stream O) at the nominal concentration of 400 $\mu g \cdot L^{-1}$. This behaviour was compared with the
270 typical behaviour of a clam in the control (C) stream (Fig. 1C1) and with the natural water temperature
271 (Tw) change during the same period (Fig. 1B2). Visually, the response to oil was characterized by a
272 decrease in valve-opening amplitude, an increase in valve-closure duration and an increase of the
273 valve agitation index. This behavioural response to crude oil was not confounded by the behaviour of
274 the clams subjected to only natural variations despite the Tw changes in the range 12.5 – 7.5 °C for
275 stream O and 12.5 – 7.4 °C for stream C during the exposure period ($t_0 - t_{10}$). Throughout the $t_{-10} - t_{20}$
276 period, Tw varied from 6.6 – 12.5 °C (inserts, Fig. 1B2 and 1C2).
277
278 **3.2. Behaviour of clam groups**
279
280 In this part, we compared the hourly valve-opening amplitudes (VOA), hourly valve-closure durations
281 (VCD) and hourly valve agitation index (VAI) in all artificial streams during the reference (t_{-10} to t_{-1}),
282 the exposure (t_1 to t_{10}) and the post-exposure (t_{11} to t_{20}) periods. The 1st day of the exposure period (t_0)
283 was excluded because it was considered as a transitional day.
284
285 Figure 2 shows the average hourly valve-opening amplitudes for the 8 artificial streams during the
286 reference (Fig. 2A), the exposure (Fig. 2B) and the post-exposure (Fig. 2C) periods, with n = 240
287 mean values for each period in each stream (24 h x 10 days). The homogeneity of valve-opening
288 amplitudes in clams subjected only to natural variations within the different artificial streams during
289 the reference period is shown in Fig. 2A. During the exposure period (Fig. 2B), compared to the clams
290 of the control stream (C) subjected to natural variations only, the valve-opening amplitudes

significantly decreased in the presence of oil alone (O) ($p < 2.2e-16$), O+Ba ($p < 2.2e-16$), O+N ($p < 2.2e-16$) and O+T ($p < 2.2e-16$). The hourly valve-opening amplitudes during Ba, noise and turbidity pulse exposures alone were not different from the valve-opening amplitude in the control stream (respectively, $p = 0.864$; $p = 1$; $p = 1$). Therefore, the decrease in valve-opening amplitude induced by oil was not modified by the addition of barium, noise pollution or turbidity pulses. During the post-exposure period (Fig. 2C), compared to the clams exposed to natural variations only (C), the clams exposed to oil alone, O+Ba, O+N or O+T exhibited lower opening amplitudes (respectively, $p = 7.1e-08$; $p = 1.3e-04$; $p = 2.3e-03$ and $p = 1.4e-13$). The behaviour of clams exposed to oil was therefore still disrupted during the post-exposure period. In contrast, the opening amplitudes of clams exposed to Ba, N and T remained identical to the opening amplitudes of clams exposed to natural variations only (respectively, $p = 1$; $p = 0.13$; $p = 0.22$).

Figure 3 shows the average hourly valve-closure durations for the 8 artificial streams and for the 3 time periods, reference (Fig. 3A), exposure (Fig. 3B) and post-exposure (Fig. 3C), again with $n = 240$ mean values for each artificial stream and period. The homogeneity and/or natural changes of valve-closure duration in clams subjected to natural variations during the reference period is presented in Fig. 3A. During the exposure period, the increase in the valve-closure duration was significantly marked by the presence of crude oil (Fig. 3B). Indeed, compared to the control (C), the hourly valve-closure durations increased in the presence of O ($p < 2.2e-16$), O+Ba ($p < 2.2e-16$), O+N ($p < 2.2e-16$) and O+T ($p < 2.2e-16$), while remaining unchanged for the clams exposed to Ba, noise and turbidity pulses (respectively, $p = 0.77$; $p = 1$; $p = 1$). The increase in valve-closure duration in the presence of crude oil was therefore not confounded by the other studied disrupters (Fig. 3B). During the post-exposure period (Fig. 3C), the behaviour of the clams exposed to oil was different from those not exposed to oil. Specifically, compared to the control clams, the clams exposed to O alone, O+Ba, O+N or O+T exhibited a longer valve-closure duration (respectively, $p < 2.2e-16$; $p < 2.2e-16$; $p = 1.3e-09$; $p < 2.2e-16$). According to this parameter, the behaviour of clams exposed to oil was therefore still disrupted during the post-exposure period. In contrast, during the post-exposure period, the valve-closure duration of clams exposed to Ba, noise and turbidity pulses was not different from the control clams ($p = 1$).

Figure 4 shows the average hourly valve agitation index (i.e., the valve agitation weighted by the valve-opening amplitude) for the 8 artificial streams and for the 3 time periods, showing the reference (Fig. 4A), exposure (Fig. 4B) and post-exposure (Fig. 4C), with $n = 240$ mean values for each artificial stream and period. The homogeneity of the valve agitation index in clams subjected to natural variations during the reference period is presented in Fig. 4A. During the exposure period, the increase in the valve agitation index was significant in the presence of crude oil (Fig. 4B). Indeed, compared to the control (C), the hourly valve agitation index increased in the presence of O ($p < 2.2e-16$), O+Ba (p

328 < 2.2e-16), O+N ($p < 2.2e-16$) and O+T ($p = 6.5$ e-16). In contrast, the hourly valve agitation index of
329 the clams exposed to Ba, noise or turbidity alone were not different from the control situation
330 (respectively, $p = 0.975$; $p = 0.143$; $p = 1$). The increase in valve agitation index in the presence of
331 crude oil was therefore not confounded by the other disrupters studied (Fig. 4B). During the post-
332 exposure period (Fig. 4C), only the behaviour of the clams exposed to O+T and T were statistically
333 different from all the others.
334
335 **3.3. Response time of clams**
336
337 The first valve closure reaction or, more rarely, the beginning of a brief series of openings and
338 closings followed by a continuous valve closure reaction, appeared at between 6.3 and 7.8 h after
339 exposure to crude oil in the artificial streams for 50 % of the clams (Fig. 5). More precisely, the
340 response time for 50 % of clams was 7.8 h in O exposure, 6.3 h in O+Ba exposure, and 6.9 h in O+N
341 and O+T exposures. Without oil, the response time for 50 % of clams was 11.2 h in T exposure.
342 Furthermore, the response time for 90 % of clams was 10.9 h in O exposure, 9.9 h in O+Ba exposure,
343 12.3 h in O+N exposure and 9.8 h in O+T exposure. The response time for 10 % of clams was only 4.7
344 h in O exposure, 2.8 h in O+Ba exposure, 1.4 h in O+N exposure, 3.9 h in O+T exposure (Fig. 5).
345
346 **3.4. PAH accumulation**
347
348 Figure 6 shows the PAH contamination status at t_{10} in the gills, the foot and the adductor muscles of
349 control and oil-exposed clams. Not surprisingly, the gills were the most contaminated tissue.
350 Accumulation in the foot was intermediate, and the adductor muscles were the least contaminated ($p_{\text{gills-foot}} = 6.5e\text{-}05$; $p_{\text{gills-muscles}} = 7.5e\text{-}12$; $p_{\text{foot-muscles}} = 6.5e\text{-}05$). In the gills, the contamination was
352 significantly greater in the presence of O, O+Ba and O+N in comparison to the control (respectively, p
353 $= 1.5e\text{-}02$; $p = 1.2e\text{-}04$; $p = 1.1e\text{-}02$). In the foot, the differences were significant between control and
354 O+Ba ($p = 9.1e\text{-}04$) but also between O and O+Ba ($p = 1.3e\text{-}02$). In the adductor muscles,
355 contamination was not statistically different between the different artificial streams. However, the
356 highest median values, whether in the gills, foot or adductor muscles, were always observed under the
357 O+Ba condition.
358

4. DISCUSSION

The primary purpose of this study performed under outdoor semi-natural conditions with *C. fluminea* was to identify, if any, a reliable and therefore discriminating behavioural response to crude oil in a multistress context. The second purpose was to complement the behavioural response of *C. fluminea* by analyzing the PAH contamination of target organs, including the gills, which is a major entrance route for contaminants, and two organs involved in clam shell movements, the foot and the adductor muscles. The main finding was that the response of *C. fluminea* to crude oil in a naturally variable environment and in the presence of multiple stress exposures (cargo ship noise, turbidity pulses and barium) is clearly distinguishable and can be identified by 3 parameters: the valve-opening amplitude, valve-closure duration and valve agitation index. While a single crude oil concentration was studied (a nominal value of 400 $\mu g \cdot L^{-1}$), the PAH accumulation in the three tested tissues was quite variable, illustrating the inter-individual variability. However, the PAH accumulation was always at the maximum value when barium was added to oil, the condition under which valve agitation was also at its highest level.

4.1. Behavioural response of bivalve mollusks in the presence of crude oil alone

As discussed in the Introduction, despite behaviour being understood as a particularly sensitive marker to assess water quality and its potential use in the oil and gas field, relatively few studies have described the valve activity of bivalve mollusks during crude oil contamination. An advanced study that has been carried out is the laboratory work by Redmond et al. (2017). The authors described the behaviour of the marine mussel *Mytilus edulis* in response to light North Sea crude oil alone, under controlled conditions for 4 days, at 3 different nominal concentrations: 15, 60 and 250 $\mu g \cdot L^{-1}$ at a water temperature of 13 °C. Behavioural parameters studied were the distance travelled by the valves, the valve gap and the time spent in various valve positions. Their results showed a statistically significant reduction in valve gap (i.e., valve amplitude) at 60 and 250 $\mu g \cdot L^{-1}$. A few other studies can also be mentioned. They were all carried out under laboratory conditions with much higher oil concentrations, from 50-1000 $mg \cdot L^{-1}$ of crude oil (Hartwick et al., 1982; Staiken et al., 1976; Swedmark et al., 1973). Swedmark et al. (1973) stated that contamination with 1000 $mg \cdot L^{-1}$ of Oman crude oil (96 h; Tw = 10 ± 2 °C) did not alter the closing capacity of the scallop *Pecten opercularis* and the mussel *Mytilus edulis*. Staiken et al. (1979) reported a depression of muscle contraction with an increase in mucus secretion in the marine bivalve *Mya arenaria* exposed for 96 h to different concentrations of Southern Louisiana Crude oil (50 to 800 $mg \cdot L^{-1}$; Tw = 4 and 14 °C). Hartwick et al. (1982) carried out contamination of the clam *Protothaca staminea* for 5 h per day over 5 days with 100 and 1000 $mg \cdot L^{-1}$ of Alberta crude oil and reported two separate behavioural response panels. At 100 $mg \cdot L^{-1}$, valves were tightly closed, and when they opened the retraction reaction of siphons was

normal. At 1000 mg·L^{-1}, the retraction reaction of siphons was slower, and a wide shell gap was observed during exposure to air. This wide valve gap was followed by a closure with (sometimes) the pinching of siphons on the outside of the shell. Overall, independent of the concentration, the valve closure reaction has been proposed by most authors as a protective reaction to the sublethal effects of crude oil (Baussant et al., 2011; Cajarville et al., 1992; Swedmark et al., 1973).

4.2. Behavioural response of bivalve mollusks in the presence of barium, noise or turbidity with or without crude oil and bioaccumulation of PAH

To the best of our knowledge, the present report is the first to study the behavioural response of a bivalve mollusk exposed to crude oil in a multiple stress context. In addition, it was carried out under outdoor semi-natural conditions. Barium is a naturally occurring metallic trace element found in the environment, in drilling fluids and in produced waters as a by-product of the oil and gas industry. Barium is one of the major metals found in produced water at high concentrations (Neff et al., 1987; 2011). Enrichment factors of produced water compared to natural seawater can reach values up to 10,000 (Trefry et al., 1995) and frequently exceed values in the order of 1000 (Neff, 2002). With regard to toxicity, Spangenberg and Cherr (1996) found that the gastrula stage of the mussel, *Mytilus californianus*, was similarly affected by contamination with produced water or with barium. These results were in line with an earlier study by Higashi et al. (1992) in which the toxicity of different fractions of produced water were investigated in embryos of the same species. To our knowledge, there is no study describing the behaviour of adult bivalve mollusks in the presence of barium. The present study did not find any statistically significant observation concerning a behaviour change of *C. fluminea* in the presence of barium alone (10-day exposure to 95 ± 3.9 µg·L^{-1}, 7 times more than the measured natural geochemical background noise). However, in the presence of oil + barium, we report a larger valve agitation index and systematically higher concentrations of PAHs in the gills, adductor muscles and foot. To explain this larger contamination status, one must keep in mind that under resting conditions the gill cavity in water breathers must be considered as an antechamber with a low inspired water turnover. This is indeed the basic mechanism allowing the haemolymph in the gills to withstand low water oxygenation levels (Massabuau and Abele, 2011), setting the stage for the low blood and tissue oxygenation strategy (Massabuau, 2001). Valve agitation in bivalve mollusks means stronger back and forth water movements between the ambient water and the pallial cavity. We propose that the increased valve agitation in clams exposed to oil + barium likely led to an increase in water renewal within the gill cavity, an increase of the contamination gradient between pallial water and the haemolymph, with or without changing the exposed gill area, which therefore facilitated the tissue contamination processes. Yet, other mechanisms could exist. For example, cadmium has been reported to promote the accumulation of the PAH benzo(a)pyrene (Benedetti et al., 2007; Wang et al., 2011),

probably by altering biotransformation pathways and thus the possibility of PAH elimination by the organism (Benedetti et al., 2007). Such a mechanism could be a 2nd working hypothesis for barium.

Underwater noise pollution is responsible for adverse effects to aquatic fauna including invertebrates (for a previous discussion see de Soto, 2016). This is true for both seawater and freshwater. In the freshwaters of the Ganges River, where *Corbicula sp*. is present (Prahad, 1929), Dey et al. (2019) reported sound pressure levels ranging from 155-162 dB re 1 µpa that is above the present reported values. In the Danube River, also inhabited by *Corbicula sp*, and in the lakes Mondsee and Traunsee, Austria, Wysocki et al. (2006) recorded similar SPL. Among the impact studies, few were carried out on bivalve mollusks despite their importance to biodiversity and their sensitivity to naturally generated sounds in the environment (Charifi et al., 2017; Ellers, 1995; Lillis et al., 2013) and to anthropogenic noise (Charifi et al., 2017; Peng et al., 2016; Roberts et al., 2015; Shi et al. 2019; Solan et al., 2016; Vazzana et al., 2016; 2018;). Charifi et al. (2017) described the sense of hearing in oysters, *Magallana gigas.* The authors demonstrated the oyster hearing ability in the range from 10 – 1000 Hz through a behavioural approach based on transient valve closure reactions. In the oyster *M. gigas*, Charifi et al. (2018) studied the dual impact of cadmium (Cd) metal pollution and noise pollution (92 cargo-ship noise of 12 min per day, with a maximum SPL of 138 and 150 dBrms re 1 µPa for 14 days). Charifi et al. (2018) reported a decrease in valve activity, a decrease in Cd bioaccumulation and slower growth rates compared to oysters exposed to Cd alone. The authors suggested a depressant effect of "heavy" cargo ship noise on oysters (Charifi et al., 2018) that was confirmed by Shi et al. (2019) in blood clams. In the present study, clams were exposed to a less powerful noise stress, only 4 to 7 cargo ship noise, 16 min per day for 10 days. We did not observe any depressant effect on behaviour, and the PAH bioaccumulation of clams exposed to oil + noise did not reflect any significant difference when compared to oil alone. The exposure frequency (i.e., number of cargo ship noises per day) could be a key explanation, in addition to the possible differences in noise sensitivity between species.

The last confounding factor was turbidity episodes. Indeed, in the environment, oil and suspended particular matter (SPM) naturally aggregate. These associations are most often formed during the collision between SPM and hydrocarbons, such as PAH, in turbulent aquatic systems (Loh et al., 2018; Sun and Zheng, 2009). Loh et al. (2018) observed that despite SPM bringing PAHs down in the water column, PAH accumulation by oysters was inhibited in the presence of SPM. The excretion of SPM-PAH pellets by pseudofeces was a possible hypothesis for this finding. In *C. fluminea*, the rapid and occasional adduction of valves causes ejection of water and pseudofeces through the inhaling siphon (Britton and Morton, 1982). However, within our experimental conditions, turbidity pulses did not influence the response of clams to oil and did not lead to a statistically differential accumulation of PAHs by the clams exposed to oil alone compared to the clams exposed to oil + turbidity pulses. We suggest that in our experimental system the conditions were perhaps not favourable for the

469 establishment of adhesion mechanisms between SPM and hydrocarbons. Indeed, in addition to non-
470 chronic exposure to turbidity, adhesion mechanisms are dependent on multiple factors, such as SPM
471 concentration, temperature, mixing energy and oil types, including the oil polar hydrocarbon fraction,
472 which was less than 0.5 % in the present study (see review by Sun and Zheng, 2009).
473
474 **4.3. Behavioural ecotoxicology in bivalve mollusks and underlying physiological mechanisms**
475
476 Biomonitoring as a whole allows the study of changes in water quality, but a new goal of behavioural
477 ecotoxicology could be to evaluate the underlying disturbances in the internal medium by assessing
478 the behaviour remotely. In the present work, we studied changes in *C. fluminea* behaviour in the
479 presence of crude oil. The hourly valve-closure duration of *C. fluminea* exposed to oil was longer than
480 in unexposed animals. This indicates an increase in the adductor muscle catch-state. In bivalves, catch
481 is a passive state of smooth muscle that leads to the maintenance of valve closure (i.e., stretch
482 resistance) for long time periods with minimal energy consumption (Galler, 2008; Yamada et al.,
483 2013). It is regulated by the phosphorylation and dephosphorylation of twitchin (Funabara et al.,
484 2003). When smooth muscle is relaxed (i.e., when the valves are not closed), twitchin is
485 phosphorylated. The contraction (i.e., valves closure) is caused by the release of acetylcholine, which
486 leads to an increase in intracellular Ca^{2+} concentrations. When the Ca^{2+} concentration becomes high,
487 twitchin is dephosphorylated by a Ca^{2+}-dependent phosphatase, and the catch state is initiated. The
488 catch state is stopped by the release of serotonin, which causes an increase in intracellular cAMP and
489 cAMP-dependent protein kinase A, which in turn leads to the phosphorylation of twitchin (Funabara et
490 al., 2006; Twarog, 1954; 1960). Thus, the presently recorded increase in catch state also reflects an
491 internal change of status in the serotonergic and cholinergic systems. This is supported by the
492 observations by Cappello et al. (2015) and Maisano et al. (2017). They reported a decrease in
493 acetylcholine and serotonin neurotransmitters in gills of the marine mussel *Mytilus galloprovincialis*
494 encaged in the field and subjected to petrochemical activities. Interestingly, Hansen et al. (2017) also
495 found a decrease in activity and a reduction of the neurotransmitter acetylcholine in the copepod
496 *Calanus finmarchicus* in response to sublethal exposure to oil. Finally, the present decrease in valve
497 closure duration fits well with a narcotic effect, which should also be present. Narcosis is defined as a
498 nonspecific and reversible disruption of the functioning of biological membranes caused by the
499 accumulation of hydrophobic compounds, such as PAHs, which causes an overall decrease in activity
500 (van Wezel and Opperhuizen, 1995).
501
502 To conclude, the present study supports the interest of studying and using the behaviour of bivalve
503 mollusks to follow global water quality in the field. This is especially true in the context of operational
504 biomonitoring in the oil industry (Andrade et al. 2016; Blanc et al. 2018; Massabuau et al. 2015). The
505 changes of behaviour in the presence of crude oil in water were reproducible and were not influenced

by the other environmentally relevant stressors studied here: noise pollution, turbidity pulses, barium concentration and water temperature. In the future, the response pattern to crude oil should be studied in other bivalve species, comparing freshwater and seawater, and at different contamination pressures. However, the literature shows that the present results and analysis are already quite coherent.

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ABSTRACT

Aquatic ecosystems are subject to many anthropogenic disturbances, and understanding their possible impacts is a real challenge. Developing approaches based on the behaviour of bivalve mollusks, an integrating marker of the state of the organisms, and therefore of their environment, is relevant, whether within a natural ecosystem or an ecosystem subject to industrial activities. The main objective of this study was to identify by HFNI Valvometry a reliable and reproducible clam behavioural response in the presence of crude oil in a multistress context. To closely replicate actual field conditions, *Corbicula fluminea* was exposed in outdoor artificial streams that were subject to natural variations and were continuously fed by fresh water from the Gave de Pau (S.W. France). After a period of 26 days in these artificial streams, the clams (n = 14-16 per condition) were separately exposed for 10 days to crude oil alone, crude oil and barium, crude oil and noise pollution, crude oil and turbidity pulses, barium alone, noise pollution alone, turbidity pulses alone or natural changes alone. The secondary objective was to characterize the accumulation of polycyclic aromatic hydrocarbons (PAHs) in 3 tissues (gills, adductor muscles and foot) in clams exposed for 10 days to

crude oil alone or under multistress conditions (n = 5 clams per condition) and then to compare the accumulation and behaviour of clams under these conditions. The response of clams to crude oil alone or under multistress conditions was visually and statistically significant and not confounded by the other disturbances tested, despite large variations in water temperature. In the presence of crude oil, the behaviour of clams was characterized by an increase in valve-closure duration, a decrease in valve-opening amplitude and an increase in valve agitation index. In the presence of crude oil, the clam behaviour showed no direct relationship with PAH accumulation in the gills, adductor muscles or foot, although hypothetical mechanisms are discussed. This work supports the growing interest in studying the behaviour of bivalve mollusks in the context of biomonitoring of the aquatic environment surrounding oil facilities.

Keywords

Bivalve mollusks
Biomonitoring
Crude oil
outdoor mesocosm
PAH
HFNI Valvometry

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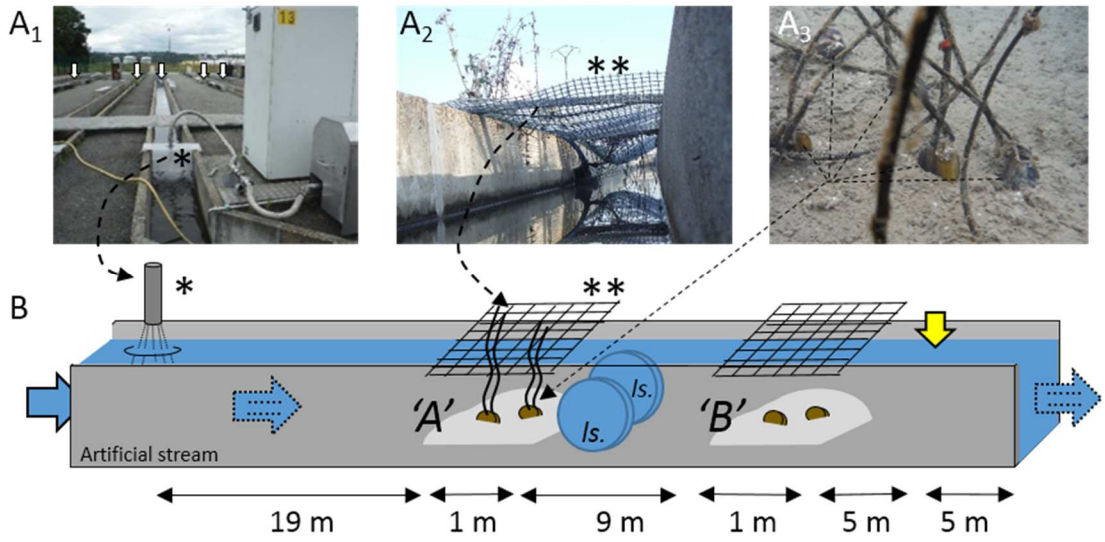


Figure 1. Outdoor mesocosm and semi-natural conditions along the Gave de Pau river (Lacq, France). A: Experiments were performed in 8 parallel artificial streams (40 x 0.5 x 0.5 m; white arrows on Fig $1A_1$ show 5 of them) supplied by an open circuit from the Gave de Pau River. B, schematic view showing the organization in each stream. From left to right: water arrival; * injection point for oil, barium or turbidity (Fig $1A_1$); *'A'* and *'B'*, two sand zones covered by anti-bird nets** (Fig. $1A_2$) for *Corbicula* equipped for behaviour recordings in zone *'A'* (Fig. $1A_3$) and for tissue samplings in zone *'B'*. Two underwater loudspeakers *ls*. were mounted facing each other at 0.2-1 m from clams in zone 'A' to reproduce noise pollution. Yellow arrow, location of the sampling point for water analyses. See Fig. 1 in Cailleau et al (2019) for an overall view.

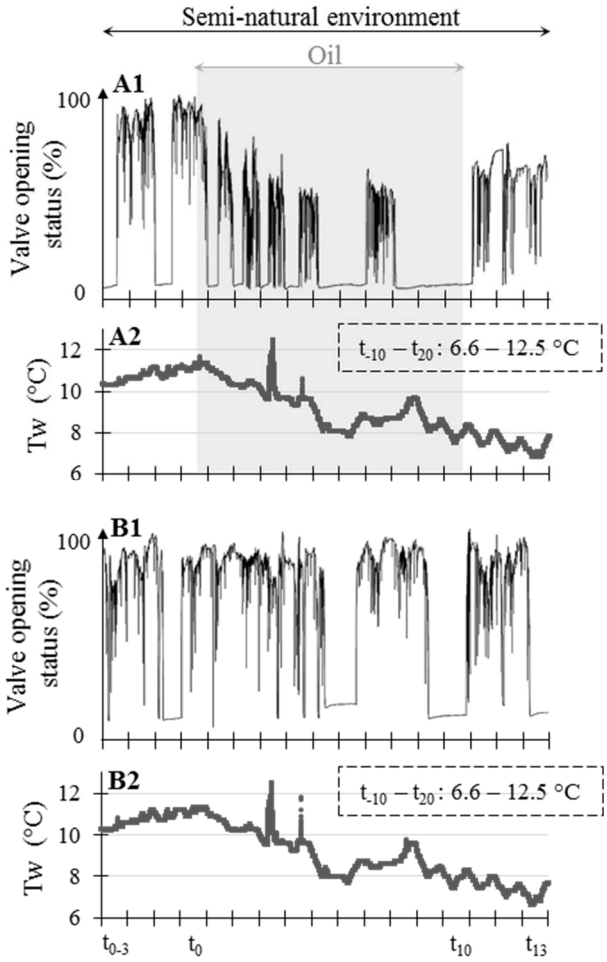
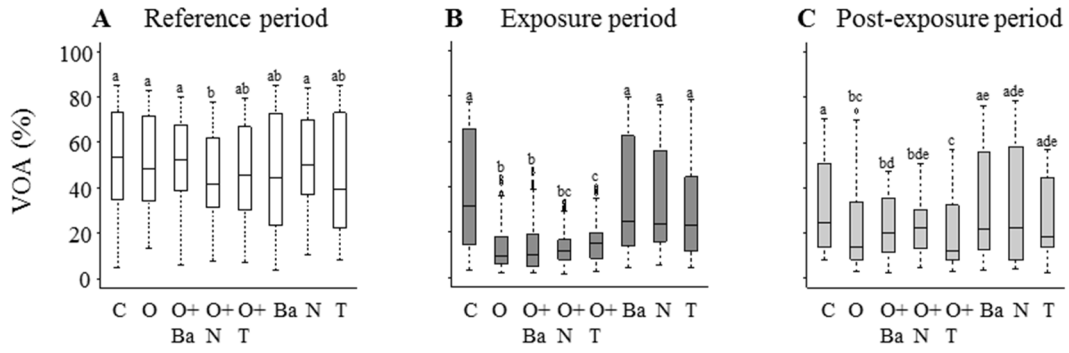
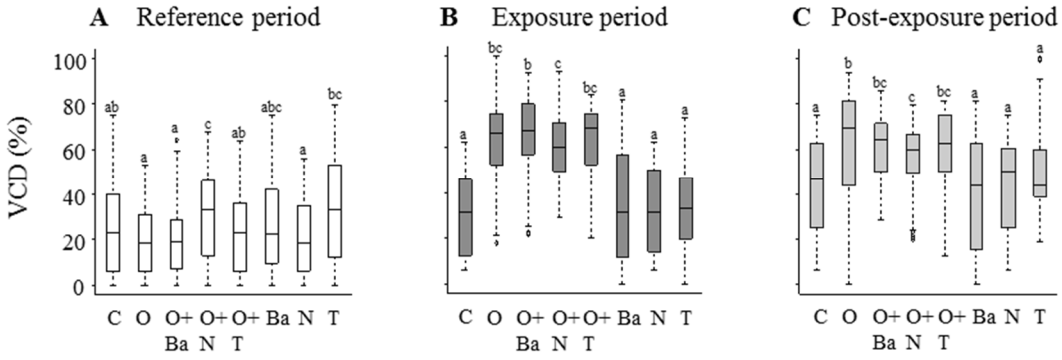


Figure 2. Typical behaviours of clams with or without crude oil in outdoor artificial streams subjected to natural variations. (A1) the typical behaviour of a clam before, during and after exposure to crude oil; (A2) Tw, water temperature (insert, the range of Tw during acclimation, exposure and post exposure, t_{-10} to t_{20}). (B1) the parallel behaviour of a reference clam unexposed to crude oil; (B2) Tw over the same time period (insert, the range of Tw between t_{-10} to t_{20}).



790 Figure 3. Comparison of the average hourly valve-opening amplitude (%) between artificial streams
791 for each period. Mean hourly valve amplitude (%) for 14-16 clams. (A) the reference period (t_{-10} to t_{-1}),
792 (B) the exposure period (t_1 to t_{10}) and (C) the post-exposure period (t_{11} to t_{20}) for each artificial stream
793 (C, control; O, oil; O + Ba; oil and barium; O+N, oil and noise pollution; O+T, oil and turbidity
794 pulses; Ba, barium; N, noise pollution; T, turbidity pulses). Boxplot (n = 240 values; 24 h x 10 days).
795 Different letters indicate significant differences ($p < 0.05$; Dunn Test with Holm adjustment).



796 Figure 4. Comparison of the average hourly valve-closure duration (%) between artificial streams for
797 each period. Mean hourly valve-closure duration (%) for 14-16 clams. (A) the reference period (t_{-10} to
798 t_{-1}), (B) the exposure period (t_1 to t_{10}) and (C) the post-exposure period (t_{11} to t_{20}) for each artificial
799 stream (C, control; O, oil; O + Ba; oil and barium; O+N, oil and noise pollution; O+T, oil and turbidity
800 pulses; Ba, barium; N, noise pollution; T, turbidity pulses). Boxplot (n = 240 values; 24 h x 10 days).
801 Different letters indicate significant differences ($p < 0.05$; Dunn Test with Holm adjustment).

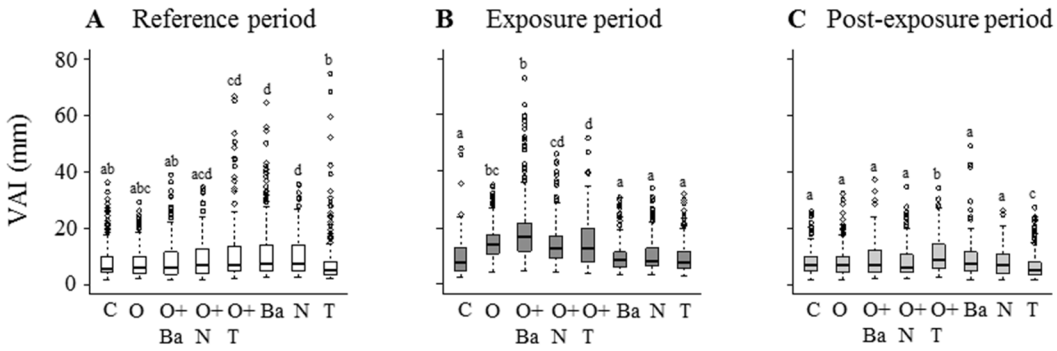


Figure 5. Comparison of the hourly valve agitation index (mm) between artificial streams for each period. Distance travelled by valves during opening states for 14-16 clams during (A) the reference period (t_{-10} to t_{-1}), (B) the exposure period (t_1 to t_{10}) and (C) the post-exposure period (t_{11} to t_{20}) for each artificial stream (C, control; O, oil; O + Ba; oil and barium; O+N, oil and noise pollution; O+T, oil and turbidity pulses; Ba, barium; N, noise pollution; T, turbidity pulses). Boxplot (n = 240 values; 24 h x 10 days). Different letters indicate significant differences ($p < 0.05$; Dunn Test with Holm adjustment).

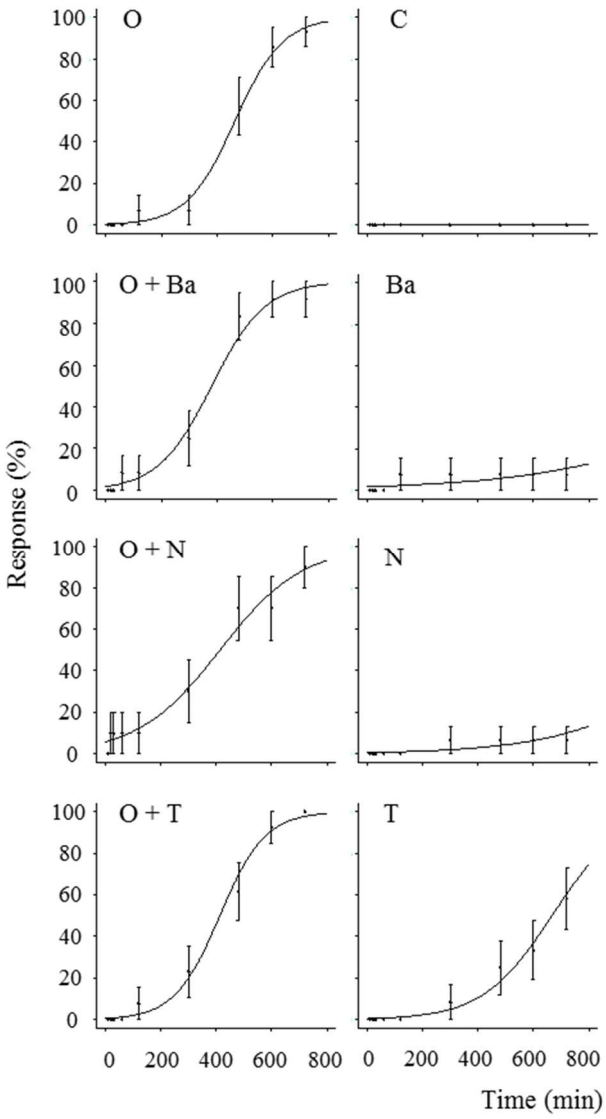


Figure 6. Response time under mono- and multistress conditions. A logistic regression of response time for the clams in each artificial stream (C, control; O, oil; O + Ba; oil and barium; O+N, oil and noise pollution; O+T, oil and turbidity pulses; Ba, barium; N, noise pollution; T, turbidity pulses). The response percentage is estimated for the group of clams using individual responses from the start of exposure (t_0; 03:15 PM; GMT+1) at different integration times (10; 20; 30; 60; 120; 300; 480; 600 and 720 min).

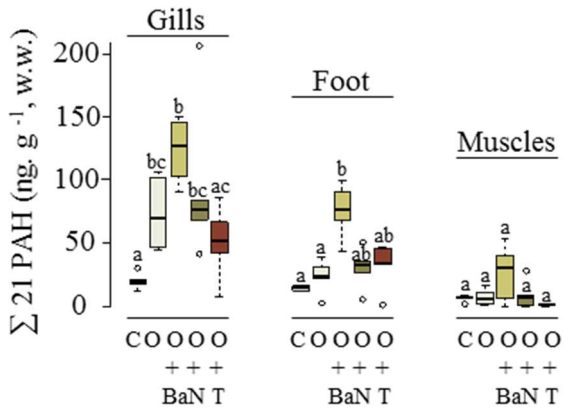


Figure 7. Comparison of PAH concentrations (ng. g^{-1}, w.w.) in clam tissues between artificial streams after 10 days of exposure. Sum of the individual concentrations of the 21 PAHs analyzed in the gills, foot and adductor muscles of 5 clams in each artificial stream (C, control; O, oil; O + Ba; oil and barium; O+N, oil and noise pollution; O+T, oil and turbidity pulses; Ba, barium; N, noise pollution; T, turbidity pulses) after 10 days of exposure (t_{10}). Boxplot (n = 5 clams per artificial stream). Different letters indicate significant differences, independent for each tissue ($p < 0.05$; Conover test with Holm adjustment).