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Feeding inhibition in *Corbicula fluminea* (O.F. Muller, 1774) as an effect criterion to pollutant exposure: Perspectives for ecotoxicity screening and refinement of chemical control

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ABSTRACT

Bivalves are commonly used in biomonitoring programs to track pollutants. Several features, including its filter-feeding abilities, cumulatively argue in favour of the use of the Asian clam (*Corbicula fluminea*) as a biosentinel and an ecotoxicological model. Filtration in bivalves is very sensitive to external stimuli and its control is dictated by regulation of the opening/closure of the valves, which may be used as an avoidance defence against contaminants. Here, we investigate the filter-feeding behaviour of the Asian clam as an endpoint for assessing exposure to pollutants, driven by two complementary goals: (i) to generate relevant and sensitive toxicological information based on the ability of *C. fluminea* to clear an algal suspension, using the invasive species as a surrogate for native bivalves; (ii) to gain insight on the potential of exploring this integrative response in the refinement of chemical control methods for this pest. Clearance rates and proportion of algae removed were measured using a simple and reproducible protocol. Despite some variation across individuals and size classes, 50–90% of food particles were generally removed within 60–120 min by clams larger than 20 mm. Removal of algae was sensitive to an array of model contaminants with biocide potential, including fertilizers, pesticides, metals and salts: eight out of nine tested substances were detected at the $\mu g\,l^{-1}$ or $mg\,l^{-1}$ range and triggered valve closure, decreasing filter-feeding in a concentration-dependent manner. For most toxicants, a good agreement between mortality (96 h − LC_{50} within the range 0.4–5500 $mg\,l^{-1}$) and feeding (2 h − IC_{50} within the range 0.005–2317 $mg\,l^{-1}$) was observed, demonstrating that a 120-min assay can be used as a protective surrogate of acute toxicity. However, copper sulphate was very strongly avoided by the clams (IC_{50} = 5.3 $\mu g\,l^{-1}$); on the contrary, dichlorvos (an organophosphate insecticide) did not cause feeding depression, either by being undetected by the clams' chemosensors and/or by interfering with the valve closure mechanism. Such an assay has a large potential as a simple screening tool for industry, environmental agencies and managers. The ability of dichlorvos to bypass the Asian clam's avoidance strategy puts it in the spotlight as a potential agent to be used alone or combined with others in eradication programs of this biofouler in closed or semi-closed industrial settings.

1. Introduction

Bivalves are commonly used as biosentinels to track pollution (O'Connor, 2002), including contamination by metals (Graney et al., 1983), polycyclic aromatic hydrocarbons (Phelps, 2016), pharmaceuticals (Brandão et al., 2014), and microparasites (Miller et al., 2005), both in freshwaters and estuarine or marine ecosystems (Oehlmann and Schulte-Oehlmann, 2003). Various authors (King et al., 2004; Tracey and Hansen, 1996) consider that bivalves can act as models to address the effects of contaminants in the benthos. In freshwaters, the Asian clam (*Corbicula fluminea*) has been proposed as a suitable sentinel in monitoring programs (Diniz et al., 2007; Phelps, 2016). Studies with this clam have focused on: (i) metal dynamics in the water column (Jou et al., 2009; Liao et al., 2005; Rosa et al., 2014a; Silva et al., 2016a), sediment (Luoma et al., 1990; Villar et al., 1999) and clam tissues (Luoma et al., 1990; Rosa et al., 2014a; Silva et al., 2016a; Villar et al.,

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1999); (ii) biochemical responses following exposure to pharmaceuticals (Brandão et al., 2014), pesticides (Beltran and Pocsidio, 2010; Ramos et al., 2012), and metals (Bigot et al., 2011; Diniz et al., 2007).

The Asian clam is one of the 100 worst invasive species in European freshwaters (DAISIE European Invasive Alien Species Gateway, 2008), having spread massively over the last century from its native distribution in Asia, Africa and Australia into North and South America, as well as Europe (Rosa et al., 2011; Sousa et al., 2008a; Sousa et al., 2008b). Despite the invasive nature and negative ecological impact of the species (Bódis et al., 2014; Ilarri et al., 2012; Sousa et al., 2008a), its ubiquitous distribution supports attempts of using the Asian clam as a sentinel and a model in environmental assessments. Additional advantages include its sedentary habit and filter-feeding abilities, the availability of large populations in invaded areas, and its capacity to bioaccumulate organic and inorganic contaminants (Doherty, 1990; Graney et al., 1983; Rosa et al., 2014a; Silva et al., 2016a; Sousa et al., 2008a). These features cumulatively argue in favour of the use of the Asian clam as a model in monitoring and toxicological studies, enhanced by the ease of its maintenance in the laboratory, its small size and reduced ethical constraints to manipulation. As a disadvantage, it is often more tolerant to stressors than other bivalves (Bidwell et al., 1995; Liao et al., 2008; Oliveira et al., 2015). Provided this is taken into account, the Asian clam may serve as a surrogate laboratory alternative to native bivalves to study the impact of pollutants, since many freshwater bivalves are endangered (Lopes-Lima et al., 2014). This is especially true if one takes advantage from sensitive features in the habit, behaviour and life history of the Asian clam, such as its filter-feeding behaviour, following evidence from other suspension-feeding *taxa* (Caro et al., 2015; Duquesne and Küster, 2010).

Filter- or suspension-feeding is an intricate phenomenon that mainly involves the gills (ctenidia) in filter-feeding bivalves. Ciliary bands generate an inflow of water and suspended particles, with the latter being retained by gill filaments and then transported to the mouth (Jørgensen, 1996). Some particles are rejected on the basis of particle size or density and excreted as pseudofaeces (mucus-bound particles). Filtration is sensitive to external stimuli (Jørgensen, 1996), and its control is dictated by regulation of the opening/closure of the valves (Jørgensen, 1996; Riisgård and Petersen, 2004). This behavioural response (known as gape adjustment) reflects environmental conditions and can be used as an avoidance defence against external stressors, including contaminants (Doherty et al., 1987; Jou et al., 2009). This behaviour has been observed in the Asian clam in response to cadmium, zinc (Doherty et al., 1987) and chlorine (Ham and Peterson, 1994). Additionally, several studies have documented the rapid opening and closure of the valves of *C. fluminea* as a response to inorganic metals (Jou et al., 2009; Tran et al., 2007), which became the basis of an early warning system for chemical contamination (Liao et al., 2005; Tran et al., 2003). Valve closure carries associated costs that can affect survival and growth; in *C. fluminea*, a reduction of up to 90% of the metabolic rate may occur when valves are closed, and long-term closure (> 4–6 h) promotes the onset of anaerobic respiration (Ortmann and Grieshaber, 2003).

The pioneer studies on the Asian clam's valve closing behaviour (see previous paragraph) were somewhat limited, as they were mostly restricted to metals and did not explore the mechanisms behind the observed avoidance response (valve closure behaviour). By focusing on contaminants with different modes of action, additional insight may be obtained about the mechanistic explanation for the avoidance behaviour and associated feeding depression. In a broader perspective, such data may expand our knowledge on the Asian clam's ability to cope with stressors, which is linked to its success as an invader and a biofouler. In a more applied perspective, such toxicological information may also contribute to the refinement of chemical control strategies targeted at the pest, especially in confined industrial settings (Mackie and Claudi, 2009; Rosa et al., 2015; Silva et al., 2016b). In fact, biofouling by the Asian clam in water-dependent industries (power plants, water-treatment plants and irrigation systems) is a serious economic threat worldwide (Pimentel et al., 2005; Rosa et al., 2011), and chemical control is still the method of choice (Rosa et al., 2015), despite the quest for eco-friendly alternatives (Pereira et al., 2016). Still, although a given control agent may have proven efficacy against the pest, the scope for treatment efficiency should be likely improved if the clam's avoidance behaviour to the chemical is known.

The purpose of this study was to investigate the filter-feeding behaviour of the Asian clam as an endpoint for assessing exposure to pollutants, driven by two complementary goals: (i) to generate relevant and sensitive toxicological information based on a integrative trait, using *C. fluminea* as a surrogate for native bivalves; (ii) to gain insight on the potential of exploring this response in the refinement of chemical control methods of the pest. We hypothesize that the avoidance response of valve closure (intermittent or prolonged) in *C. fluminea* depends on the detection of waterborne substances, which trigger a subsequent muscular reaction (valve closure requires contraction of the adductor muscle; Willmer et al., 2004). As such, we expect three possible outcomes of the interaction with pollutants: (a) contaminant is detected and triggers valve closure, leading to feeding depression or inhibition; (b) contaminant is undetected and does not trigger valve closure; (c) contaminant interferes with chemo-sensing, neurotransmission, or muscle contraction and thus inhibits the behavioural avoidance response. To test this, we followed a tiered experimental approach: first, we optimized the procedure for a feeding trial based on the clearance method (Riisgård, 2001); second, nine model substances differing in mode of action (including a neurotoxicant, a paralysing agent, a molluscicide, metals and others) were tested under the optimized experimental design. This allowed testing the sensitivity of feeding inhibition in the Asian clam, by comparing it to other endpoints and test organisms, as well as finding out which chemicals are able to bypass the clam's avoidance strategy.

2. Material and methods

2.1. Clam and microalgae maintenance

Asian clams (shell length between 10 and 25 mm) were collected in a shallow freshwater ditch near Mira (in the littoral centre of Portugal; N 40° 24′ 55", W 8° 45′ 04"), which holds a well-established population with densities reaching more than 2000 ind m^{-2} (Rosa et al., 2014b). The clams were collected by sieving the sediment through a 5 mm mesh bag and sorted by shell size class (in 5-mm intervals). They were transported to the laboratory in plastic buckets filled with local water, and gradually acclimated for one week to dechlorinated tap water. Clams were kept in the laboratory in 20-l plastic vessels with dechlorinated tap water under permanent aeration at controlled temperature (20 ± 2 °C) and photoperiod (16 h^L:8 h^D) regimes. Twice a week, water was renewed and clams were fed an *ad libitum* ration of green microalgae (*Raphidocelis subcapitata*). Dead animals were regularly removed from the vessels. Prior to any experiments, the performance of the batch (survival and feeding) was followed during a two-week quarantine period to confirm good health of the clams.

Concentrated suspensions of the green microalga *R. subcapitata* were used as a source of food for maintaining *C. fluminea*, as well as a source of suspended particles for clearance assays (see below). These suspensions were obtained by centrifugation of non-axenic batch cultures, reared in the laboratory in Woods Hole MBL medium (Stein, 1973) under permanent fluorescent light, at 20 ± 2 °C. New batch cultures were started by inoculating microalgae harvested during the exponential growth phase in fresh medium; cultures were ready for centrifugation after one week, in the latter stage of the exponential growth phase. Algal density of the concentrated suspensions was adjusted spectrophotometrically (λ = 440 nm) on the basis of a previously established calibration curve between optical density and cell density.

2.2. Stage 1–Optimization of clearance assay

2.2.1. Rationale and pre-test calibration

Feeding trials were based on the decline in the concentration of suspended particles (microalgae) through time caused by the filter-feeding activity of the clams, using the clearance method (Riisgård, 2001). Briefly, this method involves taking water samples at fixed intervals and measuring particle concentration (density of microalgae in our case). Although the method presents some disadvantages (Riisgård, 2001), it is simple and reliable enough for the purpose of our study, which intends to use feeding inhibition as a screening tool of the short-term behavioural effects of contaminants. This method is an indirect way to assess filtration rates of bivalves (Riisgård, 2001).

Optical density at 440 nm (OD_{440nm}) was used as a surrogate for microalgal cell density in clearance assays. To assure good correspondence between the OD_{440nm} measurements and cell density, we established a specific calibration curve for this study. Optical density measurements were performed in a Shimadzu UV-1800 spectrophotometer (Shimadzu Scientific Instruments) and microscopic cell counts were carried out in a Neubauer chamber. Samples covered an OD_{440nm} range between 0.010 and 0.334, and the resulting calibration curve (Eq. (1)) achieved a good fit ($R^2 = 0.99$).

$$Cell\ density(cells\ ml^{-1}) = 6931 + 23179166 \times OD_{440nm} - 9972459 \times OD_{440nm} \quad (1)$$

2.2.2. General procedure and calculations

The day before setting up each set of experimental units and treatments (see sections 2.2.3 and 2.3.3), clams were transferred from culture buckets into glass vessels filled with 500 ml of dechlorinated tap water. They were fed once with a single *R. subcapitata* ration of 8×10^4 cells ml^{-1} (Bidwell et al., 1995) and starved for the next 24 h (until the experiment began). After this period, clams from this group were randomly assigned to each experimental unit, which consisted of 1 clam in a 200-ml glass vessel filled with 100 ml dechlorinated tap water. Taking into account the area of the vessel, this corresponded to a density of 500 individuals m^{-2}, which is a common field density (Pereira et al., 2017; Rosa et al., 2014b; Sousa et al., 2008b). Clams were left to acclimate to test conditions for 1 h with aeration, before adding food (suspended algae); this was found to be sufficient to prevent negative reactions of the clams to manipulation.

Experiments started with the addition of 2 ml of concentrated microalgal suspensions to each test vessel (see section 2.2.1), which corresponded to an initial cell density of approximately 3.5×10^6 cells ml^{-1} and an initial OD_{440nm} range of 0.141–0.179. A blank control (vessel with no clam) was always also included to account for eventual growth of the microalgae during the test period. After adding the suspended algae to the vessels, a sample was immediately taken as the initial OD_{440nm} reading (t_0). Consecutive OD_{440nm} measurements were taken every 10 min for 2 h (t_{10} to t_{120}). During this period, clams were normally able to clear the water; furthermore, extending the test duration further (> 2 h) led to an increased production of pseudofaeces that confounded OD readings.

Using Eq. (1), OD_{440nm} measurements were converted to microalgal cell density, which allowed the calculation of the proportion of algae removed (Eq. (2)), and clearance rates (Riisgård, 2001). Because the decay in OD_{440nm} was not constant, nor did it start at the same point in time for all individuals (see example in Suppl. Fig. S1), we calculated maximum clearance rates by narrowing the analysis to the time frame where filtration was maximum (i.e., maximum slope in the decay, see Suppl. Fig. S1). In this way, the *per capita* maximal clearance rate (Eq. (3)) and the weight-specific maximal clearance rate (Eq. (4)) were calculated using 20-min periods (i.e., from three consecutive readings). Calculations for the proportion of algae removed were corrected for algal growth (in the absence of clams) using data from the blanks (see above). This was not necessary for maximal clearance rates, due to the narrow time frame.

$$Algae\ removal\ (\%) = 100 \times \frac{V \cdot (C_i - C_f) - V \cdot (B_i - B_f)}{V \cdot C_i} \quad (2)$$

$$Per\ capita\ CR_{max}\ (ml\ min^{-1}\ clam^{-1}) = \frac{V}{n \cdot \Delta t} \times \ln \frac{C_i}{C_f} \quad (3)$$

$$Weight{-}specific\ CR_{max}\ (ml\ min^{-1}\ g^{-1}) = \frac{V}{DW \cdot \Delta t} \times \ln \frac{C_i}{C_f} \quad (4)$$

where V corresponds to test volume (100 ml); C_i and C_f represent initial and final density of microalgae in the test vessel (clam present), respectively; B_i and B_f correspond to initial and final density of microalgae in the blank treatment (clam absent), respectively; n stands for the number of clams in the test vessel ($n = 1$); Δt is the corresponding time frame (120 min in Eq. (2), and 20 min for Eq. (3) and (4)); finally, DW represents the dry weight of each clam (in g), estimated through the length-weight relationship established for by Rosa et al. (2014b) for the winter season (Eq. (5)).

$$DW = 165 \times L^{2.49} \quad (5)$$

2.2.3. Influence of clam size class on clearance rates and removal of algae

The purpose of this assay was to test the experimental procedures and to compare the clearance performance of three size classes of clams (15–19, 20–24 and 25–30 mm in shell length). No toxicants were used at this stage and the whole experiment was carried out using the same batch of clams (i.e., clams that were collected in the same day and kept in the laboratory in the same conditions). The experiment was run in blocks, because we could not carry out measurements in all the experimental units at the same time. Blocks differed in the time of the day the experimental units were tested and in the batch of microalgal suspensions used. Ten blocks were used, with two blocks being measured each day. Each block consisted of four experimental units (vessel with an individual clam) assigned to each treatment level (clam size classes), plus three blank controls (vessels with no clams): 4 vessels × 3 treatments + 3 blanks = 15 experimental units. The decrease in algal concentration was monitored in the 150 experimental units (10 blocks × 15) during 120 min, following the above procedures (see section 2.2.2). For each experimental unit, we quantified removal of algae as well as the *per capita* and weight-specific maximal clearance rates (Eqs. (2)–(3)).

Removal of algae and clearance rates were analysed as a blocked design, testing for differences across block (random factor) and size class (fixed factor), assuming no block × size class interaction (reduced model). This allowed removing the random variation due to blocks (e.g., caused by different algal suspensions or time of the day) from the error term. Even though we assumed no interaction, it was formally tested using a full ANOVA model. Prior to analysis, clearance rates were *log*-transformed to accommodate the ANOVA assumptions of normality and homoscedasticity. No transformation was successful for the removal of algae; untransformed data were used and ANOVA was assumed to be robust enough to cope with such minor deviations. When the effect of size class was significant, a Tukey test was applied as a way to compare the different size classes. A significance level of 0.05 was used in all analyses.

2.3. Stage 2–Toxicity screening

2.3.1. Chemicals

We tested the toxicity of nine chemicals (Table 1), covering model chemicals (for which there is previous ecotoxicological information) and biocides that have been traditionally considered as suitable control chemicals against the Asian clam. The array of toxicants also covered different molecular structures and expected mechanisms of toxic action.

Table 1
Chemicals and respective concentrations used in stage 2 (toxicity screening), in acute (lethal) and clearance (feeding inhibition) assays with Asian clam (*C. fluminea*). Additional information on the chemicals (chemical class, mode of action) is reported as "Notes".

Chemical substance			Acute assay		Clearance assay	
Name and supplier	Identifier	Notes	mg l^{-1}	μM	mg l^{-1}	μM
Ammonium nitrate NH_4NO_3 (Sigma-Aldrich)	CAS 6484-52-2 EC 229–347-8	Fertilizer; membrane disruptor and respiration inhibitor	59.3 to 300[a]	741–3748[a]	6.38–204	79.6–2548
Cadmium sulphate $CdSO_4$ (Sigma-Aldrich)	CAS 10124-36-4 EC 233–331-6	Non-essential metal; oxidant; inflammatory stressor	0.179–3.00	0.858–14.4	0.122–0.927	0.586–4.45
Copper sulphate $CuSO_4{\cdot}5H_2O$* (Sigma-Aldrich)	CAS 7758-99-8 EC 231–847-6	Essential metal; oxidant; general enzyme inhibitor	12.6–48.3	78.7–302	0.0024–7.53	0.015–47.2
Dichlorvos $C_4H_7Cl_2O_4P$ (Sigma-Aldrich)	CAS 62–73-7 EC 200–547-7	Organophosphorous insecticide; ChE inhibitor	1.21–12.7[b]	5.48–57.6[b]	0.0035–5.81	0.016–26.3
Niclosamide $C_{13}H_8Cl_2N_2O_4$ (Sigma-Aldrich)	CAS 50–65-7 EC 200-056-8	Molluscicide; mitochondria disruptor	0.08–3.0[a]	0.245–9.17[a]	0.016–0.489	0.048–1.53
Potassium chloride KCl (VWR)	CAS 7447-40-7 EC 231-211-8	Salt; membrane disruptor and paralysing agent	45–850[a]	604–11,402[a]	18.8–600	252–8048
Potassium dichromate $K_2Cr_2O_7$ (Panreac)	CAS 7778-50-9 EC 231–906-6	Essential metal; oxidant	3.07–35.0	10.4–119	8.00–151	27.2–514
SDS $C_{12}H_{25}NaO_4S$ (Sigma-Aldrich)	CAS 151-21-3 EC 205–788-1	Surfactant; Cell membrane disruptor	3.79–121	13.1–421	2.00–64.0	6.94–222
Sodium chloride NaCl (Merck)	CAS 7647-14-5 EC 231–598-3	Salt; natural osmoregulation stressor	3950–20,000	67,591–342,231	387–4060	6622–69,473

[a] Data from Rosa et al. (2015).
[b] Data from Silva et al. (2016b).
* Mass concentrations of copper sulphate refer to $CuSO_4$ and not $CuSO_4{\cdot}5H_2O$.

Laboratory-grade chemicals (> 98% w/w purity) were used to prepare concentrated stock solutions in dechlorinated tap water. All reported toxicant concentrations and LC_{50} or IC_{50} values (see below) refer to the "parent" compound itself, except for copper sulphate, for which concentrations refer to $CuSO_4$ and not to $CuSO_4{\cdot}5H_2O$. Due to the lack of satisfactory analytical methods for some substances, aqueous concentrations of the toxicants were not confirmed for the sake of coherence and cost-effectiveness. Thus, all reported concentrations are nominal. In mortality tests (see section 2.3.2.), some degradation is expected for the less persistent chemicals (dichlorvos and niclosamide); still, their half-life is > 96 h (Hazardous Substances Data Bank, https://toxnet.nlm.nih.gov/). In feeding trials (see section 2.3.3), most toxicants are likely to adsorb to particulate matter (namely algal cells), so this will constitute a major route of exposure. Because the purpose of our study was mainly exploratory, the use of nominal concentrations does not impair comparisons concerning the sensitivity of the endpoints (feeding vs. mortality) or the relative toxicity of substances.

2.3.2. Mortality tests

In order to assess the sensitivity of feeding inhibition as a toxicity endpoint, lethality data for the tested substances was needed for comparative purposes. Some information was retrieved from other sources (Rosa et al., 2015; Silva et al., 2016b), but most acute toxicity data had to be determined, as described below.

Mortality tests were conducted under static conditions for 96 h, in controlled temperature (20 ± 2 °C) and photoperiod (16 h^L: 8 h^D) regimes, and under permanent aeration (dissolved oxygen levels kept above 7 mg l^{-1}). We used clams from the same batch, between 15 mm and 25 mm shell length. Clams were exposed to a control treatment (dechlorinated tap water) and a geometric range of chemical concentrations (Table 1) established by direct spiking of the test vessels from concentrated stock solutions. The experimental design was similar to that of Rosa et al. (2015), with three replicate vessels being assigned to each treatment level, and each replicate consisting of 10 clams in 500 ml of the respective test solution. Clam mortality was assessed daily, by visually checking their siphoning activity; inactive clams were scored as dead if they did not offer resistance when valves were forced to open with a blunt dissection needle. Dead clams were immediately discarded.

Mortality was modeled as binomial data using a special case of the *log*-logistic dose-response model, where the asymptotes of the curve are fixed to be 0 (all organisms are alive) and 1 (all organisms are dead), following the rationale of Ritz (2010). This allowed estimating the median Lethal Concentrations (LC_{50}) for each toxicant and 95% confidence intervals (delta method), using the *drc* package (Ritz and Streibig, 2005) for R version 3.3.2 (R Core Team, 2012). Data from Rosa et al. (2015) and Silva et al. (2016b) were re-analysed in this way for consistency purposes.

2.3.3. Feeding inhibition tests

The purpose of this array of tests was to assess the effect of the toxicants in the filter-feeding activity of *C. fluminea*, using an optimized version of the described clearance assay. For logistical reasons, independent experiments were performed for each toxicant, but each experiment used the same batch of clams (i.e., clams that were collected in the same day and kept in the laboratory in the same conditions). A single size class of clams was used (20–24 mm) and the test volume was set to 100 ml. The final range of toxicant concentrations (Table 1) was set up on the basis of the results from mortality assays and preliminary range-finding tests; the goal was to select a range of concentrations eliciting a depression in clearance of algae by the clams.

The experiment was run in blocks, using the previously described rationale and procedures (see section *2.2.3.*) with minor adjustments. Four blocks were used, with two blocks measured out each day. Each block consisted of five experimental units (vessel with an individual clam) plus a blank control (vessels with no clam) assigned to each treatment level (control + 6 toxicant concentrations – Table 1): (5 test vessels + 1 blank) × 7 treatments = 42 experimental units. Blank controls were important to account for the potential growth of algae during the test period (see section 2.2.2), but also to account for any hypothetical effects of the toxicant on algal growth. The decrease in algal concentration was monitored in the 168 experimental units (42 × 4 blocks) during 120 min (as in section 2.2.2), and removal of algae (Eq. (2)) was quantified in each experimental unit.

Data of removal of algae from each experiment were analysed as a blocked design, testing for differences across block (random factor) and toxicant concentration (fixed factor), assuming no block × size class interaction, using a type III sum of squares. When the effect of toxicant concentration was significant, a Dunnett test was applied as a way to compare each concentration relative to the control and to estimate the

lowest observed effect concentration (LOEC). In parallel, median Inhibitory Concentrations (IC_{50}) were estimated per block after fitting a three-parameter log-logistic model (Ritz, 2010), using the *drc* package (Ritz and Streibig, 2005) for R version 3.3.2 (R Core Team, 2012). We modeled the removal of algae as a continuous variable. Average IC_{50} for each contaminant (along with corresponding confidence intervals) was calculated as the geometric mean of the IC_{50} estimates for the individual blocks. Correlation analysis (Pearson's *r*) was used to establish an association between mean IC_{50} and LC_{50} values (after a log_{10} transformation).

3. Results

3.1. Stage 1–Optimization of clearance assay

In general, clams started filtering as soon as food was added and removed a large proportion of algae within the first 30–45 min. After this intense filtration period, the removal of algae was reduced and the absorbance curve reached an asymptote (Suppl. Fig. S1). Therefore, in most cases, a period of intense filtering (maximum filtration) could be identified, usually occurring within the initial 60 min. However, a few clams responded later, overcoming a more prolonged initial lag-phase; in this case, the removal of algae continued until the end of the 120-min period. High variability was observed, both among and within blocks (Fig. 1). A few clams did not filter at all or filtered poorly within the observation period (Suppl. Fig. S1). Such cases (< 20% removal of algae) were removed from further analysis, in a total of 4 for the smallest size class (15–19 mm), 1 for intermediate-size clams (20–24 mm), and 3 for the largest ones (25–30 mm). Because we had no accurate method to determine the health of the individual clams, we assumed that clams filtering poorly were unhealthy and used this criterion henceforth for clams that were not exposed to contaminants.

Significant differences were found among size classes, pooling over blocks, for proportion of algae removed ($F_{(2,\ 100)} = 73.5$, $P < 0.001$), *per capita* CR_{max} (reduced ANOVA model, $F_{(2,\ 100)} = 46.7$, $P < 0.001$), and weight-specific CR_{max} ($F_{(2,\ 100)} = 4.48$, $P = 0.014$). Significant interactions between block and size class were found for *per capita* (full ANOVA model, $F_{(18,\ 82)} = 2.08$, $P = 0.013$) and weight-specific CR_{max} ($F_{(18,\ 82)} = 2.33$, $P = 0.005$), but not for removal of algae ($F_{(18,\ 82)} = 1.16$, $P = 0.314$) – see Suppl. Fig. S2. Since blocks were random artificial units (experimental repetitions), these interactions were interpreted as having no biological significance; thus, analyses proceeded with the reduced model (blocked design, assuming no interaction). More algae were significantly removed by intermediate and larger clams (Tukey test, $P < 0.05$), compared to smaller clams (Fig. 1). *Per capita* CR_{max} was significantly different among all size classes (Tukey test, $P < 0.05$) with clearance rates increasing with clam size (Fig. 1). Weight-specific CR_{max} inverted this tendency (Fig. 1), and significant differences were found only between the smallest and the largest size classes (Tukey test, $P < 0.05$).

Table 2
Toxicity benchmarks estimated for acute (LC_{50}) and feeding assays (IC_{50} and LOEC) with the Asian clam (*C. fluminea*), respectively. LC_{50} and IC_{50} are shown along with corresponding 95% confidence intervals. ND stands for not determined.

	Acute assay	Clearance assay	
Chemical	96 h – LC_{50} (mg l^{-1})	2 h – IC_{50} (mg l^{-1})	LOEC (mg l^{-1})
Ammonium nitrate	202 (189–216)	22.8 (14.6–35.7)	12.8
Cadmium sulphate	2.89 (1.67–4.11)	0.631 (0.347–1.15)	0.618
Copper sulphate	9.52 (6.04–13.0)	0.00529 (0.0022–0.013)	0.0241
Dichlorvos	2.40 (2.15–2.66)	ND	1.32*
Niclosamide	0.467 (0.397–0.538)	0.195 (0.149–0.256)	0.250
Potassium chloride	353 (234–472)	132 (73.5–235)	75.0
Potassium dichromate	10.9 (8.62–13.2)	35.8 (18.8–68.4)	25.9
SDS	38.0 (25.4–50.6)	29.2 (8.51–100)	32.0
Sodium chloride	5501 (4853–6151)	2317 (1697–3164)	2539

* Stimulation effect.

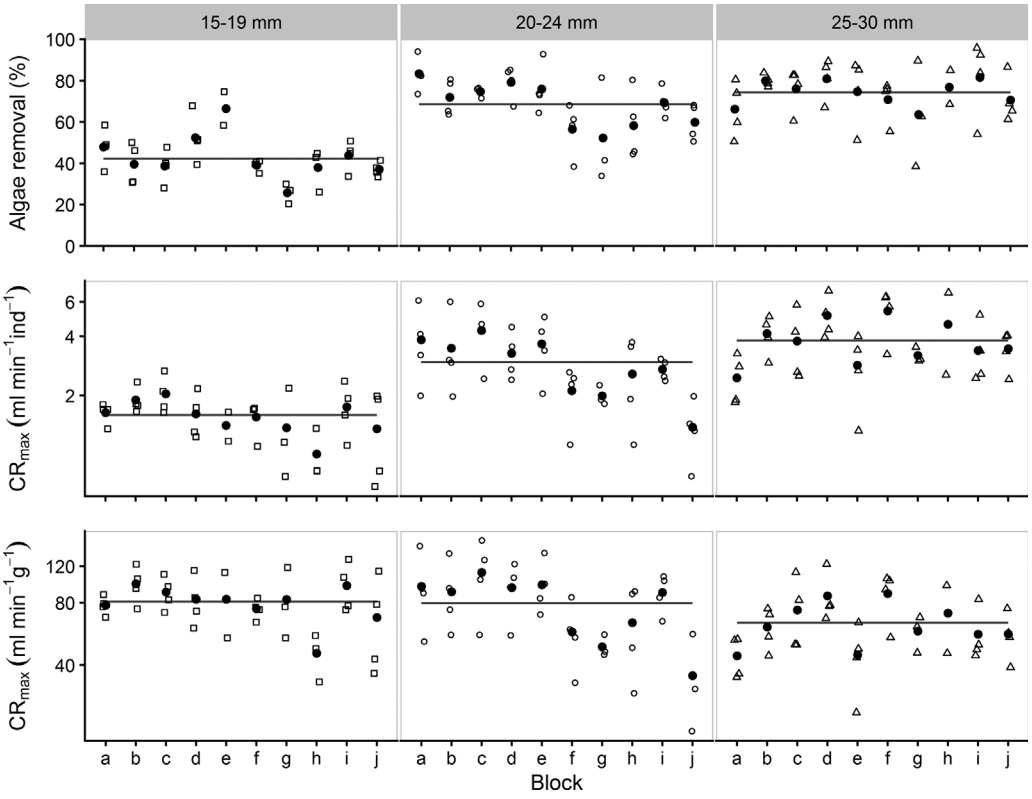

Fig. 1. Filtration parameters of Asian clams (*Corbicula fluminea*) fed with suspensions of green microalgae (*Raphidocelis subcapitata*): proportion of algae removed (top), *per capita* maximum clearance rate (middle, *log* scale), and weight-specific maximum clearance rate (bottom, *log* scale). The experiment was repeated 10 times (blocks a–j) for a set of small (15–19 mm), intermediate (20–24 mm) and large (25–30 mm) clams in a randomised block design. Empty symbols represent individual clams and black circles show the mean within each block; horizontal lines represent the overall mean for each size class, pooling all blocks.

Table 3
ANOVA summary table for the feeding assays conducted for each contaminant, using a blocked design. Test (*F*) statistics (and degrees of freedom) are shown; bold figures depict statistically significant differences across concentrations. $MS_{residual}$ stands for the mean squares (= variance) of the error term in the ANOVA.

Contaminant	Source of variation	Test statistic	*P*
Ammonium nitrate	Block	$F_{(3,\ 130)} = 4.78$	0.003
	Concentration	$F_{(6,\ 130)} = 54.3$	< **0.001**
	Residual	$MS_{residual} = 238$	
Cadmium sulphate	Block	$F_{(3,\ 129)} = 8.69$	< 0.001
	Concentration	$F_{(6,\ 129)} = 19.8$	< **0.001**
	Residual	$MS_{residual} = 258$	
Copper sulphate	Block	$F_{(3,\ 127)} = 1.34$	0.263
	Concentration	$F_{(6,\ 127)} = 144$	< **0.001**
	Residual	$MS_{residual} = 105$	
Dichlorvos	Block	$F_{(3,\ 129)} = 20.9$	< 0.001
	Concentration	$F_{(6,\ 129)} = 7.53$	< **0.001**
	Residual	$MS_{residual} = 264$	
Niclosamide	Block	$F_{(3,\ 129)} = 13.9$	< 0.001
	Concentration	$F_{(6,\ 129)} = 92.6$	< **0.001**
	Residual	$MS_{residual} = 182$	
Potassium chloride	Block	$F_{(3,\ 129)} = 1.23$	0.301
	Concentration	$F_{(6,\ 129)} = 48.1$	< **0.001**
	Residual	$MS_{residual} = 322$	
Potassium dichromate	Block	$F_{(3,\ 129)} = 5.12$	0.002
	Concentration	$F_{(6,\ 129)} = 83.9$	< **0.001**
	Residual	$MS_{residual} = 116$	
SDS	Block	$F_{(3,\ 130)} = 2.93$	0.036
	Concentration	$F_{(6,\ 130)} = 19.2$	< **0.001**
	Residual	$MS_{residual} = 326$	
Sodium chloride	Block	$F_{(3,\ 130)} = 3.12$	0.029
	Concentration	$F_{(6,\ 130)} = 34.6$	< **0.001**
	Residual	$MS_{residual} = 306$	

3.2. Stage 2–Toxicity screening

All toxicants were lethal to *C. fluminea* in a concentration-dependent fashion (Suppl. Fig. S3), generally at levels within the $mg\,l^{-1}$ range (Table 2). Niclosamide was the most toxic, causing substantial mortality under $1\,mg\,l^{-1}$. LC_{50} values for metals (cadmium sulphate, copper sulphate, and potassium dichromate) and the insecticide dichlorvos were within the range of $1–11\,mg\,l^{-1}$. SDS occupied an intermediate position in terms of toxicity ($LC_{50} = 38\,mg\,l^{-1}$), whereas ammonium nitrate and potassium chloride were much less toxic ($LC_{50} > 100\,mg\,l^{-1}$). Sodium chloride was the least toxic, with its LC_{50} being at the $g\,l^{-1}$ level (Table 2).

All tested contaminants also significantly affected the feeding behaviour of *C. fluminea* (Table 3), and all but one toxicant (dichlorvos) reduced the proportion of algae removed by the clams in a clear concentration-dependent pattern (Fig. 2). We visually confirmed that this was due to a reduction in the filter-feeding activity of the clams, which closed their valves for some periods when exposed to the chemicals. For some contaminants, clams remained closed during the entire assay at higher concentrations. Dichlorvos was the exception to this feeding inhibition pattern, as its effect was actually slightly stimulatory. No obvious feeding inhibition was observed up to $5.8\,mg\,l^{-1}$ of dichlorvos, a value more than two times higher than the LC_{50}; as a result, no IC_{50} could be determined (Suppl. Fig. S4). In follow-up experiments, we exposed Asian clams to dichlorvos concentrations as high as $96\,mg\,l^{-1}$, which caused mortality without causing feeding inhibition.

For most chemicals, filtration was more sensitive than mortality – compare LC_{50} vs. IC_{50} values in Table 2 and Fig. 2. This was especially notable for copper sulphate, to which the clams responded very promptly by reducing filtering activity from $2\,\mu g\,l^{-1}$ upwards (Fig. 2); in fact, the IC_{50} for this metal was 1800 times lower than the 96-h LC_{50} (see also Fig. 3). SDS, potassium chloride and potassium dichromate inhibited feeding at concentrations close to those causing mortality (or even higher, in the case of potassium dichromate – Fig. 2); in these cases, feeding can be considered at least as sensitive as mortality.

Dichlorvos (no feeding inhibition) and copper sulphate (feeding inhibition at very low concentrations) were the exceptions to what was an otherwise good correspondence between filtration and mortality endpoints (Fig. 3). Indeed, a strong and highly significant correlation between *log* LC_{50} and *log* IC_{50} ($r = 0.94$, $P = 0.001$) was found, when both dichlorvos and copper sulphate were not considered. When copper sulphate was included in the analysis, the correlation became weaker ($r = 0.78$, $P = 0.021$) – see also Fig. 3. Dichlorvos could not be included in the analysis, because no IC_{50} could be determined.

4. Discussion

As a behavioural trait, filter-feeding is an integrated phenotypic response, reflecting biochemical, cellular and physiological changes (Willmer et al., 2004); however, the consequences of feeding depression or inhibition go beyond that, and are likely to propagate to the population level (Agatz et al., 2013; Castro et al., 2004; Duquesne and Küster, 2010). Behavioural regulation of filter-feeding in Asian clam, via intermittent or prolonged valve closure (i.e., gape regulation), represents a trade-off between protection from the stressor and physiological performance, since long periods of valve closure decrease feeding, metabolic rate and promote anaerobic respiration (Ortmann and Grieshaber, 2003). Hence, there is great relevance in the inclusion of this type of complex, yet sensitive response in ecotoxicological assessments; also, this makes them interesting targets for the improvement of chemical control methods of the Asian clam in fouled systems. This dual goal was successfully achieved in our study.

We showed that filter-feeding can be measured using a simple and reproducible protocol, which allowed estimating the normal feeding activity of the Asian clams. *Per capita* clearance rates (CR_{max}) varied between less than $2\,ml\,min^{-1}\,ind^{-1}$ (in smaller individuals) to slightly over $6\,ml\,min^{-1}\,ind^{-1}$ (in larger individuals); weight-specific CR_{max} ranged from 40 to $120\,ml\,min^{-1}\,g^{-1}$. Values in the literature are highly variable (with high maxima; Beaver et al., 1991) because experimental conditions vary. However, records that are comparable in terms of experimental conditions are within the same order of magnitude as the ones we report: $0.7–5.8\,ml\,min^{-1}\,ind^{-1}$ (Haines, 1979; Buttner and Heidinger, 1981; Cohen et al., 1984; Way et al., 1990) and $25–342\,ml\,min^{-1}\,g^{-1}$ (Buttner and Heidinger, 1981; Liu et al., 2009). The clearance rates of bivalves are often higher than those of other filter-feeders (e.g., DeMott, 1982; Marroni et al., 2017), and *C. fluminea* is a more potent filter-feeder than other invasive bivalves (Sylvester et al., 2005). The validation of the Asian clam as a powerful filter-feeder even under a highly controlled experimental setting is critical to support the use of the inhibition of its filtration rates as a suitable ecotoxicological endpoint in a reproducible test system.

The first experimental phase (stage 1) was also useful to support a series of changes that were implemented in the definitive assay with the toxicants. We observed higher variability in the clearance rates when compared to the removal of algae, which led to significant block and size class interactions for the *per capita* and weight-specific CR_{max}. Although maximal clearance rates are important in ecological and physiological studies (Riisgård, 2001; Riisgård and Petersen, 2004), the estimates of removal of algae responded more consistently (see Fig.S1) and are more intuitive to non-specialists (algal removal is conceptually and ecologically self-evident). Weighing all together, the removal of algae became the choice of toxicological endpoint for the definite trial and it is our recommendation for toxicity screening purposes. Because all that is needed for estimating this parameter is the optical density at t_0 and t_{120}, a simplified approach could be the measurement only of these two time points. However, we do not recommend this, because the time-integrated response (i.e., measurements at 10 min intervals) of the individual clams allows quality control checks of clam performance, correcting for interferences (e.g., pseudofaeces) and discarding potential outliers. Additionally, and as expected, results from stage 1 revealed positive size-dependence in the *per capita* CR_{max} and in algal removal,

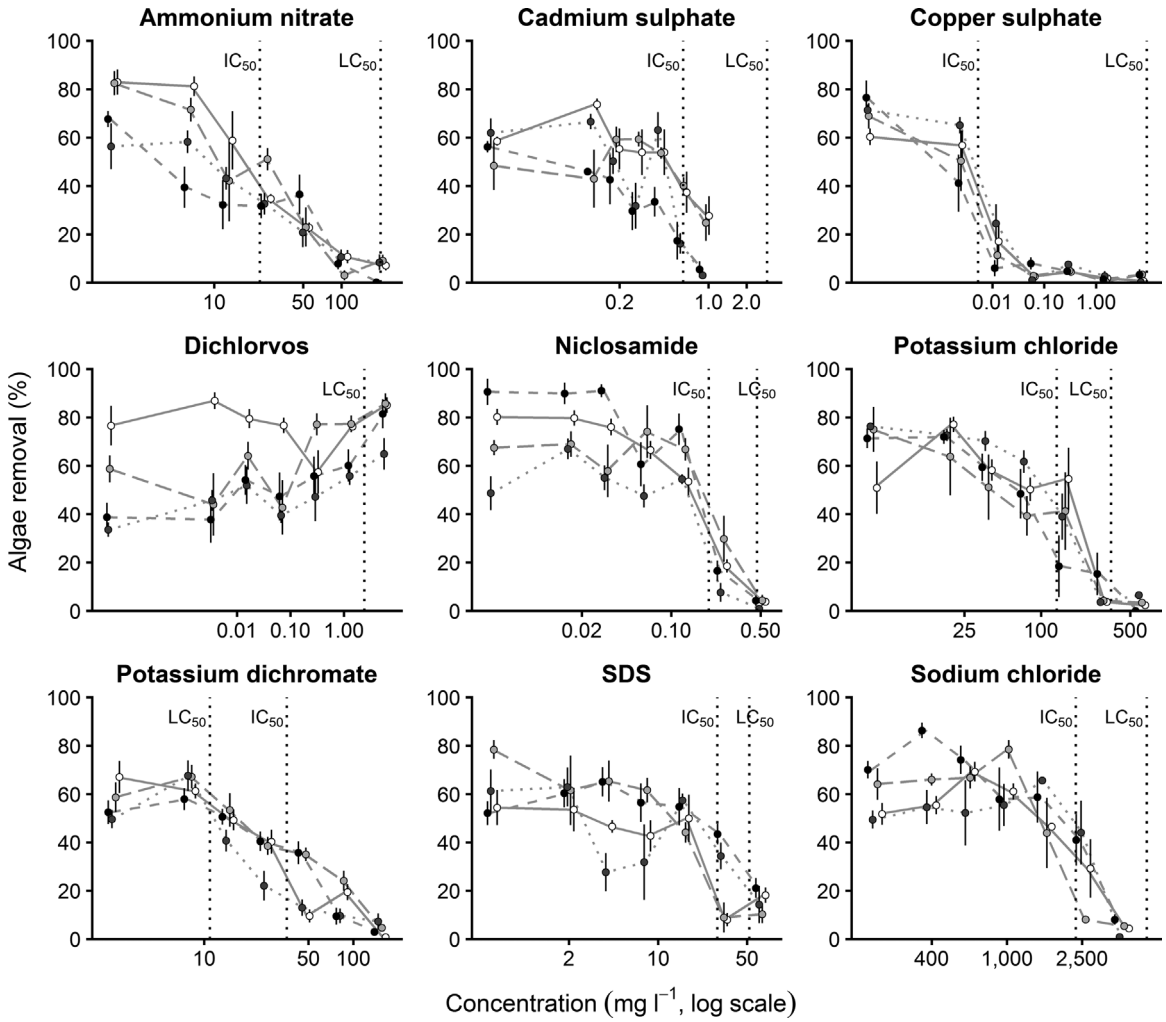

Fig. 2. Asian clam (*Corbicula fluminea*) filtration patterns along a geometric series of concentrations of selected chemicals. Each chemical was tested in a randomised block design, and each of the four blocks is represented by a different symbol × line combination (lines were added for visualization purposes and do not represent any fitted model – see Suppl. Fig. S4). Error bars represent the standard error for individual clams within each block and concentration (n = 5). Vertical dotted lines represent the feeding inhibition (2 h − IC_{50}, this assay) and mortality (96 h − LC_{50}) benchmarks.

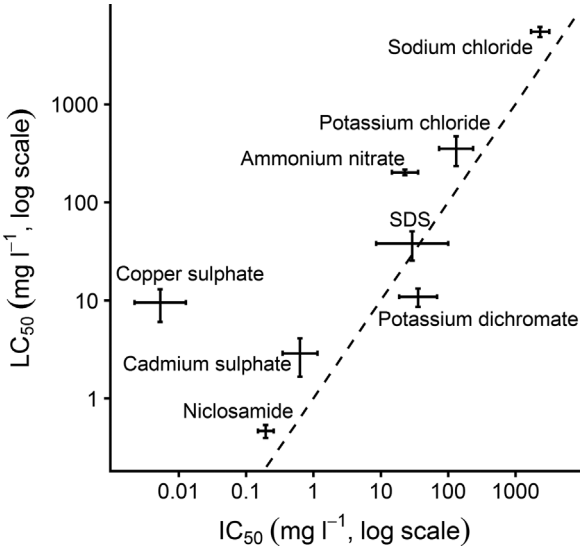

Fig. 3. Association between mortality (96 h − LC_{50}) and feeding inhibition (2 h − IC_{50}) endpoints for the tested chemicals. Error bars represent estimated 95% confidence intervals (delta method) for both endpoints. The dashed line represents a theoretical *log-log* match between LC_{50} and IC_{50} (i.e. slope = 1 and intercept = 0). Dichlorvos is not represented because no reliable IC_{50} could be estimated.

but also a greater investment (effort per unit mass) in filtration in the small and intermediate size classes. The intermediate size class (20–24 mm) of clams displayed high and consistent clearance rates and was therefore our choice for the definite assay, although smaller individuals are often more common in field populations (Pereira et al., 2017; Sousa et al., 2008b). Additional decisions on experimental design concerned the adjustment of the number of blocks and experimental units per block (see Material and Methods), mostly due to logistical reasons.

Results from stage 2 demonstrated that the filter-feeding behaviour of *C. fluminea* is a sensitive endpoint to contaminant exposure, as previously shown by other authors, mostly for metals (see Introduction for a review). Our approach, however, allowed broadening to other contaminant classes and demonstrated that this is a general response, highlighting the relevance and usefulness of filter-feeding behaviour of *C. fluminea* as a simple screening tool for ecotoxicologists or environmental managers. Indeed, clams were able to detect toxicants that subsequently triggered valve closure, eliciting a concentration-dependent response in all but one chemical (dichorvos). In one case (copper sulphate), this avoidance response was very strong, occurring at very low concentrations. We had hypothesized that this functional response could be sensitive to pollutant concentrations that would be considered harmless by traditional endpoints (following Caro et al., 2015; Duquesne and Küster, 2010; McWilliam and Baird, 2002). Indeed, this

was the case for most of the chemicals tested in our study. In Fig. 3, all data points but potassium dichromate and dichlorvos (not shown) are located to the left of the theoretical match between LC_{50} and IC_{50} (see also dashed vertical lines in Fig. 2). This clearly demonstrates that a 120-min assay can be used as a protective surrogate of acute toxicity for most chemicals. Furthermore, IC_{50} values from our study were more protective than acute EC_{50} estimated with other bivalves (Suppl. Tables S1–S9), again with the exception of potassium dichromate and dichlorvos. In the case of copper, the IC_{50} was also much more protective than most acute endpoints estimated with other invertebrates, including feeding inhibition in *D. magna* (Lari et al., 2017).

The high sensitivity of the clam's filter-feeding behaviour to copper sulphate was striking, as it was three orders of magnitude lower than the corresponding LC_{50}. A possible explanation is that the Asian clam is adapted to the presence of this compound, which has been historically used as an anti-foulant, agrochemical pesticide, as well as a biocide in water treatment facilities (Srinivasan and Swain, 2007; Vincent et al., 2016; Willis and Bishop, 2016). Indeed, microevolution to chronic metal exposure does not take much time to occur (Hochmuth et al., 2015; Pedrosa et al., 2017). As the sampling site is embedded in a humanized (urban and agricultural) landscape, the avoidance response could be part of a strategy of evolved tolerance to metal pollution – namely copper pesticides; indeed, traceable levels of copper were observed in soft tissues and shells of clams from this site (Silva et al., 2016a), suggesting actual exposure in the field. However, when comparing *Daphnia* populations from reference and historically contaminated sites, Lopes and co-workers (Lopes et al., 2004) showed that the earliest and most sensitive avoiders actually came from non-contaminated sites. This contradicts the hypothesis of a rapid avoidance response in tolerant populations and suggests the existence of other mechanisms of evolved tolerance. An alternative hypothesis could have to do with the chemosensory system of the clams, and its high sensitivity for metals (either waterborne or bound to suspended algae). Although little is known about the chemosensory system of the Asian clam, invertebrates with simpler nervous systems can detect and avoid chemical substances in their environment (Hilliard et al., 2002; Sambongi et al., 1999). A sensitive response to metals was also observed in freshwater mussels, namely *D. polymorpha* (Kraak et al., 1992) and *Anodonta cygnae* (Kádár et al., 2001; Moëzzi et al., 2013). Furthermore, valve closure/opening rhythms in response to metals were observed in early studies with the Asian clam (Doherty et al., 1987), and several authors have explored the physiology of this avoidance mechanism (Jou et al., 2009; Liao et al., 2007, 2005; Tran et al., 2003). Our study distinctly shows that the Asian clam can detect very low concentrations of copper ($< 5\,\mu g\,l^{-1}$ $CuSO_4$, which corresponds to $< 2\,\mu g\,l^{-1}$ Cu^{2+}), and promptly trigger an avoidance response.

The response of the clams to dichlorvos was also remarkable, lying in the opposite end from the observations with copper sulphate. Clams were exposed to very high levels of this organophosphate pesticide, but feeding inhibition was never observed. These findings are contrary to most studies with neurotoxic substances, especially cholinesterase-inhibitors such as dichlorvos (Donkin et al., 1997; Jadhav and Rajini, 2009). Feeding inhibition due to organophosphate exposure is often attributed to narcosis, inhibition of muscle contraction (e.g., pharyngeal pumping), or both, and these effects are normally linked to cholinesterase inhibition and accumulation of acetylcholine in synaptic clefts. In the Asian clam, dichlorvos causes cholinesterase inhibition at concentrations lower than those that cause mortality (Silva et al., 2016b). Therefore, we must assume that dichlorvos was either undetected by chemosensors or interfered with one of the steps of the avoidance response (sensing, neurotransmission, or muscle contraction). A study in a marine mussel exposed to dichlorvos (Donkin et al., 1997) demonstrated partial feeding inhibition along with a poorer tonus of adductor (shell-closing) muscles, giving the impression of a relaxed state. Thus, it is possible that the dichlorvos concentrations tested in our study impaired adductor muscle contraction (valve closure) without affecting the gill musculature (water pump) – see discussion in Donkin et al. (1997). However, the limited knowledge on the chemosensory mechanisms in the Asian clam does not allow excluding effects in terms of dichlorvos sensing.

Irrespective of the mechanism of action, the ability of dichlorvos to bypass the clams' avoidance strategy puts it in the spotlight as a potential agent to use or to be combined with others in eradication programs of the pest in closed or semi-closed industrial settings. In our study, lethal concentrations of dichlorvos were used without eliciting any avoidance response, which warrants the success of the toxicant as a biocide. On the contrary, the other toxicants – and particularly copper sulphate – were less efficient, because the clam is able to partially isolate itself from the external medium. Although long-term closure of the valves and complete shutdown of the water current and filtration is not possible (see Ortmann and Grieshaber, 2003), a partial closure of the valves will certainly reduce the efficiency of toxicant delivery. For example, the high discrepancy between feeding inhibition and mortality discussed above for copper sulphate could be explained – in part – by the need of a higher concentration than theoretically expected to kill the clams, as the result of the valve closure (i.e. avoidance) mechanism. Given the ability of dichlorvos to bypass this avoidance response, a future avenue of applied research could be the combination of dichlorvos (and eventually other cholinesterase-inhibitors or muscle relaxants) with some of the other biocides, especially those that are toxic at lower concentrations (such as niclosamide) or of lesser environmental concern (such as copper sulphate). Such mixtures could provide improved chemical control methods of the pest, profiting from the synergistic action of the mixtures (Silva et al., 2016b). However, the efficiency of such an approach is dependent on the ability of dichlorvos to nullify the avoidance response elicited by the other substance(s). This joint effect is worth investigating to clarify the effect of dichlorvos in the valve closure behaviour, since more work is needed towards a mechanistic understanding of the clams' chemosensorial perception and its linkage to muscular response.

Two main aspects were highlighted by our work. First, it is reasonable to say that behavioural avoidance of chemicals via valve closure (and associated feeding depression) is a candidate endpoint to integrate testing batteries for environmental screening purposes (see also Jou et al., 2009; Liao et al., 2005; Tran et al., 2003). Although arguably too simplistic, this is a sensitive and fast biosensor that may be used for rapid screening of toxins, contaminants or nuisance algae in drinking water or industrial effluents, and it may also aid ranking substances or effluents according to their toxicity (in a preliminary evaluation tier). Second, research on the clams' behavioural responses to chemicals can foster the development of more efficient chemical methods for the control of this biofouler in water-dependent facilities. Such applications are relevant to industry, environmental agencies and managers. Adding this to the potential of the species as a biofilter (Rosa et al., 2014a; Silva et al., 2016a), it is likely that the Asian clam becomes more important in biosensing, biomonitoring, and bioremediation. However, this should never justify its use or translocation to non-invaded natural sites, and measures should be undertaken to prevent its local and global spread.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2018.01.002.

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