Adverse effects of wastewater discharges in reproduction, energy budget, neuroendocrine and inflammation processes observed in marine clams Ruditapes philippinarum

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A B S T R A C T

The present study investigated possible adverse outcomes in the marine clams Ruditapes philippinarum exposed to sediment affected by wastewater discharges at the Bay of Cádiz (SW, Spain). Six locations representing five cities were chosen for the sediment sampling during winter and summer seasons: P1 — Chiclana de la Frontera, P2 — Puerto Real, P3 — Cadiz, P4 and P5 — El Puerto de Santa María, P6 — Rota (reference site). Biochemical biomarkers were explored in clams after 14-days of exposure under controlled conditions, that included changes in cellular energy status (total lipids content — TLP and mitochondrial electron transport activity — MET), gametogenic activity (dopamine and ALP levels), metabolism of monoamines (monoamine oxidase activity — MAO), inflammation and spawning properties (cyclooxygenase activity — COX). Wastewater discharges induced energy budget alterations, as suggested by MET decrease (P4 and P5) and accumulation of TLP (P1, P2 and P3) in gonads. ALP levels (P1, P2 and P3), dopamine (P2) and COX activity (P1, P2, P3, P4 and P5) decreased in clams after the exposure to summer sediments. MAO increased in clams exposed to winter (P1 and P2) and summer (P3 and P4) sediments. Wastewater discharges composition changed between different seasons, mainly leading to oxidative stress, inflammation (COX activity and ALP levels) and spawning delay in summer. This study highlights the importance of considering reproduction of marine biota when assessing adverse effects of wastewater discharges. Continuous release of wastewater adequately threatened or not, in aquatic ecosystems may culminate in adverse effects to the local benthic biota.

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1. Introduction

Wastewater discharges and sewage outfalls drive large amounts of effluents to estuaries and marine environments, which contain a plethora of contaminants including metals, polycyclic aromatic hydrocarbons (PAH), pesticides, steroids, surfactants, nutrients and pharmaceutical and personal care products (PPCP). Marine and estuarine environments are the major receptor of wastewater discharges from coastal areas, where the most urban populated areas in the world are located (Lara-Martín et al., 2014). Although a variety of PPCP have been currently detected in the marine environment (Lara-Martín et al., 2014; Long et al., 2013), only few studies have addressed the wastewater exposure and effects on non-target species (Vidal-Dorsch et al., 2014; Beyer et al., 2013; Mariya et al., 2012).

The Water Framework Directive (WFD) recommends the use of biota for assessing contamination trends in water bodies (Gust et al., 2014). Sensitivity of the molluscs to mixture of contaminants has been explored to evaluate chronic adverse effects of municipal effluents as biochemical and immunochemical responses (Box et al., 2007; Solé et al., 2009; Franzellitti et al., 2010; Gagné et al., 2011). Functional changes at cellular levels as neuroendocrine effects (monoamine oxidase activity — MAO, dopamine levels), inflammation (cyclooxygenase activity — COX), changes in reproduction (ALP levels) and energy status (total lipids content —
TLP, mitochondrial electron transport – MET) were previously assessed in freshwater molluscs. Wastewater treatment plant (WWTP) effluents produced inflammation measured by cyclooxygenase activity (COX) in mussels Elliptio complanata (Gagné et al., 2008a, 2007a, 2005) and snails Lymnaea stagnalis (Gust et al., 2013). Neuroendocrine toxicity and oestrogenic activity of primary-treated municipal effluents were assessed on E. complanata through the determination of monoamine oxidase (MAO) activity (Gagné et al., 2007a,b) and Vtg-like proteins applying ALP assay (Gagné et al., 2007b). Evidence of feminization was confirmed in E. complanata exposed to municipal effluents through the measurements of COX activity, serotonin, dopamine and Vtg-like proteins (Gagné et al., 2011). Freshwater organisms exposed to effluents increased energy expenditure determined by mitochondrial electron transport activity (MET) and total lipids (TLP) reduction (Gagné et al., 2007a; Smolders et al., 2004).

Marine bivalves, such as clams Ruditapes philippinarum are considered as sensitive bioindicators exposed to chronic water/sediment pollution. This species has been extensively used in biomonitoring (Morales-Caselles et al., 2008; Moschino et al., 2011) and sublethal studies (Buratti et al., 2010, 2012; Coughlan et al., 2009; Martín-Díaz et al., 2008b, 2007, 2005). Research on neuroendocrine responses in marine bivalves to stressors and their application in biomonitoring studies is not as extensive on biochemical biomarkers (Gagné et al., 2008b, Matozzo and Marin, 2007). Reproduction aspects have particular relevance to monitor population dynamics in contaminated environments (Solé et al., 2003; Martín-Díaz et al., 2008a; Matozzo et al., 2008).

The aim of the present study was to evaluate sublethal stress responses of bivalve sentinel organisms exposed to sediment contaminated by wastewater discharges, and to discuss the suitability of this bioindicator. Five different locations (P1 – P5) representing four cities (SW, Spain) were selected under directly influence of wastewater discharges and compared with a reference site (P6). 14-days bioassay with marine clams (R. philippinarum) was performed under laboratory conditions. Changes in total lipids (energy reserves), mitochondrial electron transport (energy consumption), cyclooxygenase activity (inflammation properties), monoamine oxidase activity and dopamine levels (neuroendocrine effects), and ALP levels (reproduction) were determined in gonads taking into account seasonality and contamination.

2. Material and methods

2.1. General approach

The Bay of Cádiz (SW, Spain) comprises a population of 460,000 inhabitants, but during the summer season the population increases by 30% compared with winter owing to tourism (INE, 2011). There is an increase of some PPCPs use (e.g. sunscreen in summer) in each season, and the decrease of others (e.g. cold medications in summer). Summer in South Spain is a dry season, and consequently, water consumption increases. However, winter comes with strong storms, which deal with sediment resuspension in the studied areas, and as a consequence, contaminants accumulated in the sediment compartment tend to be available to the aquatic biota. Main industries located in this zone are related with ship, offshore, car and aerospace manufacturing. Agriculture and tourism are also important socio-economic activities at the Bay of Cádiz.

Six points were chosen at the Bay of Cádiz (SW, Spain) (Fig. 1) for the assessment of sediment toxicity. Five of them were under the influence of wastewater discharges from urban areas: P1 – Chiclana de la Frontera, P2 – Puerto Real, P3 – Cádiz, P4 and P5 – El Puerto de Santa María. Previous studies recognized these areas as directly affected by wastewater discharges (Lara-Martín et al., 2008; Carrasco et al., 2003; Ponce et al., 2000), also regarded to maps provided by Junta de Andalucía (Spanish Government). P6 was located at Rota, and chosen as reference site.

- Chiclana de la Frontera (P1): located at Iro River. Previous study speculated the discharge of anticholinergic agents, such as pesticides (Solé et al., 2009). This river receives water from agricultural sources as well as urban wastewater discharge. Although the wastewater is treated in a WWTP before the discharge, a high level of contamination by nutrients and pathogens were measured in the aquatic system (Garrido-Pérez et al., 2002);
- Puerto Real (P2): characterized by WWTP discharge, moderate metal contamination (Carrasco et al., 2003) and significant shipping activity;
- Cádiz (P3): this area support a seasonal wastewater pumping and storage station, that send the wastewater to be treated in another WWTP located at San Fernando. However, there are occasional discharges of wastewater from this station to the Bay of Cádiz;
- El Puerto de Santa María (P4 and P5): P4 is characterized by seasonal wastewater pumping, and also receives the effluents coming from the upper part of the Guadalete River. SAS were previously detected in sediment in this area which correlated their usage and the presence of wastewater discharges (Lara-Martín et al., 2006). Some marinas and small harbours are located in this point. P4 might be considered the main receiving
point for wastewater generated in the upstream regions of the Cádiz province. P5 is located near a sewage outfall in the north of the Bay of Cádiz, at Puerto Sherry, which receives WWTP ef-
fluents from the city. There are only occasionally untreated discharges since WWTP has existed for several years (Lara-
Martin et al., 2006).
- Rota (P6): far from known wastewater discharges, with a high water renewal, near the Chorillo sandy beach.

2.2. Sediment sampling

Sediment was sampled during winter and summer 2011, from an inflatable launch on an ebbing tide by means of Van Veen grab (when it was possible) or scuba divers help, taking the topmost 10 cm layer of the sediments. Samples were brought to the laboratory, sieved to remove large debris and other animals, and kept at 4 °C in the dark for maximum two days until the clams’ exposure. Sediment samples were placed in 20-L aquarium filled with filtered seawater.

Physical chemical characteristics of the sediment sampled at P1 – P6 in winter and summer seasons were described in Tables 1 and 2. For sediment grain size analysis, an aliquot of dry sediment was analysed following the methodology recommended by USGS (2000). Total organic carbon (TOC) and organic matter (OM) content were evaluated (USEPA, 2002). Aqua regia extraction (ISO11466, 1995) was applied for metal analyses. Concentrations of metals (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn) were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES). Hg concentrations were determined with LECO AMA 254 analyser. Results were checked using MESS-1 NRC reference material. Such metals were selected based on sediment quality guidelines (SQG): USEPA (2004), CCME (1995), CEDEX (1994) and Riba et al. (2004).

Concerning organic compounds, polycyclic aromatic hydrocarbons (PAHs) in sediment samples were analysed according to USEPA SW-846 method 8270/8082 (2007). PAH fractions were chosen based on Riba et al. (2004). The sum of PAH fractions was used for the integrative analysis.

Selected 34 pharmaceuticals and secondary alkane sulfonates (SAS) were measured following methods proposed by Jelic et al. (2009) and Baena-Nogueras et al. (2013). Pharmaceuticals determined were anti-inflammatory agents (acetaminophen, diclofenac, fenoprofen), anti-hypertensive (atenolol, propranolol), lipid regulators (clofibric acid, gemfibrozi), psychiatric drugs (carbamazepine, fluoxetine, amitriptyline, caffeine), antibiotics (chloramphenicol, cefdinir, tiamulin, erythromycin, clarithromycin, azithromycin, roxithromycin, lincomycin, clindamycin, flumequine, sparfloxacin, novobiocin, metronidazole, ornidazole, sulfadiazine, sulfamethoxypyridazine, sulfathiazole, trimethoprim, monensin), antacids (famotidine, ranitidine) and others (glibenclamide, hydrochlorothiazide). The sum of each group of pharmaceuticals was used for the integrative analysis. For additional information about chemicals analysis, please see Maranho et al. (2015, 2014).

2.3. Clams exposure and tissues preparation

Specimens of R. philippinarum were bought from an aquaculture farm located at Chiclana de la Frontera (SW, Spain). Samples consisted of clams with 35–45-mm shell length. These specimens were acclimated in the laboratory for 7 days before exposure. After the acclimation period, 28 individuals were placed to each aquarium (day-0). The bioassay was performed in duplicate. Conditions of the bioassay were verified each two days (pH: 8.1 ± 3, dissolved oxygen: 7.3 mg L⁻¹ ± 2, salinity: 35.3 ± 1.4). 1:3 of the
A portion of homogenate was centrifuged at 3,000 g at 4 °C. Luminescence plates (Microlite 2, Thermo Fisher Scientific) were filled with filtered seawater (18 ± 2 °C) for deproteinisation. Twelve clams from each duplicate (n = 24 clams) were chosen randomly for the biomarker analysis. Gonad mass was dissected out, pooled (n = 4 gonads) and homogenized on ice using Teflon pestle tissue grinder apparatus in homogenization buffer (pH 7.5) containing 140 mM NaCl, 25 mM Hepes-NaOH, .1 mM EDTA and .1 mM dithiothreitol (DTT) (Gagné et al., 2003). Levels of inorganic phosphates liberated from NaOH treatment were determined by phosphomolybdate assay (Stanton, 1968). Absorbance was measured at 444 nm (some cases with interferences in the colour, the reading was taken at 815 nm). Data were expressed as ng g⁻¹ of horseradish peroxidase.

### 2.5. Oestrogenic activity assessment

Alkali-labile phosphate (ALP) method was applied in homogenized extracts as described by Gagné et al. (2003). Proteins were precipitated by aceton (35% v/v), centrifuged at 10,000 g for 5 min at 4 °C and washed in 50% acetone. After this, the sample was re-centrifuged, resuspended in 100 μL of 1M NaOH and incubated for 30 min at 60 °C. Levels of inorganic phosphates liberated from NaOH treatment were determined by phosphomolybdate assay (Stanton, 1968). Absorbance was measured at 444 nm (some cases with interferences in the colour, the reading was taken at 815 nm). Data were expressed as μg ALP/mg proteins.

### 2.6. Inflammation biomarker

Cyclooxygenase (COX) activity was tracked by measuring the oxidation of 2, 7 – dichlorofluorescein in the presence of arachidonate in S₁₅ fractions (Fujimoto et al., 2002). The S₁₅ fraction was incubated in 50 mM Tris—HCl, 0.5% Tween-20, 50 μM arachidonate, 2 μM dichlorofluorescein and .1 μg ml⁻¹ horseradish peroxidase. Fluorescence was measured at 485 nm and 530 nm. COX activity was expressed as RFU/min/mg proteins.

### 2.7. Energy budget

Total lipids (TLP) were determined in homogenate samples according to the phosphovanillin method (Friggs et al., 1972). Samples were incubated for 10 min at 80 °C in the presence of H₂SO₄ and phosphovanillin reagent. The appearance of pink color was measured at 540 nm. Standard solutions of olive oil were used for calibration. Data were expressed as μg TLP/mg proteins.

### 2.8. Data analysis

Normality and homogeneity of biomarker data were tested using Shapiro Wilk’s and Bartlett’s tests, respectively. Data were subjected to one-way Analysis of Variance (ANOVA) followed by post-hoc Tukey’s tests.
Dunnett’s t test. Significance was set at p < 0.05. Spearman’s rank correlation was performed to detect significant trends between biomarker responses determined in clams exposed to each sediment sample point and between seasons. Significance was set at p < 0.05. Two separate PCAs were conducted on the biological and chemical results, one for winter, and the other one for summer. Only the variables whose coefficient was ≥ 5 (Comrey’s, 1973) were considered to be components of the factors. All responses were analysed using the SPSS/PC 21.0 + statistical package.

3. Results

3.1. Physical chemical data

Grain size distribution indicated three overall groups predominated: essentially clayed silt sediments (P1, P2 and P4), medium sand (P3) and predominance of sand (P5 and P6) with fairly significant gravel and sand content. P2 showed the highest levels of TOC and OM during winter and summer. To evaluate potential ecotoxicological effects associated with observed concentrations of metals and PAHs, different published Sediment Quality Guidelines (SQGs) were consulted. Contaminants that exceeded any SQGs were highlighted in Table 1. The letter superscripted indicated which SQGs were surpassed for metals (USEPA, Marine Screening Benchmarks, 2004; CCME, 1995; CEDEX, 1994, Riba et al., 2004) and PAHs (CCME, 1999). Chemical results indicated P1, P2, P3, P4 and P5 were contaminated by organic and inorganic contaminants (metals and PAHs). P1 both seasons and P4 summer showed the highest concentration of SAS (Table 2). Anti-inflammatory concentrations were higher in sediment sampled at P1 than the other sites for both seasons. P5 winter also showed the highest concentration of psychiatric drugs and antibiotics.

3.2. Biomarker responses

Neuroendocrine parameters, energy budget and inflammation state of clams exposed to marine sediments contaminated by effluent discharges from WWTPs located at the Bay of Cádiz (SW, Spain) were investigated. Although there was no mortality of clams exposed to sediment sampled in the summer season, increasing of clam’s mortality was observed during the winter season: P1 − 32.1%, P2 − 21.4%, P3 − 26.7%, P4 − 50%, P5 − 7.14%, P6 − 15% in the reference site.

Biomarker responses were examined in pooled samples of clams exposed to sediment samples affected by wastewater discharges (Figs. 2–7). There was no significant difference of dopamine levels in winter season (Fig. 2). Clams exposed to sediments from P2 showed significantly lower dopamine levels (2-fold) compared with the reference site during the summer season (p < 0.05).

During the winter season, clams exposed to sediments affected by wastewater discharges P1 and P2 showed significant MAO activity reaching 2-fold higher compared with the reference site (p < 0.05) (Fig. 3). However, clams exposed to sediments from P3 and P4 significantly increased MAO activity (p < 0.05) in summer, which was found 1.9 and 2.6-fold higher than the reference site, respectively.

ALP levels were higher in summer than winter (Fig. 4). P1 (2.2-fold), P3 (1.6-fold) and P4 (2.2-fold) were significantly lower compared with the reference site (p < 0.05) during winter. COX activity (Fig. 5) increased in summer compared with winter including clams exposed to sediment sampled at the reference site. Nevertheless, COX activity was significantly decreased compared with the reference site (p < 0.05) during the summer season for all stations (P1: 2.3-fold, P2: 3.5-fold, P3: 1.9-fold, P4: 2.9-fold, P5: 1.9-fold).

Clams exposed to sediments sampled in winter showed higher TLP content (Fig. 6), being P1 (1.9-fold), P2 (2.5-fold) and P3 (1.9-fold) significantly increased compared with the reference site (p < 0.05). Clams showed lower TLP in summer compared with winter, however, P1 (2.4-fold) showed significantly higher TLP content compared with control clams (p < 0.05).

Respiratory metabolism of clams exposed to marine sediments affected by wastewater discharges were determined by mitochondrial electron transport (MET) activity (Fig. 7). During winter,
there was no significant difference for any station compared with the reference site. However, P4 and P5 (2.1-fold) showed significant decrease in MET activity in summer ($p < 0.05$).

3.3. Correlation between biomarker responses

Concerning P1 in winter, there was no correlation between biomarker responses. In summer, TLP was positively correlated with dopamine, ALP levels and MAO activity ($r = 1, p < 0.01$). TLP and COX activity were negatively correlated ($r = -1, p < 0.01$).

Dopamine was also positively correlated with MET activity ($r = 1, p < 0.01$).

Biomarker responses determined in clams exposed to P2 in winter showed positive correlation of MET activity, TLP ($r = 0.9, p < 0.05$) and COX activity ($r = 1, p < 0.01$). MET activity was also negatively correlated with MAO activity ($r = -1, p < 0.01$). Dopamine and ALP levels were negatively correlated ($r = -1, p < 0.01$). Clams exposed in summer showed positive correlation between COX activity and TLP ($r = 0.9, p < 0.05$). ALP levels were positively correlated with MAO activity ($r = 1, p < 0.01$), and negatively
correlated with dopamine \((r = -1, p < 0.01)\).

Positive correlation was observed between TLP and dopamine in clams exposed to sediment sampled at P3 in winter \((r = 1, p < 0.01)\). No correlation was observed in summer season.

Clams exposed to sediment sampled at P4 in winter showed positive correlation between dopamine, MET and COX activities \((r = 1, p < 0.01)\). In summer, COX activity was negatively correlated with ALP levels and MET activity \((r = -1, p < 0.01)\). MAO activity was positively correlated with dopamine and MET activity \((r = 1, p < 0.01)\), and negatively correlated with ALP levels \((r = -1, p < 0.01)\).

In winter, clams exposed to P5 showed positive correlation between ALP levels and COX activity \((r = .9, p < 0.05)\), and negative correlation with MET activity \((r = -9, p < 0.05)\). In summer, MAO activity and ALP levels were negatively correlated \((r = -1, p < 0.01)\).

P6 was considered the reference site. Clams exposed to this sediment samples showed positive correlation between COX and MAO activities \((r = 1, p < 0.01)\). However, MAO and MET activities, and TLP and ALP levels were negatively correlated \((r = -1, p < 0.01)\). In summer, TLP was positively correlated with dopamine, MAO and MET activities \((r = -1, p < 0.01)\). MET activity was also positively correlated with ALP levels \((r = 1, p < 0.01)\).

### 3.4. Integrated approach

The PCA analysis was performed with chemical data and biomarker responses from winter and summer (Table 3). In winter, three factors axes explained 90.34% of the total variance. Positive correlations of factor 1 explained 32.55% of variance, and related TLP and dopamine levels with contamination by PAHs, metals (Al, Cu, Ni, Zn, Pb) and pharmaceutical products (others) associated with TOC and OM in the sediment. Negative correlations linked MET and COX activities with psychiatric drugs associated with % of fines in the sediment. Factor 2 accounted for 31.51% of variances, and positive correlations explained the relationship between MAO activity and dopamine levels related with contamination by metals (Al, Fe, Mn, Cr, Zn, Ni, As), pharmaceuticals (anti-inflammatories, lipid regulators, others) and SAS associated with OM content in the sediment. Factor 3 explained 26.28% of the variance. Positive loadings linked COX activity with contamination by metals (Cr, Cu) and pharmaceutical products (anti-inflammatories, anti-hypertensive, lipid regulators). Negative loadings grouped ALP levels with PAHs and pharmaceutical products (antibiotics) associated with % of fines in the sediment.

The second PCA was concerning summer data. Factor 1 explained 31.65% of the total variance. Positive loadings represented contamination by PAHs, metals (Al, Fe, Cr, Ni, Zn), SAS and pharmaceutical products (others) associated with TOC and OM in the sediment. Negative loadings of the factor 1 showed the relationship between dopamine, MET and COX activities due contamination by Cd. Factor 2 accounted for 27.30% of the original variance and it grouped, with positive loadings, TLP, metals (Al, Fe, Cr, Ni) and pharmaceutical products (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacids, others) concentrations. Factor 3 explained 24.02% of the total variance. Positive loadings of factor 3 showed metal (Cu, Zn, Pb) and pharmaceutical products (anti-hypertensive drugs and others) presented in the sediment related to TOC, which affected MET activity. Negative loading showed ALP levels related with metals (Mn, As) and % of fines in the sediment.

The weight of factors at each sample site concerning both seasons was shown in Fig. 8. In winter, factor 1 exhibited a positively predominant score for P2 and a negatively predominant score for P5. While factor 2 was positively dominant in the case of P1 and P2, P3 showed the negative predominance. Factor 3 grouped variables with positive coefficients predominant in P1 and P4, and negative variables predominant in P2 and P5. In summer, factor 1 variables with positive loadings were important in P2, and negative loadings

### Table 3

Principal component analysis (PCA) based on chemical contamination and biomarker responses of clams exposed under controlled conditions to sediments affected by wastewater discharges at the Bay of Cádiz (SW, Spain) in winter and summer.

<table>
<thead>
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<th>Variables</th>
<th>Winter</th>
<th>Summer</th>
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<tr>
<td></td>
<td>#1 32.55%</td>
<td>#2 31.51%</td>
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<tr>
<td>TLP</td>
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<td>MET</td>
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<td>MAO</td>
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<td>Dopamine</td>
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<td>COX</td>
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<tr>
<td>Fe</td>
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<td>.756</td>
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<tr>
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<td>.686</td>
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were important in P3. Factor 2 was positively dominant in P1, and negatively dominant in P2 and P3. Factor 3 showed positive scores and high dominance in P3. Factor 3 score was negatively predominant in P5.

4. Discussion

According to data from the Ministry of Environment of the Junta de Andalucía (Spain), Cádiz and San Fernando spend up water during summer an average of 59% over winter months which reflect directly to the volume of wastewater discharges. Wastewater treatment consists of a set of physical, biological and chemical operations that seek to eliminate organic matter and contaminants before discharge; however levels of contamination remaining in the treated effluents meet the existing legal limits. Nevertheless, even after treatments, wastewater presented toxicity to organisms exposed to effluents (Gagné et al., 2007a,b, 2008a,b). Sediment sampled at P4 in winter showed high mortality of clams, which could be related with contamination by Hg and pharmaceuticals as anti-inflammatory, antihypertensive, psychiatric drugs and antibiotics, when comparing winter and summer seasons.

Marine sediment sampled near different WWTPs discharges at the Bay of Cádiz affected the clams in a different way depending of the locations and seasons. Seasonal changes in the biochemical components have been studied in R. philippinarum (Fernandez-Reiriz et al., 2007) in North Spain. Fernandez-Reiriz et al. (2007) observed that clams R. philippinarum used their own energy reserves (carbohydrates and glycogen) for sexual development in high temperature situations (18°C), while on low temperature conditions (14°C) was produced an accumulation of reserves. In the present study, R. philippinarum increased the lipids content (TLP) in gonads during winter when compared with clams exposed during summer. Therefore, positive energy balances permitted clams gonadal development and reserves accumulation of both low and high temperature conditions (Fernandez-Reiriz et al., 2007).

In general, the pattern followed by each biomarker response observed in clams exposed to the reference site (P6) was closely related to the state of sexual maturity and the use of previously stored reserves. Clams exposed to sediment sampled at reference site showed different correlations according to seasons. In winter, COX and MAO activities were positively correlated (p < 0.01). MAO activity has the function to break down neurotransmitters such as noradrenaline, serotonin and dopamine. Increase of MAO enzymatic activity can provide free radicals due to the oxidation of neurotransmitters, culminating on harm the neurons that produce dopamine. Evidence for MAO activity in bivalve molluscs is compelling based on previous studies (Stokey, 2004). Increase of COX activity deals with inflammatory properties, but also with spawning phase which normally occurs during summer for this specie. Decrease of COX activity could be due the increase of NSAIDs use for the population during summer.

Bivalve molluscs have been shown seasonal changes in biomass of soft tissue and its relation to the reproductive cycle reflects a complex interaction between external environmental factors and endogenous response (regulated by neuroendocrine system). Morthorst et al. (2014) applied ELISA to one freshwater bivalve species and compared the results with ALP methods. In fact, ELISA seems more sensitive method than ALP. However, Porte et al. (2006) brings many examples of the application of ALP methods in bivalves, mainly because there are few specific antibodies developed against bivalve Vtg or Vtg-like molecules, and these antibodies usually show low cross-reactivity across species (Li et al., 2008; Blaise et al., 1999; Kang et al., 2003; Osada et al., 2003; Park and Choi, 2004). Matozzo et al. (2012) observed that Triclosan was not oestrogenic to the clam R. philippinarum through the application of ALP methods. Therefore, seasonality studies in different bivalve molluscs have shown that ALP levels follow the same trend of the gametogenic cycle in females (Blaise et al., 1999; Ortiz-Zarragoitia and Cajaraville, 2005).

Within the same species, periods of reserves mobilization may change between locations, once the stages of reproductive cycle and energy reserves may be influenced by exogenous factors. Lipids form part of the reserves and are also important component of bivalve oocytes. Invertebrate reproduction is energy intensive (Brooks et al., 2003). Their maximum levels occur in the pre-spawning period before summer. The accumulation of reserves, allocation of stored energy to somatic growth or to the germinal pathway, and importance of each biochemical response to the reproductive process play a special role in the strategies to environmental stresses. Although there is a positive correlation between ALP levels, TLP and MET, according to Baek et al. (2014) lipids are important to maintaining the embryonic and larval development, while glycogen is used for gamete production and metabolic maintenance. Glycogen content should bring additional information about reproduction effects in clams. Temperature and food availability influences clams’ reproduction (Fernandez-Reiriz et al., 2007; Ojea et al., 2004). In the present study, clams were exposed under controlled temperature and there was no scarce of food resources, since the sediment affected by wastewater discharges were fulfilled of nutrients. However, wastewaters discharges produced different effects at all levels examined within the same season on different sites due to contamination.

The Bay of Cádiz (SW, Spain) is an area with great ecological and economic relevance. Differences on biochemical responses between sites in the same season should be due the different wastewater treatments. Volume of wastewater discharged per day by each WWTP or pumping stations was different. However, volume along the seasons in the year of 2011 did not vary significantly, because the rain in winter might be compensated by the increase of population and water discharge during the summer. Composition
of the wastewater discharges changed, being the main stress for the organisms, even the volume kept around the same along the year. Such information can be found at https://www.apemsas.valores-de-depuracion, http://www.aqualia-infraestructuras.es/referencias.cfm?idArtCat=12, https://chilcananatural.com/datos-y-estadisticas/agua/.

To extrapolate the same behaviour for *R. philippinarum*, and considering P6 as a good reference site, clams exposed to sediments sampled at P1 (Chiclana de la Frontera) did not show correlation between biomarker responses during winter. In summer, COX activity was negatively correlated with TLP (p < 0.01), which was positively correlated with ALP levels (p < 0.01). However, the energy required to support anti-inflammatory properties by endogenous or exogenous inputs of NSAID contaminants represents expenditure for bivalves, as corroborated by the negative correlation between COX activity and TLP.

In summer, there was no significant increase of ALP levels compared with the reference site. The same happened at the Seine estuary, since ALP levels in mussels from polluted sites were not significantly induced compared with the least-polluted site (Gagné et al., 2008b). Vtg-like proteins are the precursor of the energy reserves for the embryos, composed by lipid phosphorylated proteins and the phagocytic efficiency index involved in the immunocompetence of mussels exposed to municipal effluents. Vtg-like proteins have been associated with infection-resistant response which plays an integrative function in regulating immunity via its pleiotropic effects on both recognizing pathogen-associated molecular patterns and promoting macrophage phagocytosis in fish (Li et al., 2008).

MAO activity was negatively correlated with ALP levels (p < 0.01) in clams exposed to sediment from P5 in summer. MAO activity significantly increased in clams exposed to P1 and P2 in winter, and P3 and P4 in summer (p < 0.05). Previous study showed that MAO activity was induced in caged mussels exposed to urban effluent plume (Gagné and Blaise, 2003). The main role of MAO activity is the oxidative catabolism of important amine neurotransmitters, including serotonin, dopamine and adrenaline. Monoamines (e.g., serotonin and dopamine) are important mediators of gamete maturation and spawning (Gagné et al., 2007b). Selective serotonin reuptake inhibitor (SSRIs), as anti-depressive drugs, works as MAO inhibitors. The increase of MAO activity can be due to progestrone products, therefore, reduce dopamine levels, increase free radicals and oxidative stress (Edmondson et al., 2009), and it is associated to oocyte maturation (Brooks et al., 2003).

Indeed, the correlation between dopamine levels and ALP levels in winter suggests gametogenesis where a neuroendocrine interaction between signalling and dopamine metabolism in clams exposed to sediment affected by WWTP effluents (P2 and P4). At the end of gametogenesis, dopamine levels drop and serotonin increases to finalize gamete maturation, to initiate egg release as with COX activity (Gagné et al., 2011). However, COX activity significantly decreased in summer for clams exposed to all stations compared with the reference site (p < 0.05). Oestrogenic signalling may keep the clams in gametogenesis stage rather than spawning (Matozzo et al., 2008). This could be due the fact that municipal effluent contaminants such as oestrogenic compounds and non-steroidal anti-inflammatory drugs (NSAIDs) present in wastewater discharges could inhibit COX activity in summer, which could result in negative impacts on spawning. Previous studies showed the presence of NSAID contaminants in wastewater discharges (Hernando et al., 2006; Scheut et al., 2005; Loffler and Ternes, 2005). The class of compounds detected at highest concentrations in sediments sampled in Ebro River were analgesics/anti-inflammatory drugs followed by β-agonists and some antibiotics, which ibuprofen was the most abundant pharmaceutical product detected (Da Silva et al., 2011).
High mortality rates in winter and impaired reproduction mainly in summer should affect the equilibrium of the population. Based on this information, it is expected less individuals and delay in reproduction, which can result in the reduction of the clams' population in the future. To evaluate the bioavailability and help to elucidate which one(s) of the contaminant from the sediment are really engaged in the observed effects, efforts must take into account bioaccumulation, sex identification and histology of gonads.

5. Conclusion

This study underscores the contamination of the Bay of Cádiz by anthropic activities and the impact of wastewater discharges, responsible for effects related to reproduction of the bivalve exposed. Such effects are associated to oxidative metabolism, stress endpoints and the reproductive stage, and could culminate in long-term changes affecting the population. Wastewater discharges composition changed between different seasons, as the biochemical responses, mainly leading to oxidative stress and inflammation (COX activity). Observed effects are consistent with the occurrence of pharmaceutical products, PAHs, metals and surfactants. The exposure to municipal effluents seems to keep clams in a gametogenesis stage and delayed spawning based on the presented results. Further investigation should encompass the spawning activity in clams exposed to municipal effluent and to determine whether this process is disrupted. Thus, the continuous release of wastewater discharges adequately treated or not in aquatic ecosystems poses a reproduction risk to the clams' R. philippinarum.

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