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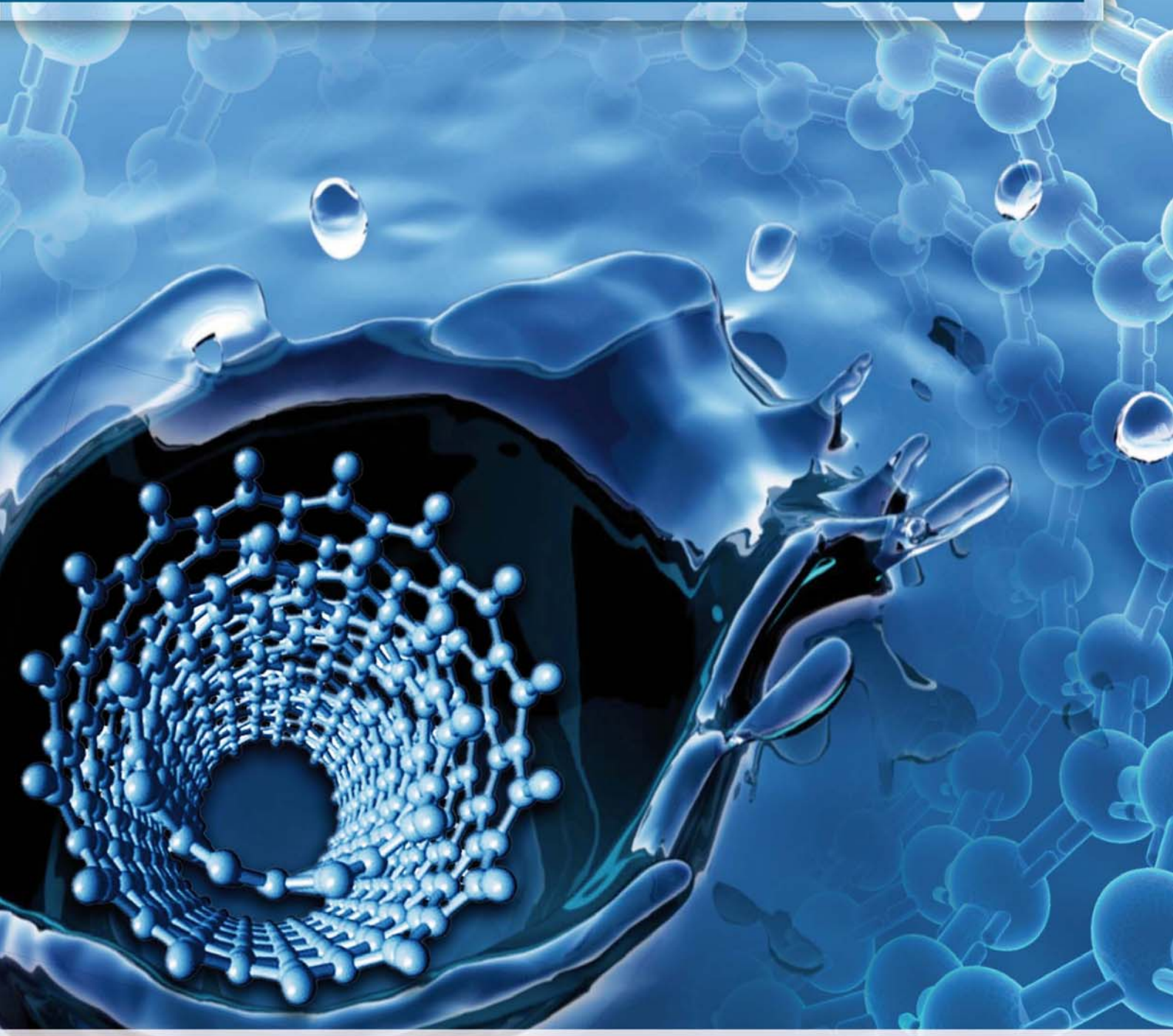

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Using lysosomal membrane stability of haemocytes in *Ruditapes philippinarum* as a biomarker of cellular stress to assess contamination by caffeine, ibuprofen, carbamazepine and novobiocin

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Abstract

Although pharmaceuticals have been detected in the environment only in the range from ng/L to μg/L, it has been demonstrated that they can adversely affect the health status of aquatic organisms. Lysosomal membrane stability (LMS) has previously been applied as an indicator of cellular well-being to determine health status in bivalve mussels. The objective of this study is to evaluate LMS in *Ruditapes philippinarum* haemolymph using the neutral red retention assay (NRRA). Clams were exposed in laboratory conditions to caffeine (0.1, 5, 15, 50 μg/L), ibuprofen (0.1, 5, 10, 50 μg/L), carbamazepine and novobiocin (both at 0.1, 1, 10, 50 μg/L) for 35 days. Results show a dose-dependent effect of the pharmaceuticals. The neutral red retention time measured at the end of the bioassay was significantly reduced by 50% after exposure to environmental concentrations ($p < 0.05$) (caffeine = 15 μg/L; ibuprofen = 10 μg/L; carbamazepine = 1 μg/L and novobiocin = 1 μg/L), compared to controls. Clams exposed to these pharmaceuticals were considered to present a diminished health status (retention time < 45 min), significantly worse than controls (96 min) ($p < 0.05$). The predicted no environmental effect concentration (PNEC) results showed that these pharmaceuticals are very toxic at the environmental concentrations tested. Measurement of the alteration of LMS has been found to be a sensitive technique that enables evaluation of the health status of clams after exposure to pharmaceuticals under laboratory conditions, thus representing a robust Tier-1 screening biomarker.

Key words: neutral red; pharmaceuticals; bioassay; haemolymph; bivalves; health status; Manila clam
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Introduction

The marine environment has been subjected to increasing numbers of stressors associated with the human population, including metals, hydrocarbons and persistent organic pollutants, among others. However, in recent years, active pharmaceutical compounds and their metabolites, directly and continuously released into the environment through municipal effluents, have caused serious new environmental concerns (Brain et al., 2004; Daughton and Ternes, 1999; Halling-Sørensen et al., 1998; Nakada et al., 2006). Even though many drugs have been detected in the environment at relatively low concentrations, ranging from ng/L to μg/L (Daneshvar et al., 2010; Fent et al., 2000; Gagné et al., 2006; Gómez et al., 2007; Halling-Sørensen et al., 1998: Yoon et al., 2010). It has been demonstrated that these environmental concentrations of pharmaceuticals may cause sublethal effects in aquatic biota (Binelli et al., 2009a; Canesi et al., 2007; Fent et al., 2000; Ferrari et al., 2003; Quinn et al., 2008a, 2008b; Martín-Díaz et al., 2009a, 2009b; Quinn et al., 2011). While acute toxicity incidents (e.g., death) are clearly visible, it is more complex to detect potential long-term effects of chronic stress. Given their properties (e.g., lipophilicity and persistence), pharmaceuticals can bioaccumulate (Halling-Sørensen et al., 1998) and cause toxic effects in non-target organisms (Grung et al., 2007). To date, there is scarce toxicity data for the majority of pharmaceuticals in use, thus impeding their adequate risk assessment. It is also of great importance to know the effects of pharmaceuticals compounds pose to aquatic life, so as to allow better risk assessment and environmental

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protection before the effects are irreversible or costly to remedy (Ringwood et al., 2003).

The critical issues include not only determining if organisms are adversely affected, but also identifying the effects of chronic stress on an organism's health (Rinwood et al., 2003). For these tasks, sensitive and effective tools are needed to facilitate diagnosis when environmental conditions adversely affect the integrity of organisms, before the effects become irreversible or costly to remedy (Rinwood et al., 2003). To establish the status of marine ecosystems and organism health, the use of biomarkers of exposure and effect has been suggested (Broeg et al., 2005; Depledge and Fossi, 1994; Lam, 2009; Lam and Gray, 2001; Van der Oost et al., 2003; Viarengo et al., 2007).

Several standardized short-term, sensitive and cost-effective biomarkers have been developed and applied for assessing the chronic toxicity of an aquatic environment for exposed organisms (Martín-Díaz et al., 2004; Viarengo et al., 2007). Furthermore, the use of these biomarkers is considered of great value since these responses provide the initial screening, and act as early warning indicators to provide the first evidence of organisms' responses to toxic chemicals (Wepener, 2005). The measurement of lysosomal membrane stability (LMS) as a general indicator of cellular well-being in eukaryotes (Moore et al., 2000) is a recognized screening biomarker that has previously been applied to assess the health status of a variety of organisms (Moore et al., 2000; Viarengo et al., 2007) and is employed for the Tier-1 approach in wide-scale biomonitoring programmes. The usefulness of this biomarker derives from the fact that lysosomes are conserved cellular organelles (Lowe and Moore, 1992; Lowe and Fossato, 2000) that are capable of sequestering, accumulating and detoxifying organic contaminants including toxic metals (Dailianis et al., 2003; Moore, 1985; Moore et al., 2004; Rashid et al., 1991). In addition, lysosomes are easy to visualize and are present in all nucleated cells (Viarengo et al., 2007).

The neutral red retention assay (NRRA) applied for LMS analysis uses the fact that healthy lysosomes retain the dye longer than perturbed ones; lysosomal damage can provoke leakage of the dye into the cytosol, possibly indicating more severe damage leading to cell death (Viarengo et al., 2007). Therefore, the retention time of the neutral red dye in living haemocytes provides a reliable health index of the organism (Moore, 2002; Moore et al., 2006; Norton, 2000).

LMS has been used as valuable indicator of cellular damage index in fish and shellfish (Allen and Moore, 2004; Dailianis et al., 2003; Harding et al., 2004; Ringwood et al., 2003; Lowe et al., 1992, 1995; Moore, 1994; Moore et al., 2006; Parolini et al., 2010). Moreover, NRRA applied specifically in the bivalve mollusc has been included in the general guidelines for monitoring the effects of contaminants, because this organism has the ability to bioaccumulate and bioconcentrate xenobiotics present in its environment (Martín-Díaz et al., 2009a). However, to the authors' knowledge little is known about this methodology applied in the Manila clam *Ruditapes philippinarum*; in this regard Buratti et al. (2010) have applied this technique in clams exposed to contaminated sediment while Coughlan et al. (2009) used the LMS to evaluate the effect of salinity in the health status of this species. This species has been used previously as a bioindicator species in ecotoxicological studies in order to assess organic and metal pollution under laboratory and field conditions, and has been shown to be a suitable bioindicator species in marine environment quality assessment (Martín-Díaz et al., 2004, 2007, 2008a, 2008b; Morales-Casselles et al., 2008; Rodríguez-Obeso et al., 2007).

In the present study four frequently-used pharmaceuticals, caffeine, ibuprofen, carbamazepine and novobiocin, were chosen to conduct laboratory experiments with *R. philippinarum*. Caffeine is a common psychoactive stimulant drug applied worldwide (Fent et al., 2006; Palo and Choudhury, 2006). Its concentrations in the environment have been detected in the range from 0.07 to 293 μg/L. Ibuprofen is an anti-inflammatory drug used for general pain relief; concentration of this drug have been recorded ranging from 0.01 to 24.6 μg/L. Moreover it has shown relatively high mobility in aquatic environments, but a low persistence, compared to other pharmaceuticals (Buser et al., 1999). Carbamazepine is prescribed for anticonvulsant and mood-stabilizing treatment, has been found in the environment at concentrations ranging from 0.03 to 6.3 μg/L. Studies of carbamazepine and its degradation products in water indicate high persistence, which is found more frequently and at higher concentrations than other pharmaceuticals in municipal effluents (Cunningham et al., 2010). Novobiocin is a potent antibacterial agent found in the environment at concentration of 0.33 μg/L. To the authors' knowledge, fate of this antibiotic has not been reported.

The objective of this study is to investigate cellular well-being in living haemocytes in order to evaluate the suitability of LMS for assessing the health status of *R. philippinarum* exposed to environmental concentrations of caffeine, ibuprofen, carbamazepine and novobiocin. In parallel, it is intended to validate the LMS measured through the neutral red retention assay as a biomarker, applied in *R. philippinarum* haemolymph, with a view to recommending this as a screening tool for the assessment of pollution by pharmaceuticals in marine environments, being used initially with a complementary battery of biological response measurements for assessing contaminated environments.

1 Materials and methods

1.1 Acclimation and maintenance conditions of clams

Manila clams *R. philippinarum* were purchased from an

aquaculture farm. All individuals used in this research were of similar size and length (42.00 ± 0.9 mm). Once in the laboratory, clams were acclimated for one week in 300 L tanks supplied with constant aeration; parameters such as pH 7.8–8.2, temperature (15 ± 1°C), salinity (33.8‰ ± 0.3‰), dissolved oxygen (> 5 mg/L) and saturation of dissolved oxygen (60%) were strictly controlled and maintained under a light:dark photoperiod (12 hr:12 hr).

1.2 Bioassay

The bioassay was performed in duplicate. After a one week acclimation period, six clams were placed in 20 L glass aquaria, aerated and exposed to different dissolved concentrations of pharmaceuticals (caffeine 0, 0.1, 5, 15, 50 μg/L, ibuprofen 0, 0.1, 5, 10, 50 μg/L, novobiocin 0, 0.1, 1, 10, 50 μg/L and carbamazepine 0, 0.1, 1, 10, 50 μg/L) during 35 days, in an static renovation system. Clams were fed every two days with phytoplankton (*Tetraselmis* sp., T-150 and *Chaetoceros* sp.). After the feeding process, the water was siphoned out. Waste food, faeces, and any other debris were carefully removed and the water renewed. Then a volume of the stock solution of pharmaceuticals prepared in DMSO (0.001%, *V/V*) was added to each 20 L aquarium in order to expose organisms to the pharmaceutical concentration required. Sublethal toxicity assessment for this solution was also undertaken in parallel with the bioassay.

1.3 Pharmaceuticals

Pharmaceuticals were purchased from Sigma-Aldrich (Spain). Doses of these chemicals were selected based on environmental concentrations reported in studies carried out in municipal effluents, sewage treatment plants effluents, waste water treatment plant effluents, surface water, rivers, creeks, lakes and sea water, worldwide (**Table 1**).

1.4 Lysosomal membrane stability

Lysosomal membrane stability was analyzed using NRRA following the methodology reported in detail by Martínez-Gómez et al. (2008) and adapted to the species used in this study. Clam physiological saline (436 mol/L NaCl, 10 mmol/L KCl, 10 mmol/L $CaCl_2$, 53 mmol/L $MgSO_4$ and 20 mmol/L Hepes sodium salt adjusted to pH 7.3 with 1 mol/L (NaOH) was prepared following the protocol of Lowe et al. (1995) and Marchi et al. (2004). Clam haemolymph was extracted from abductor muscle with a 1-mL syringe containing 0.1 mL physiological saline. Two replicates of 40 μL samples were transferred onto microscope slides previously treated with 5 μL of the coating agent poly-L-lysine and incubated in a lightproof humidity chamber, where the haemocytes were left to settle and attach to the slide surface during 30 min. The excess solution was removed carefully letting the liquid run off and 40 μL aliquot of neutral red solution (0.2 mmol/L) was added. The slides were incubated again into the humidity chamber during 15 min and the excess solution was removed by tilting the slides before placing a coverslip. Finally, the retention time was examined under microscope at 400× magnification at 15, 30, 60, 90, 105, 120 and 135 min. After each inspection the slides were returned to the dark chamber. The test was stopped when the dye loss was evident in more than 50% of the haemocytes (assessed qualitatively) and the times were recorded. Results were expressed as destabilization time, representing the time at which more than 50% of the lysosomes have released the dye into the cytosol. The time taken for the dye to leak out into the cytosol is related to the degree of membrane damage. In case lysosome membranes are severely damaged the dye will leak out within 15 min of incubation, whereas healthy lysosomes retain it for up to 180 min (Lowe and Pipe, 1994). LMS was evaluated in clam haemocytes at the beginning of the assay (day 0) and every 7 days during the course of the experiment.

1.5 Statistical analysis

Data were analysed using the SPSS/PC + statistical package. Significant differences between exposed and control samples were determined using a one-way ANOVA, followed by the Dunnett's multiple comparison test. Pair wise correlations were obtained through the Pearson's rank order correlation test. The significance level was set at $p < 0.05$. EC_{50} values were estimated by the statistical program TOXCALC®.

2 Results and discussion

The mean value of neutral red retention time (NRRT) observed in lysosomes from control clams (n = 12) at the beginning of the assay (day 0) was (106 ± 27) min, while NRRT from control organisms (n = 12) at the end of the experiment (day 35) was (94 ± 7) min. No significant differences were observed between measurements performed on day 0 and on day 35 for control organisms. The test undertaken with DMSO showed a NRRT of (98 ± 9) min (n = 12), and no significant differences were observed between control organisms, organisms from day 0 and organisms exposed to 0.001% of DMSO, therefore results of this research will be compared with controls. The physiological saline solution applied in this research had no effect in LMS of manila clam as mentioned previously by Coughlan et al. (2009). The baseline NRRT level of this study, taking into consideration the values observed in control organism before and after the experiment, is above the range of those results measured in the same species by Buratti et al. (2010) who recorded a NRRT of (70 ± 10) min in the control organisms.

However, since comparison with other studies carried out with this biomarker in *R. philippinarum* in laboratory is impossible because of the lack of data, the control of

Table 1 Analysis assessment based on measured environmental concentration (MEC)/predicted no environmental effect concentration (PNEC) results

Pharmaceutical	MEC (μg/L)	Place of study	Reference	PNEC	MEC/PNEC	Assessment
Caffeine	0.01	SW	Weigel et al., 2004a	0.00237	4.219	Yes
	0.07	STP	Weigel et al., 2004a	0.00237	29.915	Yes
	0.09	SW	Weigel et al., 2004a	0.00237	37.974	Yes
	0.16	Creek	Yoon et al., 2010	0.00237	68.376	Yes
	0.25	River	Yoon et al., 2010	0.00237	106.837	Yes
	1.9	WWTP	Kosma et al., 2010	0.00237	811.956	Yes
	3.6	ME	Ternes, 1998	0.00237	1538.462	Yes
	4.42	WWTP	Santos et al., 2005	0.00237	1888.888	Yes
	6	WWTP	Kolpin et al., 2002	0.00237	2564.103	Yes
	10	ME	Gagné et al., 2005	0.00237	4273.504	Yes
	12	STP	Gómez et al., 2007	0.00237	5128.205	Yes
	13.9	WWTP	Kosma et al., 2010	0.00237	5940.171	Yes
	22.2	WWTP	Gagné et al., 2006	0.00237	9487.180	Yes
	293.0	STP	Weigel et al., 2004a	0.00237	123628.692	Yes
Ibuprofen	0.01	Lake	Tixier et al., 2003	0.00071	14.085	Yes
	0.01	ME	Thomas and Foster, 2004	0.00071	14.085	Yes
	0.01	SW	Weigel et al., 2004a	0.00071	14.085	Yes
	0.03	STP	Weigel et al., 2004b	0.00071	42.254	Yes
	0.05	River	Yoon et al., 2010	0.00071	70.423	Yes
	0.15	Surf. W	Gros et al., 2006	0.00071	211.268	Yes
	0.3	Creek	Yoon et al., 2010	0.00071	422.535	Yes
	0.5	WWTP	Kosma et al., 2010	0.00071	704.225	Yes
	0.7	STP	Weigel et al., 2004b	0.00071	985.915	Yes
	0.8	WWTP	Gros et al., 2006	0.00071	1126.761	Yes
	1.0	WWTP	Kolpin et al., 2002	00.0071	1408.451	Yes
	1.3	WWTP	Tixier et al., 2003	0.00071	1830.986	Yes
	2.1	STP	Carballa et al., 2004	0.00071	2957.747	Yes
	2.3	River	Roberts and Thomas, 2005	0.00071	3267.606	Yes
	2.6	WWTP	Kosma et al., 2010	0.00071	3661.972	Yes
	3	WWTP	Roberts and Thomas, 2005	0.00071	4225.352	Yes
	6.3	WWTP	Santos et al., 2005	0.00071	8873.239	Yes
	7.1	STP	Gómez et al., 2007	0.00071	10000.000	Yes
	10.1	WWTP	Santos et al., 2005	0.00071	14225.352	Yes
	20.0	STP	Weigel et al., 2004b	0.00071	28169.014	Yes
	24.6	WWTP	Miège et al., 2009	0.00071	34647.887	Yes
Carbamazepine	0.03	WWTP	Gagné et al., 2006	0.00032	93.750	Yes
	0.04	Lake	Tixier et al., 2003	0.00032	125.000	Yes
	0.07	River	Yoon et al., 2010	0.00032	218.7500	Yes
	0.1	Surf. W	Gros et al., 2006	0.00032	312.500	Yes
	0.1	ME	Gagné et al., 2005	0.00032	312.500	Yes
	0.1	STP	Clara et al., 2004	0.00032	312.500	Yes
	0.1	River	Daneshvar et al., 2010	0.00032	312.500	Yes
	0.13	STP	Gómez et al., 2007	0.00032	406.25	Yes
	0.16	Creek	Yoon et al., 2010	0.00032	500.000	Yes
	0.44	WWTP	Daneshvar et al., 2010	0.00032	1375.000	Yes
	0.63	WWTP	Gros et al., 2006	0.00032	1968.75	Yes
	0.75	WWTP	Santos et al., 2005	0.00032	2343.7500	Yes
	0.7	STP	Metcalfe et al., 2003	0.00032	2187.500	Yes
	0.87	STP	Ferrari et al., 2003	0.00032	2718.75	Yes
	0.95	WWTP	Tixier et al., 2003	0.00032	2968.75	Yes
	1.1	WWTP	Kosma et al., 2010	0.00032	3437.500	Yes
	1.5	STP	Andreozzi et al., 2003	0.00032	4687.500	Yes
	1.5	WWTP	Clara et al., 2004	0.00032	4687.500	Yes
	2.3	WWTP	Miège et al., 2009	0.00032	7187.500	Yes
	4	STP	Metcalfe et al., 2003	0.00032	12500.00	Yes
	6.3	ME	Ternes, 1998	0.00032	19687.500	Yes
Novobiocin	0.33	ME	Gagné et al., 2006	0.00316	104.403	Yes

ME: municipal effluent, STP: sewage treatment plant effluent, WWTP: wastewater treatment plant effluent, Surf. W: surface water, SW: sea water.

this study has been compared with other laboratory studies with bivalve molluscs. The results obtained in this research approach are in good agreement with those obtained by Martín-Díaz et al. (2009a) who established a NRRT of 90 min in haemolymph of *Mytilus galloproviancialis* (Mediterranean mussel) controls and Nicholson (2003), who reported 60 min NRRT in haemolymph of *Perna viridis* (philippine green mussel) control organisms, while NRRTs of 84 and 90 min were recorded in haemolymph of *Dreissena polymorpha* (zebra mussel) (Binelli et al.,

2009a, 2009b). In addition, studies with other bivalve species, such as *Ostrea edula* (flat oyster), have reported 80 min in haemolymph of control organisms (Hauton et al., 1998). The effects on lysosomal membrane stability of *R. philippinarum* determined after exposure to caffeine, ibuprofen, carbamazepine and novobiocin were compared first to those organisms on day 0, in order to evaluate changes over the course of the 35-day experiment.

It was observed that most of the organisms analyzed in this experiment on day 35, after exposure to increasing concentrations of the selected pharmaceuticals, presented less than 50% of the LMS compared to clams on day 0 ($p < 0.05$) except those exposed to ibuprofen at 0.1 which presented 53% of LMS (NRRT = 56 ± 8 min) (**Fig. 1**). At the end of the experiment, environmental concentrations of caffeine (15 μg/L (**Fig. 1A**) and carbamazepine (1 μg/L (**Fig. 1C**) reduced significantly ($p < 0.05$) the LMS to 20% (NRRT = 19 ± 7 min) and 36% (NRRT = 34 ± 14 min) respectively, compared to control clams. Our results agrees with Martín-Díaz et al. (2009a) who observed a 60% reduction of LMS in haemocytes from mussels (*M. galloproviancialis*) exposed during 7 days to a carbamazepine concentration of 0.1 μg/L. Furthermore NRRT at the end of the experiment was significantly low ($p < 0.05$) in clams exposed to highest concentration of caffeine, ibuprofen, carbamazepine and novobiocin, compared to control clams.

Adopting the LMS criteria of Viarengo et al. (2007) applied in fish, and of Martínez-Gómez et al. (2008) applied in molluscs and following the criteria of Aguirre-Martínez et al. (2010) employed in crabs, the threshold values used specifically in this study were as follows: clams were considered to be healthy if NRRT was ⩾ 80 min; they were considered stressed but compensated if NRRT was < 80, but ⩾ 45 min; and to present diminished health status if NRRT was < 45 min. The clams exposed to the following concentrations: 50 μg/L caffeine (24 ± 10 min), 10 μg/L ibuprofen (30 ± 12 min), 50 μg/L ibuprofen (23 ± 9 min); 10 μg/L carbamazepine (34 ± 8 min), 50 μg/L carbamazepine (23 ± 9 min), 10 μg/L of novobiocin (30 ± 12 min), and 50 μg/L of novobiocin (19 ± 8 min) were significantly destabilized ($p < 0.05$), compared to control organisms (94 ± 7 min). Results obtained in this assay showed a dose-dependent effect ($p < 0.01$) of pharmaceuticals. Significant negative relationships ($p < 0.05$) were observed between pharmaceutical concentrations and

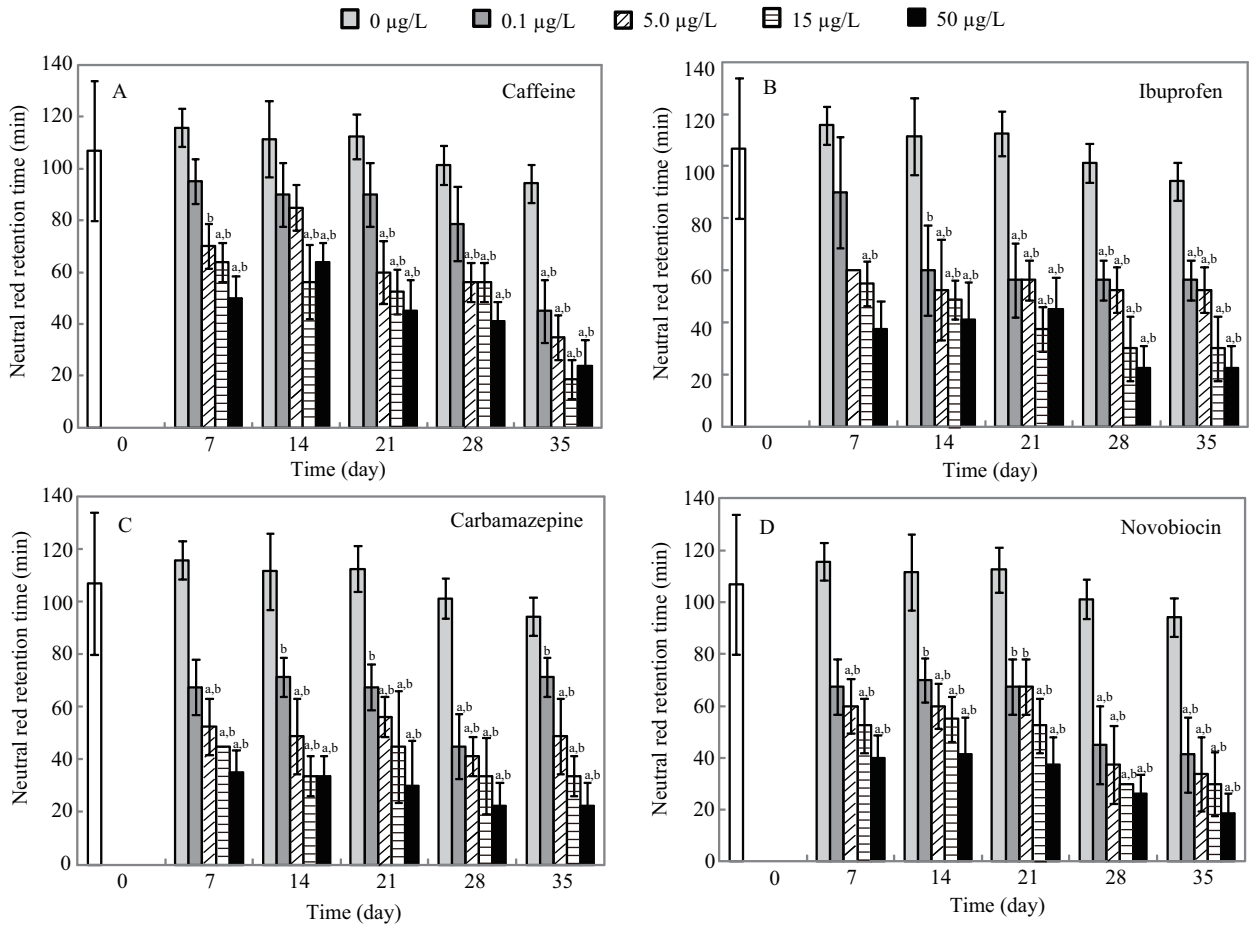

Fig. 1 Lysosomal membrane stability evaluated in clam haemocytes exposed for 35 days to caffeine (A), ibuprofen (B), carbamazepine (C), novobiocin (D) by the application of neutral red retention assay (NRRA) (mean ± SE, $n = 12$). The a indicates significant differences between means compared with clams on day 0 ($p < 0.05$); b indicates significant differences between means compared with control organisms.

LMS (specifically, caffeine $r = 0.68$, ibuprofen $r = 0.71$, carbamazepine $r = 0.62$, and novobiocin $r = 0.72$).

Bearing in mind that in this experiment organisms that presented NRRT > 90 min were considered as healthy organisms, specimens of *R. philippinarum* analyzed after exposure to environmental concentration of caffeine (15 μg/L), ibuprofen (5 μg/L), carbamazepine (1 μg/L) and novobiocin (1 μg/L) were considered either to present a diminished health status or to be stressed; these findings are significantly different ($p < 0.05$) from those in respect of the controls. These results indicate that the pharmaceutical gradient tested in this research is liable to affect the lysosomal membrane stability not only in an overall dose-dependent manner ($p < 0.01$) but also in a time-dependent manner ($p < 0.01$, $r = 1$) (caffeine: $r = 0.75$, ibuprofen: $r = 0.78$, carbamazepine: $r = 0.74$, and novobiocin: $r = 0.76$). These dose-dependent and time-dependent responses have been also reported by Binelli et al. (2009) from their study of the NRRT in zebra mussel (*Dreissena polymorpha*) exposed to the antibacterial agent triclosan.

Changes observed in haemocyte stabilization of manila clams after exposure for 35 days to the concentrations of caffeine, ibuprofen, carbamazepine and novobiocin tested in this study confirmed that these drugs presented effects at the environmental range tested (**Fig. 1**). The variation in membrane stabilization found in this research is in agreement with a previous study made by Canesi et al. (2007), who confirmed the sensitivity of LMS of mussels exposed to increasing concentrations of estrogenic chemicals and showed a clear dose-dependent response. The NRRTs obtained in this research therefore reflect stress and damage in haemocyte cells in the organisms following pharmaceutical exposure. Lysosome damage is consequently the result of their ability to concentrate contaminants (Moore, 1990), since the lysosomes are the target for the toxic action of pollutants (Lowe and Pipe, 1994). The study of lysosomal alteration is of major importance because it indicates the cellular well-being of the organism, which can be traduced to the organism's overall health status, considering that disease and death in animals occur primarily at the cellular level (Grisham and Smith, 1984).

Effective concentration (EC_{50}) at 95% significance level was calculated in order to determine which of the concentrations of pharmaceuticals tested in this study reduced NRRT by < 45 min in 50% of the organisms. The EC_{50} for caffeine was 2.37 μg/L, for ibuprofen 0.71 μg/L, for carbamazepine 0.32 μg/L and for novobiocin 3.16 μg/L (**Table 2**). Based on the chronic EC_{50} results, and following the EU Directive 93/67/EEC intended to classify substances according to their measured effective concentration (MEC) (CEC, 1996), the four pharmaceuticals tested can be classified as extremely toxic ($EC_{50} < 100$ μg/L) (**Table 3**). These findings agree with previous studies performed by Aguirre-Martínez et al. (2010, 2013b), who reported that caffeine, ibuprofen, carbamazepine and novobiocin have a significant ($p < 0.05$) effect on the lysosomal membrane stability analyzed in the haemolymph of the crab *Carcinus maenas*; these pharmaceuticals are toxic in the range of 0.1–50 μg/L ($EC_{50} < 100$ μg/L). Our findings also agree with those of Owuor et al. (2009), who demonstrated that these pharmaceuticals have a significant teratogenic effect in the sea urchin *Paracentrorus lividus* when exposed to same pharmaceutical gradient tested in this study ($EC_{50} < 100$ μg/L). However, those authors proved that their effect was not toxic in fertilization tests with *P. lividus*, nor in tests of the bioluminescence response in bacteria *Vibrio fischeri*, when applying the same concentrations tested in this research ($EC_{50} > 100{,}000$ μg/L) similarly Aguirre-Martínez et al., (2013a) showed that concentration tested in this study is not toxic for *Isocrhysis galbana* when applying growth inhibition test. On the other hand, Quinn et al. (2008b), who measured the chronic EC_{50} effects of 11 pharmaceuticals tested in the cnidarian *Hydra attenuatta*, based on the classification from the EU Directive 93/67/EEC, classified ibuprofen as toxic (EC_{50} < 100–1000 μg/L) and carbamazepine and novobiocin as harmful (EC_{50} < 10,000–100,000 μg/L) while caffeine was classified as non-toxic ($EC_{50} > 100$ μg/L) at environmental concentrations (**Table 3**). Other research studies carried out with a variety of phyla have demonstrated the detrimental toxic effect of carbamazepine in aquatic organisms (Ferrari et al., 2003; Jones et al., 2002; Repetto et al., 2003).

The predicted 'no environmental effect' concentration (PNEC) has been calculated with the object of obtaining the environmentally-relevant concentration of selected pharmaceuticals, and following EU regulations for Tier-2 guidance (Quinn et al., 2008a). PNEC was calculated dividing the EC_{50} value by an assessment factor of 1000. In addition, the MEC was divided by the calculated PNEC. If MEC/PNEC values are < 1 this indicates that the pharmaceutical tested at an environmental concentration does not need further evaluation, while MEC/PNEC > 1 indicates the need for a deeper assessment, consequently Tier-3 should be performed (Quinn et al., 2008a). The calculated MEC/PNEC values are presented in **Table 1**; these show that caffeine, ibuprofen, carbamazepine and novobiocin at environmental concentrations require deeper evaluation, or that a Tier-3 study should be carried out. Similarly, after toxicity tests with *H. attenuata* (survival and teratogenic endpoints), Quinn et al. (2008a) suggested further toxicity assessment for ibuprofen, which was considered as potentially toxic for the development of *H. attenuata* at environmental-relevant concentrations.

The level of toxicity calculated for caffeine, ibuprofen carbamazepine and novobiocin indicated that concentrations measured in the environment in the μg/L range appear to be extremely toxic for lysosomal membrane stability of clams. However, following the E.U.-ECOSAR toxicity

Table 2 Effective concentration (EC_{50}) of pharmaceuticals that affect LMS in 50% of clam haemolymph analyzed

Pharmaceutical	EC_{50} (μg/L)	Measured environmental concentration MEC range (μg/L)	Reference
Caffeine	2.37	0.01–293	Weigel et al., 2004a
Ibuprofen	0.71	0.01–24.6	Thomas and Foster, 2004; Miège et al., 2009
Carbamazepine	0.32	0.03–6.3	Gagné et al., 2006a; Ternes, 1998
Novobiocin	3.16	0.33	Gagné et al., 2006

Table 3 Toxicity level of pharmaceuticals, based on chronic EC_{50} results from clam *Ruditapes philippinarum* in this study compared with other studies based on the classification from E.U. Directive 93/67/EEC

Pharmaceutical EC_{50} (μg/L)	Extremely toxic < 100	Very toxic < 100–1000	Toxic 1000–10,000	Harmful 10,000–100,000	Non toxic > 100,000
Caffeine	*R. philippinarum* (LMS) *P. lividus* (embryogenesis) *C. maenas* (LMS)				*H. attenuata* (feeding) *V. fishery* (bioluminescence) *P. lividus* (fertilization) *I. galbana* (growth inhibition)
Ibuprofen	*R. philippinarum* (LMS) *P. lividus* (embryogenesis) *C. maenas* (LMS)		*H. attenuata* (feeding)		 *V. fishery* (bioluminescence) *P. lividus* (fertilization) *I. galbana* (growth inhibition)
Carbamazepine	*R. philippinarum* (LMS) *P. lividus* (embryogenesis) *C. maenas* (LMS)			*H. attenuata* (feeding)	 *V. fishery* (bioluminescence) *P. lividus* (fertilization) *I. galbana* (growth inhibition)
Novobiocin	*R. philippinarum* (LMS) *P. lividus* (embryogenesis) *C. maenas* (LMS)			*H. attenuata* (feeding)	 *V. fishery* (bioluminescence) *P. lividus* (fertilization) *I. galbana* (growth inhibition)

R. philippinarum (this study); *P. lividus* and *V. fishery* (Owour et al., 2009); *H. attenuata* (Quinn et al., 2008b); *C. maenas* (Aguirre-Martinez et al., 2013b), *I. galbana* (Aguirre-Martinez et al., 2013a).

prediction model, developed for the U.S. EPA, if the predicted effective concentration is < 0.01 μg/L, no further test or evaluation is required; in contrast, the results of this study indicated the need for further assessment. The results of this study also confirmed the sensitivity of this assay.

Biomarkers of cellular distress, such as lysosomal damage evaluation, have been suggested as a bio-monitoring tool (Dailianis et al., 2003), and as a tool for assessing the general condition of key invertebrate species (Bowen and Depledge, 2006). The present research study has demonstrated not only the sensitivity of this assay at environmental concentrations for the range of pharmaceuticals tested, but has also confirmed that the NRRA applied in *R. philippinarum* haemolymph constitutes a good biological biomarker of cellular distress (Coughlan et al., 2009). The NRRA is a short-term, sensitive and costeffective screening biomarker to be applied as an early warning of toxic chemical effects on biota (Viarengo et al., 2007; Bowen and Depledge, 2006).

3 Conclusions

The objectives of the study is to analyze the cellular well-being of *R. philippinarum*, in order to indicate health status after exposure to different environmental concentrations of pharmaceuticals, using LMS as biological indicator of exposure. The pharmaceutical gradient tested in this research (0–50 μg/L) is able to destabilize lysosomal membrane, in an overall dose-dependent and time-dependent manner. Lysosomal damage, evaluated in *R. philippinarum* haemolymph, constitutes a sensitive tool for evaluating exposure to environmental concentrations of caffeine, ibuprofen, carbamazepine and novobiocin, under laboratory conditions. LMS is considered a suitable biomarker for pharmaceutical contamination in aquatic environments, given its ability to demonstrate a detrimental contaminant effect; and LMS provides a robust Tier-1 screening biomarker for Rapid Assessment of Marine Pollution and Environmental Impact Assessments based on the 2-tier approach. Environmental concentrations of the drugs examined in this assay may nevertheless affect other aquatic organisms; therefore more research should be carried out in coastal environments, and using other ecotoxicological responses to evaluate the effects of carbamazepine and ibuprofen. Finally, the data reported in this study represent important ecotoxicological information and will provide a useful reference for the assessment of pharmaceutical exposure in marine organisms, using the manila clam as the bioindicator species and with a Tier-testing approach.

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