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The effect of the insecticide diazinon on the osmoregulation and the avoidance response of the white leg shrimp (*Penaeus vannamei*) is salinity dependent

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ABSTRACT

Diazinon is one of the insecticides that represent a high risk for Costa Rican estuarine environments due to its widespread use in pineapple plantations. In estuaries, organisms are frequently submitted to stress caused by natural factors (e.g., continuous changes in salinity levels) and, additionally, to stress due to contamination. Therefore, the driving question of this study was: will organisms be more susceptible to suffer the deleterious effects caused by diazinon because of the stress resulting from the salinity changes? The estuarine shrimp *Penaeus vannamei* was used as the model organism and two responses were measured: osmoregulation (the physiological effect after a forced and continuous 24 h-exposure) and avoidance [the behavioural effect after a short (3 h) non-forced, multi-compartmented exposure]. Juveniles were exposed to diazinon (0.1, 1, 10 and 100 μg/L) at three different salinities (10, 20 and 30). Disruption in the capacity to regulate the haemolymph osmotic pressure was observed at a salinity of 30 in individuals exposed to diazinon and methanol (used as vehicle). At that salinity, the ability of shrimps to detect and avoid the highest diazinon concentrations was impaired. *P. vannamei* juveniles inhabit environments with a high variation in salinity, but with an optimum osmotic point close to a salinity of 20; therefore, the higher the salinity, the greater the vulnerability of shrimps to the effects of diazinon. From an ecological point of view, this combined effect of salinity and contamination might also limit the spatial distribution of the organisms.

1. Introduction

Costa Rica is one of the countries with the highest pesticide use per cropland area in the world (FAO, 2019). Some of the crops produced more intensively for exportation, like banana and pineapple, are located in lowlands where their runoff is known to affect estuarine ecosystems (Arias-Andrés et al., 2018). The compounds utilized in those crops represent a risk of toxicity for non-target biota inhabiting those recipient habitats. Regarding the insecticides used for the aforementioned crops, diazinon is the one used most intensely; this is the case in pineapple production where more than 5 Kg of active ingredient ha^{-1} yr^{-1} is used (Bravo et al. 2013).

Diazinon [O,O-diethyl-O-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] phosphorothioate] is an organophosphate compound, used as an insecticide, acaricide and repellent. It is widely applied for pest control on various crops and in supplies for veterinary use (Lewis et al., 2016). The presence of diazinon residues has been detected in streams and rivers from all continents (Brunetto et al., 1992; Sosan et al., 2008; Ashauer et al., 2011; Derbalah et al., 2019; Hageman et al., 2019). The environmental concentrations are normally in the order of ng/L; however, exceptionally high concentrations have been recorded in freshwater ecosystems such as rivers and fishponds. For instance, 31 μg/L in a fishpond in Bangladesh (Hasanuzzaman et al., 2017), 70 μg/L in effluent from a pesticide factory in Egypt (Abdel-Halim et al., 2006), 96 μg/L in a river in Mexico (Arellano-Aguilar et al., 2017) and even 768 μg/L in a river in Iran (Fadaei et al., 2012). Further, some studies have detected diazinon in coastal environments influenced by agricultural areas (Arellano-Aguilar et al., 2017; Triassi et al., 2019) all of which provide evidence that this compound can pose a risk to estuarine ecosystems.

In Costa Rica, contamination by diazinon represents a serious environmental problem, as it has been among the 16 most imported pesticides in the period of 2000–2009 (de la Cruz et al., 2014) with widespread use in pineapple plantations (Echeverría-Sáenz et al., 2018).

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In Costa Rican freshwater ecosystems, the concentrations of diazinon do not usually surpass 3 μg/L (Echeverría-Sáenz et al., 2012) while in one coastal lagoon the concentrations recorded were below 0.1 μg/L (Arias-Andrés et al., 2018). However, the potential acute toxicity of diazinon for aquatic biota can be considered as being from moderate to very high (Rämö et al., 2018), especially if native species of crustaceans (i.e. *Macrobrachium digueti* and *Daphnia ambigua*) are considered (Arias-Andrés et al., 2018).

Diazinon is an inhibitor of cholinesterase (Sparling and Fellers, 2007); however, several types of damage, apart from neurotoxicity, have been observed in organisms exposed to it. Effects on neurodevelopment have been recorded in cell models (Flaskos, 2012); teratogenic effects (Aronzon et al., 2014) and changes in enzymatic activities (Ezemonye and Tongo, 2010) have been reported in amphibians; as well as changes in demographic parameters in daphnids (Stark and Vargas, 2003). In fish, endocrine disruption (Maxwell and Dutta, 2005), histological alterations (Dutta and Maxwell, 2003), reduced reproduction (Flynn et al., 2018) and imbalance in the levels of protein and nucleic acid (Ansari and Kumar, 1988) have been associated with exposure to diazinon. Despite the common presence of this compound in aquatic environments and its potential toxicity, studies in countries of Central America such as Costa Rica are incipient, especially in coastal waters where most marine species use estuaries as a nursery area at the juvenile stages (González-Ortegón et al., 2015).

Estuarine environments are among the most stressful aquatic biotopes, where salinity changes continuously (González-Ortegón et al., 2006). Therefore, marine species need to regulate their osmotic pressure to remain in a good physiological condition and cope with the natural variations in salinity (Péqueux, 1995; González-Ortegón et al., 2006). Any failure in the internal ionic regulation might affect their physiological homeostasis. Osmotic regulation in crustaceans requires some energy investment (Mantel and Farmer, 1983), which might increase when organisms must face stressful conditions like contamination (González-Ortegón et al., 2013). Previous studies with insecticides, such as diazinon, and the estuarine shrimp *Penaeus (=Litopenaeus) vannamei* (Boone, 1931) showed that the osmoregulation capability was altered at the high salinity of 50 (Galindo-Reyes et al., 2000). This shrimp spends the early part of its life cycle in estuarine-lagoon environments in Central America, where it is more likely to be exposed to higher levels of insecticides (Cuevas et al., 2018). Further, under the extreme conditions existing in these ecosystems, slight changes in osmoregulatory capability could be critical for the recruitment of this species in other estuarine-lagoon areas.

Besides the physiological disruption, diazinon might cause important behavioural alterations in organisms. For instance, diazinon caused hyperkinesis, erratic swimming and non-feeding in larvae of the amphibian *Rhinella arenarum* (Aronzon et al., 2014), altered swimming patterns in the fish *Oryzias latipes* (Chon et al., 2005), *Danio rerio* and *Pimephales promelas* (Steele et al., 2018). Under the umbrella of behavioural response, the avoidance response to contamination is an early mechanism to protect organisms from the exposure to contaminants (Lopes et al., 2004; Jutfelt et al., 2017; Araújo and Blasco, 2019). In fact, many organisms have proved to be able to avoid contaminants (see reviews by Cherry and Cairns, 1982; Araújo et al., 2016a, Moreira-Santos et al., 2019), particularly using the non-forced exposure approach (Lopes et al., 2004). Although no study about avoidance in multi-compartmented exposure systems has been performed with diazinon, some level of repellency would be expected as avoidance behaviour has been described for different species when exposed to some agrochemicals, such as: abamectine, atrazine and pyrimethanil, among others (see review by Moreira-Santos et al., 2019).

There is a strong relationship between osmoregulation and avoidance, as any behaviour demonstrated by organisms is strongly influenced by their physiological condition (Hellou, 2011). Although other physiological endpoints could provide us with information about how the changes in salinity affect the shrimps in the field, osmolality measurements provide information about how the changes in salinity could specifically affect the osmotic content in the shrimps; and thus, this physiological endpoint could show how changes in salinity may disrupt the physiological mechanisms of this group of organisms. If that occurs, it would be expected that the behavioural niche in shrimps would be modified and might explain their opportunities for dispersal and their colonization of new habitats along the coast (see González-Ortegón et al., 2016). In this regard, the assessment of their avoidance response to a contaminant, with a background of physiological stress induced by alterations in salinity, should improve our understanding of how the exposure and the spatial distribution of organisms can be affected in a contaminated estuary.

Although diazinon could be considered a moderately stable compound, studies about its risk to estuarine environments are less frequent, probably because the loss of the substance is expected to occur in estuarine environments due to processes of sorption to suspended matter, degradation and volatilization (Steen et al., 2001). In this sense, the aim of the present study was, firstly, to assess how changes in salinity together with contamination by diazinon could affect the osmoregulation of the estuarine shrimp *P. vannamei*. Secondly, whether the shrimps were able to detect potentially toxic diazinon concentrations and avoid exposure was evaluated, as well as whether this avoidance response could be influenced by changes in salinity. *P. vannamei* was used as the test organism as it is a common species cultured in coastal zones of Costa Rica (Valverde-Moya and Alfaro-Montoya, 2015; Valverde-Moya and Varela-Mejías, 2018) and because previous studies have shown its susceptibility to contamination by agrochemicals including diazinon (Galindo-Reyes et al., 2002; Damm and Rico, 2018) and its ability to avoid contaminants such as copper (Araújo et al., 2016b).

2. Material and methods

2.1. Test organism

The Peneid, *P. vannamei*, is naturally distributed along the Pacific coast of America, between Mexico and Peru. The post-larvae of this species migrate into estuaries and develop in these ecosystems until reaching the adult stage. They then leave the estuaries and spend their adult life in the open ocean (FAO, 2006–2020). Individuals of *P. vannamei* (mean size: 14.8 ± 1.3 cm carapace length (CL)) were sampled from a culture pond in Punta Morales, Puntarenas, Costa Rica, during October 2019. The shrimp were transported to the laboratory in seawater (salinity of 20).

In the laboratory, the organisms were maintained in a 100 L tank containing site water at 26 ± 2 °C, with biological filtration, constant aeration and fed daily *ad libitum* with commercial dry pellets (Nicovita 28% protein). The day before the experiments, the specimens were not fed. Only juveniles were used in the experiments, since this species only uses the marshes and estuary as a nursery area during its juvenile stages.

The acclimation of the organisms for assays at different salinities was performed gradually to diminish the natural stress of salinity change. That is, in estuaries, due to the short-term tidal salinity variations, osmotic equilibrium between the organism and environment may never be attained (Kinne, 1971). However, in a previous study with the white shrimp and experimental salinity changes every 3 h, it was demonstrated that this species was characterized as a strong regulator because it adapts rapidly to the new salinities by increasing and decreasing the haemolymph's osmotic concentration (Diaz et al., 2001). Once the shrimps were acclimatized to a salinity of 20, they were transferred to water with salinities of 15 and 25 for 24 h. Finally, the organisms from the salinity of 15 were transferred to the salinity of 10 and those from the salinity of 25 were transferred to the salinity of 30. The organisms were maintained during 24 h in their respective salinities before the osmoregulation or avoidance tests, it is worth stating that this acclimation-salinity period is longer than what occurs in nature, due to the time lengths of tidal change (González-Ortegón et al., 2006). The higher salinities were obtained by adding sea salt (Ocean fish, Prodac) to the seawater, and the lower salinity by diluting seawater with filtered, UV-treated tap water (Millipore). Salinity was determined using a calibrated multi-field meter (WTW Cond 315i; ± 0.1).

2.2. Diazinon

A stock solution (764.6 μg/mL) of diazinon (active ingredient 98.8%; CAS number: 333-41-5, Sigma-Aldrich, Saint Louis, Missouri, USA) was prepared in methanol (>99.9%, Riedel-de Haen' Seetze, Germany). Aliquots of this stock solution were added to the experimental chambers for the assays, using a micropipette. Additionally, a volume of methanol was added to ensure a similar concentration in all chambers: 0.001% (v/v) for the osmoregulation experiments and 0.01% (v/v) for the avoidance experiments. This difference was due to the use of a higher diazinon concentration (see next sections) in the avoidance experiments. A sample of each exposure solution was collected at the beginning and at the end of the osmoregulation experiments and at the end of avoidance experiments in order to quantify the diazinon concentration.

The diazinon analyses were performed by solid phase extraction. The samples were extracted using a Waters Oasis® HLB cartridge, 60 mg 3CC, previously conditioned with 1 mL methanol and 1 mL ultrapure water. After the conditioning, the water was passed through the cartridge and then it was dried and eluted with 1.5 mL methanol. Some extracts were diluted and some were concentrated, depending on the nominal concentration to be verified. All methanol extracts were analysed with an Acquity UPLC Waters system coupled to a Xevo TQ-S micro (triple quadrupole) tandem mass spectrometer. The analytical column was BEH C18 (2.1 mm x 100 mm 1.7 μm) at a flow rate of 0.4 mL/min. The mobile phase consisted of a binary water (0.1% HCOOH, 1 mM ammonium acetate)/methanol water (0.1% HCOOH, 1 mM ammonium acetate) gradient. The extracts were quantified by calibration curves prepared in the same solvents. Three water samples with salinities of 10, 20 and 30, spiked with diazinon and one laboratory blank of each, were analysed along the samples for quality control (QC).

2.3. Osmolality experiments

The experimental design consisted of the exposure to three diazinon concentrations that are environmentally relevant according to the aforementioned references (0.1, 1 and 10 μg/L), two control treatments without and with solvent and three salinities (10, 20 and 30). Each treatment was tested in triplicate and 10 shrimps were exposed in each tank (3 L). The organisms were tested for 24 h in the dark and with continuous aeration. After exposure, five organisms were taken from each tank to determine the osmotic concentration of the haemolymph. A single haemolymph sample of 50 μL was extracted from the shrimps' pericardial cavity by puncturing the pericardium with a 100 μL syringe. Haemolymph and water osmolalities were measured with a freezing point osmometer (Osmomat 3000, Gonotec GmbH, Germany). The CL of animals, from postorbital to posterior carapace edge, was measured after extracting the haemolymph.

2.4. Avoidance tests

The avoidance tests consisted of the simulation of a gradient of diazinon throughout which shrimps could move freely. A multi-compartmented exposure system was employed to simulate the gradient of diazinon (Fig. 1). Initially, control tests at different salinities (10, 20 and 30) were run to verify whether the shrimps' distribution in the system was random. In this test, the compartments were filled (3 L in each compartment) with diazinon-free water (the same water used in the culture of the shrimps) and, then, four shrimps were put in each compartment. In the test with diazinon, before the addition of the pesticide, the connections between the compartments were closed using plastic plugs. Tweezers were used to introduce the plastic plugs as shown in Fig. 1. Afterwards, aliquots of the stock solution of diazinon were added to each compartment in order to have a nominal gradient of 0.1, 1.0, 10 and 100 μg/L along the system. Four organisms were immediately put in each compartment and, then, the plugs were removed. In both control and avoidance tests, three replicates were performed for each salinity. The period of exposure was of 3 h and the shrimps' distribution was recorded every 30 min. The exposure was in the dark to avoid any visual interference on the behaviour of the shrimps. A red light was used during the observations of the organisms to reduce any possible interference caused by the observer.

2.5. Statistical analysis

For the salinities tested in the osmoregulation experiments (10, 20 and 30), the relationship between the haemolymph and water osmolalities was examined by least-squares linear regression analysis. The regression equation was calculated for the haemolymph osmolality curve and the isosmotic point was obtained directly from the intersection of the regression line with the isosmotic line. Linear regression models were used to evaluate differences between salinities and treatments using the robust statistical test multcomp and the sand-wich package in R, which allows post hoc comparisons for unbalanced data (Herberich et al., 2010) to be made.

A mixed-design ANOVA was used to check how the shrimps were distributed along the compartments of the system in the avoidance tests. The data concerning the percentage of shrimps (after arcsine square root transformation) were separated as a function of time (within-subjects factor; repeated measures) and compartments (between-subjects factors). The sphericity of the repeated measures was evaluated using Mauchly's test (see Tables S1a, 2a, 3a, 4a, 5a and 5a in Supplementary Material), and if the sphericity was violated (the variances of the differences were not equal) the Greenhouse-Geisser correction for degrees of freedom was applied. When statistically significant differences ($p < 0.05$) were observed for time or compartment, the Bonferroni test was

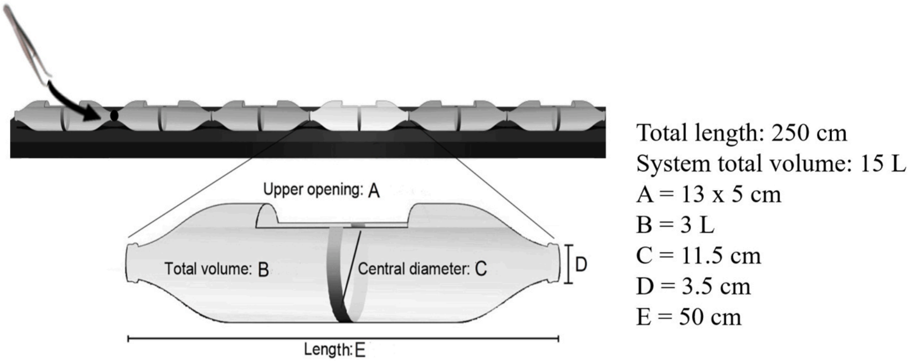

Fig. 1. Multi-compartmented exposure system used in the non-forced avoidance tests.

used. As the results of the organisms' distribution were not time-dependent (time: $p > 0.05$; see Tables S1b, 2b, 3b, 4b, 5b and 6b in Supplementary Material), data were pooled considering the means from the six observation times (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h).

The formulas proposed by Moreira-Santos et al. (2008) were applied to calculate the avoidance percentage. More details of this calculation have been previously described by Silva et al. (2018). The diazinon concentration that elicited an avoidance in x% [AC_X, ± confidence interval (CI)] of the exposed population was calculated using the PriProbit program (Sakuma, 1998).

3. Results

3.1. Diazinon quantification

During the osmoregulation experiments, the diazinon concentrations were above 60% of the nominal at the beginning of the assays in all salinities and dropped to between 35% and 59% by the end of the tests (Table S7a in Supplementary Material). Regarding the avoidance experiments, the concentrations of diazinon varied from nominal but a gradient was still maintained (Table S7b in Supplementary Material).

3.2. Osmoregulation response

At the two lowest experimental salinities (10 and 20), the haemolymph concentration of shrimps in the control treatment maintained hyperosmotic with respect to that of the medium (hyperosmoregulator), while at the high experimental salinity (30) a hypo-osmoregulatory pattern was observed. The slopes of the regression lines (R^2, Δ haemolymph osmolality *versus* Δ medium osmolality) indicated that shrimps exposed to diazinon and methanol presented low efficiency in osmoregulatory capability (Δ between 0.10 and 0.26) compared to the control treatment ($\Delta = 0.04$) (Table 1). The contrast tests showed stronger effects at a salinity of 30: the haemolymph osmotic pressure increased under diazinon and solvent control compared with shrimps in the control treatment (Fig. 2). Significant disruption in osmoregulation due to the presence of diazinon was recorded with the lowest concentration tested, and a trend towards increased osmolarity was observed in the highest diazinon concentration and the solvent control, at a salinity of 30.

3.3. Avoidance response

The control test performed to check the spatial distribution of the shrimps without contamination showed no preference (no statistically significant differences) for any compartment, either at salinity 10, 20 or 30 (Fig. 3 and Tables S1c, S2c and S3c in Supplementary Material).

The spatial distribution of the shrimps along the diazinon gradient was strongly influenced by the salinity (Fig. 3 and Tables S4c, S5c and S6c in Supplementary Material). At the salinity of 10, the shrimps were more frequently found (around 30% of the population) in the control compartment (with no diazinon). The lowest density of shrimp (14%) was recorded at the highest diazinon concentration (ca. 50 μg/L). At this salinity, a clear trend of concentration-dependent shrimp distribution was observed.

At salinity of 20, a clear preference for the uncontaminated compartment was also evidenced, with a mean of ca. 35% of the shrimp population occupying that compartment. However, no statistically significant difference was observed regarding the shrimp density between the control compartment and the highest diazinon concentration. The distribution of the shrimps was concentration-dependent in the three lowest concentrations (range from 0 to 10 μg/L), but it tended to increase in the two highest (around 30 and 50 μg/L) concentrations (Fig. 3).

At the highest salinity level (30), the avoidance behaviour of the shrimps was completely different from the results obtained in the two lower salinities. The distribution of the shrimps along the diazinon gradient was random, with no preference for any compartment, as observed in the control tests. Shrimps were not able to detect and avoid the diazinon (Fig. 3).

As the avoidance response was only clearly observed for the salinity of 10, a concentration-response sigmoidal curve has been plotted (Fig. 4). Considering that 20% would be the percentage of shrimp to be found if the shrimp distribution was similar in all the compartments (100% divided into 5 compartments) and that the percentage of avoidance was not higher than 30%, the AC_{20} value was also calculated. Data showed that in a concentration of 19 μg/L of diazinon, an avoidance of 20% of the population would be expected to occur.

Table 1
The relationship between the haemolymph concentration of *P. vannamei* (y) and water osmolality (x) from the three experimental salinities under different treatments.

Treatment	Regression Equation[a]	R^2[b]	Isosmotic point[c]	Length mean ± SD[d]
Control	$y = 0.044x + 638.2$	0.11	668 (24.7)	14.6 ± 1.1
Solvent control	$y = 0.265x + 531.1$	0.52	772 (28.5)	15.6 ± 1.1
Diazinon 0.1	$y = 0.269x + 527.2$	0.84	721 (26.6)	14.2 ± 1.0
Diazinon 1	$y = 0.102x + 622.4$	0.35	693 (25.6)	14.6 ± 0.9
Diazinon 10	$y = 0.224x + 551.7$	0.73	711 (26.3)	14.5 ± 1.2

[a] y = haemolymph concentration and x = medium osmolality.

[b] Correlation coefficient.

[c] Isosmotic point expressed in mmol/Kg and salinity (indicated in parentheses).

[d] Length of carapace (LC, in mm).

4. Discussion

This study was intended to provide an approximation of how a natural stressor such as increased salinity, in combination with the presence of one of the most common insecticides used in the world, diazinon, can affect the physiology and avoidance behaviour of a representative estuarine shrimp in Central America.

Osmoregulatory capacity has already been used in decapod crustaceans (Nieto et al., 2016) to monitor the effect of different stressful conditions, including contaminants. For instance, Charmantier and Soyez (1994) showed that sublethal levels of low oxygen tension resulted in significant declines in the osmoregulatory capacity in *Penaeus* (=*Litopenaeus*) *vannamei*. As expected and similar to other studies (Diaz et al., 2001; Chong-Robles et al., 2014), the haemolymph osmotic pressure of *P. vannamei* between the salinities 10 and 30 indicated that it is a euryhaline species with an isosmotic point at around 25 of salinity. Nevertheless, the haemolymph osmotic pressure and consequently the osmoregulatory capacity of *P. vannamei* were modified under the exposure to diazinon at 30 of salinity. In the particular case of diazinon, exposure to this compound changed the osmoregulation ability of fingerlings of the Persian sturgeon (*Acipenser persicus*) (Hajirezaee et al., 2016) and the Caspian roach (*Rutilus rutilus*) (Katuli et al., 2014). Better growth of *P. vannamei* juveniles was observed at salinities of between 15 and 25 when compared to the salinity of 30 (Huang, 1983; Valverde-Moya and Varela-Mejías, 2018). Juveniles of marine species need to maintain specific osmotic gradients between their body fluids and the ambient medium to remain in good physiological condition and cope with natural variations in salinity characterising these habitats (González-Ortegón et al., 2006; Péqueux, 1995). Our experiment was carried out using methanol as vehicle for diazinon. The concentration of the solvent in the chambers (0.0103 g/L) was four hundred times below a concentration expected to cause toxicity (4.8 g/L) to a model

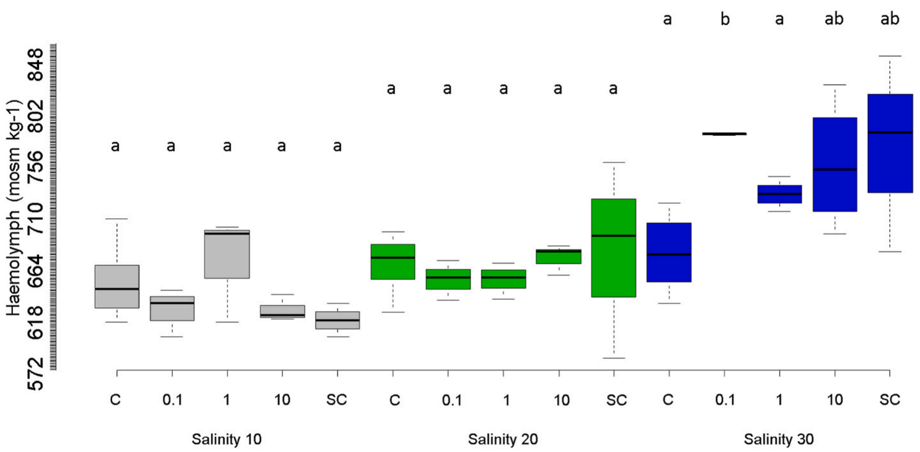

Fig. 2. Boxplot of haemolymph osmolality (mosm/kg) for each treatment and experimental salinity (10, 20 and 30) of *L. vanammei* in relation to the water salinity. Middle line = median; upper edge = 75th percentile; lower edge = 25th percentile; lines = variability outside the quartiles. The letters on top of each boxplot indicate statistically significant differences (max-t-test: $p < 0.001$) among treatments under the same salinity. C = Control; SC = Solvent Control; 0.1, 1 and 10 = concentrations of diazinon in μg/L.

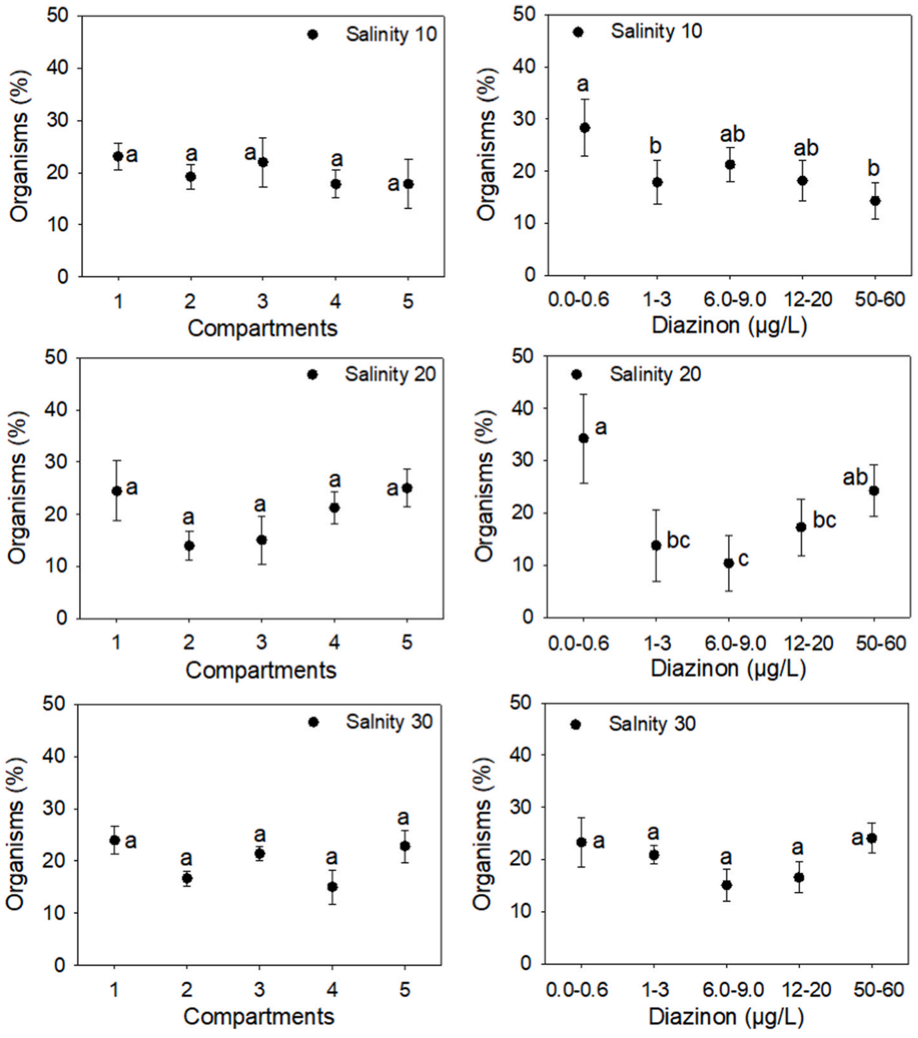

Fig. 3. Percentage of shrimps (mean of six observations: at each 30 min during 3 h) found in the different compartments (1–5 for the control tests) of the control test (graphs on the left) and the test with diazinon gradient (graphs on the right), at different salinities: 10, 20 and 30. Different letters on the means indicate statistically significant differences ($p<0.05$; Bonferronni test). Concentrations are presented as ranges such as shown in Table S7b (Supplementary Material).

crustacean species (Kaviraj et al., 2004). Further, the exposure of *Daphnia magna* to a concentration of methanol five times higher than the one used here, showed not to interfere with a metabolic biotransformation process (David et al., 2012). However, an effect of the presence of methanol on osmoregulation was observed in *P. vannamei*. The increased haemolymph osmotic pressure observed in the shrimps exposed to diazinon and methanol at the salinity of 30 was probably the consequence of a disruption of the osmotically active ion content of the haemolymph. Osmotic regulation in crustaceans requires some energy investment to compensate the regulatory mechanisms (Mantel and

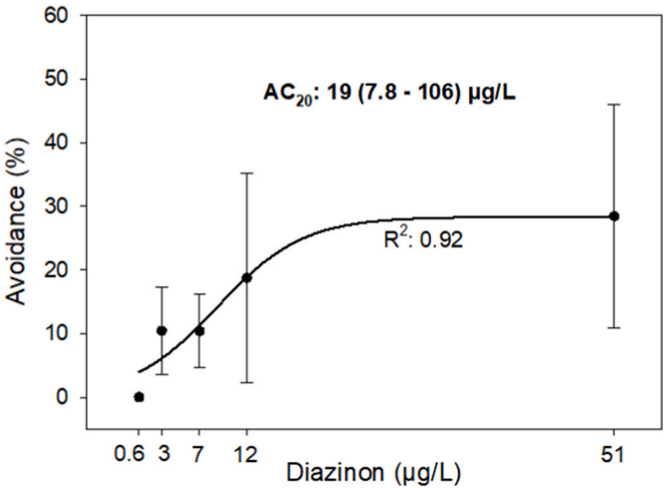

Fig. 4. Avoidance percentage of shrimps exposed to a diazinon gradient at the salinity of 10. The R^2 value of the sigmoid curve and the AC_{20} value are also presented.

Farmer, 1983). Under exposure to diazinon in the presence of methanol, the juveniles did not regulate the internal ionic concentration correctly, which may have a significant influence on the performance of the population in coastal waters in the presence of contamination. Previous studies have showed that the osmoregulation capability of *P. vannamei* was altered at the very high salinity of 50 (Galindo-Reyes et al., 2000), and the range of optimum salinities for growth is even below the isosmotic point at the salinity range 5–15 (Bray et al., 1994). In the current study, the increase in salinity seems to make *P. vannamei* more vulnerable to the effects of pollutants. The juveniles of *P. vannamei* mostly spend their time in estuarine-lagoon environments, where they are more likely to be exposed to higher levels of insecticides such as diazinon. Any environmental effect that leads to an increase in the salinity (e.g., the reduction of freshwater discharges from rivers) could be critical for the survival of this species and, thus, for its spatial recruitment.

The avoidance response was evidenced in the shrimps when exposed to diazinon. The capacity of freshwater (Araújo et al., 2019a; Vera-Vera et al., 2019) and marine (Araújo et al., 2016b, 2019b; Ehiguese et al., 2019) shrimps to perceive contamination in their environment and to escape to less polluted areas was attested previously. Regarding diazinon, behavioural alterations have also been described before in the shrimp *Metapenaus ensis* exposed to 0.1 mg/L (Chu and Lau, 1994). The capacity of *P. vannamei* to detect and avoid diazinon was affected by salinity. At the lowest level of salinity (10) tested, a level in which no evidence of osmotic stress was observed, the shrimps avoided diazinon, but at a salinity of 30, avoidance of the highest concentrations was impaired. It is known that this response is not linear, but instead it depends on the other factors due to the cost-benefits balance that organisms might make to assess the risks (De Lange et al., 2006; Araújo and Blasco, 2019). Therefore, it is expected that the avoidance response is altered by the presence of some abiotic environmental factor [either from anthropogenic origins (e.g., other contaminants) or natural (salinity)] that disrupts the organisms' capacity to sense and react to chemical stressors or simply changes the aversiveness of the ecosystem. Changes in salinity can affect the mobility of estuarine crustaceans when animals are out of their optimal salinity range (Michalec et al., 2010), although the salinity stress can also increase the activity as the organisms try to escape from unfavourable environments (Rivera-Ingraham and Lignot, 2017). This physiological imbalance might partially explain why, at a high salinity and where the shrimp were suffering osmotic stress, they were unable to avoid diazinon. This could have serious implications for estuarine species, as exposure to chemical pollutants would be augmented due to the masking effect of another environmental stressor. If a confounding factor or physiological alteration interferes with the ability to perceive a harmful environment, the organism might not avoid the contaminated site and, therefore, be more susceptible to suffer the toxic effects caused by the continuous exposure to contamination. In the present study, an increase in salinity disrupted the avoidance response by *P. vannamei* that was not able to avoid high concentrations of diazinon.

Estuaries are ecosystems that are particularly sensitive to global change. On the one hand, local variations of temperature, salinity, dissolved oxygen and other physical-chemical parameters represent a major source of stress for biota. Further, the global change-associated increase in pesticide use in upstream agricultural areas, together with changes in the rainfall patterns of heavier storms that increase runoff alternating with periods of drought that increase the salinity in estuaries is making animals cope with an increasing exposure to pollutants while suffering increased physiological stress (DeLorenzo, 2015). This is of particular interest on the Pacific coast of Costa Rica, where the impacts of the local phenomenon el Niño, as well as global climate change are reducing the rainfall and increasing the salinity of estuarine environments (Valverde-Moya and Varela-Mejías, 2018). This situation has already been related to the increment of infectious diseases, reduction of productivity and even massive mortalities in local culture ponds of *P. vannamei* (Valverde-Moya and Varela-Mejías, 2018). The present study reinforces the idea that the effects of contaminants are not only dependent on their concentration, but also on their interactions with other environmental factors. The continuous variation in salinity in coastal zones might cause intermittent stressful conditions for shrimps, which are more intense when environmental conditions (e.g., high salinity, in the current case) are more unfavourable. The integration of two different approaches of ecotoxicological studies, such as the classical approach assessing toxic effects (by using forced and mandatory exposure system) and the avoidance response that assesses the preferential displacement of organisms in a contamination gradient (by using a non-forced exposure system), provides a new vision of the role of contamination in ecosystems. The risk of contamination might be variable and dependent on the presence of contamination levels (e.g., concentrations that require detoxification) or even on the association with other potentially stressful factors (e.g., a high salinity that makes contamination more dangerous even at low concentrations). Finally, it is important to highlight that within the context of global change, we recommend testing the effects of chemical pollutants in a gradient of factors (e.g., temperature) that usually oscillate continuously for estuarine organisms susceptible to a varying environment. As well, it is important to consider that the use of other compounds such as methanol in order to better dissolve the studied compound may depend on the organism and the exposure to specific environmental conditions. This reopens a discussion in ecotoxicology when other chemical compounds are used as vehicle to actually test the target contaminant.

5. Conclusions

Juveniles of *P. vannamei* proved to be sensitive to contamination by diazinon and this sensitivity was salinity-dependent. The toxic effects were related to the loss of the ability to (i) regulate the osmotic pressure of the haemolymph in the presence of diazinon and methanol and (ii) detect and escape from potentially toxic diazinon concentrations. For both osmoregulation and the avoidance responses, the effects were more pronounced when the shrimps were tested at high salinity (30); however, data at the salinity of 30 showed an unexpected, but strong effect of the solvent methanol, regardless of the diazinon exposure. As *P. vannamei* inhabits environments with a high variation in salinities, this higher susceptibility of shrimps to the increase of salinity makes them more vulnerable to the effects of diazinon and might limit their spatial distribution according to contamination levels and salinity. The evaluation of different sub-lethal endpoints (physiology and behaviour, in this case) might improve the understanding of the effects of pollutants at environmentally relevant concentrations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111364.

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