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TOXICITY OF THREE COMMERCIAL TANNINS TO THE NUISANCE INVASIVE SPECIES *LIMNOPERNA FORTUNEI* (DUNKER, 1857): IMPLICATIONS FOR CONTROL

Patricio J. Pereyra[1,*], Gustavo Bulus Rossini[2] and Gustavo Darrigran[1]

[1] GIMIP División Zoología de Invertebrados, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Paseo del Bosque s/n, (1900) La Plata, Argentina.
[2] Centro de Investigaciones Medio Ambientales, CIC-PBA (1900) La Plata, Argentina.

ABSTRACT

Adding biocides to water is one strategy to control macrofouling organisms. A natural biocide that helps to prevent/control macrofouling of *Limnoperna fortunei* (Dunker, 1857) on human installations is one way to minimize environmental impacts of different control strategies. Laboratory tests were carried out to evaluate effects of three commercial tannis preparations (ECOTEC®-UA, ECOTEC®-L and ECOTEC®-MC) on the survival of two life-history stages (larvae and adults) of *L. fortunei*. In addition tests were performed on two non-target species, a crustacean *Daphnia magna* and a plant *Lactuca sativa,* to evaluate effects of these tannins on the aquatic environment. The larvae of *L. fortunei* were more vulnerable to the concentrations of the three tannins than adults. The two non-target species were not affected at concentrations that were effective for larvae. These results suggest that these products could be used as biocides to control macrofouling caused by *L. fortunei*.

KEYWORDS: *Limnoperna fortunei*, macrofouling, bioassays, tannins, biocide, Argentina

1. INTRODUCTION

Bivalve molluscs are one of the more important groups of organisms that cause macrofouling on human installations [1]. Among these, in the Holartic region, the zebra mussel *Dreissena polymorpha* (Pallas, 1771) and in the Neotropical region golden mussel, *Limnoperna fortunei* (Bivalvia: Mytilidae) are noteworthy [2, 3]. The invasion of *L. fortunei* in the Neotropical region began in 1990 [4] and impacts on natural and man-made structures are similar to those observed for *D. polymorpha* in the Holartic region [5-8]. This type of macrofouling is unusual in freshwater habitats in the Neotropical region since there are no native freshwater bivalves that cause it [6].

Limnoperna fortunei infects man-made structures and causes macrofouling in three places in Southeast Asia: Hong Kong [9], Taiwan [10] and Japan [11]. In South America, invasions have been recorded in various power stations (nuclear, thermal and hydroelectric) [8], water treatment plants [6], and irrigation systems [7]. Among the many problems caused by this species, the following are noteworthy: obstruction of filters and pipes, production of turbulent flow, loss of hydraulic capacity and an increase in corrosion [12].

Among the treatments for controlling macrofouling (*e.g.* manual and mechanical removal, the use of filters, manipulation of the water temperature, use of antifouling paints ultraviolet light and ozonization [13-15], addition of biocides to water is one of the most widely used techniques, both for macrofouling in general and for the golden mussel in particular [13, 16, 17]. One of the compounds most often used as a molluscicide is sodium hypochlorite [17, 18] but this compound corrodes the pipes and produces carcinogenic compounds at certain concentrations [19]. Therefore, it is important to look for alternative biocides that are less damaging than some now widely used [20-22].

Tannins are a group of plant compounds, principally glucoside complexes of catechol and pyrogallol, which consists mainly of gallic acid residues that linked to glucose by glucoside bonds. They are natural compounds common in higher plants and brown algae and are found in practically any body of water where there is a large amount of decaying vegetation. The presence of tannins is most noticeable by the presence of yellowish to brown water. Tannins are also important in industry [23, 24] and very common in food (beverages, wines, cereals and berries) [25].

Recent studies show that quebracho tannins might have antifouling properties that are effective against marine organisms such as *Balanus amphitrite* and *Polydora ligni* [26, 27].

* Corresponding author

In this context, it is believed that, the tannins' preparations of quebracho colorado (*Schinopsis balansae*, Engl) are (i) toxic to *L. fortunei*, and, (ii) the toxicity is a function of size and stage of the target individuals. Moreover, effects on non-target species are low in relation to concentrations that affect *L. fortunei.*

2. MATERIALS AND METHODS

2.1. Characteristics of tested products

Tannin preparations used in this study were obtained by a three step process: (1) grinding and diffusion; (2) primary evaporation; and (3) chemical treatment and spray-drying.

The grinding process (step 1) included cutting quebracho colorado logs and boiling them in water at 130 °C to obtain tannin extract with 10 – 11% of solid matter. The primary evaporation process (step 2) was used to obtain tannin extract of 55% solid matter. Finally, chemical treatment (step 3) of the extract was diluted with sodium bisulphate; its properties (color, percentage of salts and insoluble) were controlled, and then spray-dried to obtain the products of dust ground material (more details are provided at http://www.unitan.net/esp/unitan/index.html).

Even though all the tested products were tannins preparations, they differed in some physicochemical properties (Table 1) such as the percentage of natural polyphenols (74, 70 and 86% for ECOTEC-UA®, ECOTEC-L® and ECOTEC-MC® respectively).

TABLE 1 - Characteristics of the three tannins tested (UNITAN SAICA pers. com.).

	ECOTEC-UA®	ECOTEC-L®	ECOTEC-MC®
Tannins (% max)	74	70.0	86.5
Observed red colour	1.9 - 2.2	1.1 - 1.4	
Observed yellow colour	3.8 - 4.2	2.2 - 3.0	
pH	4.7 - 5.1	4.0 - 4.4	4.1 - 4.5
Insolubles (% max)	0.0	2.0	2.5
Ashes (% max)	7.2	5.5	2.5
Humidity (% max)	9.0	9.0	9.0

2.2. Collection of organisms

Larvae of *L. fortunei* were collected near the Argentine coast of Río de la Plata (34°49´S - 57°56´W) by filtering 1000 L water with a 45µ net. The presence of larvae was verified with a stereoscopic microscope before performing tests.

Adult specimens of *L. fortunei* were manually collected in the field. Individuals were transported to the laboratory where they were acclimatized for fifteen days in 10 L acuaria (conductivity = 1 ± 0.3 ms/cm, temperature = 23 ± 2 ºC; pH = 7 ± 0.5). Aquaria water was changed completely the first three days and then partly changed (5 L) every two days up to the end of the fifteenth day of the acclimatization period. Specimens were fed daily with TetraMin® fish food, except for the two days before the tests.

Daphnia magna neonates used in toxicity tests were obtained from cultures maintained in standard conditions (conductivity 1,0 ms/cm; hardness 215 mg/L CO_3Ca; alcalinity 180 mg/L CO_3Ca, pH range 7,6 ± 0,2, temperature 20 ± 2 ºC, photoperiod 16:8 light:darkness and fed with chlorococcal algae) [28].

The seeds of *L. sativa* (mantecosa variety) were obtained from commercial suppliers with 98% guaranteed germination.

2.3. Biocide tests

Toxicity tests with *L. fortunei* were performed on two stages of their life cycle: larvae and two sizes of adults (13 ± 1 mm and 19 ± 1 mm). All tests were carried out in triplicate using at least five concentrations and controls and using tap water for the dilutions and controls. The temperature (23 ºC, min = 21.8 ºC, max = 23.7 ºC), photoperiod (16:8 light: darkness, pH (7.31, min = 7.08, max = 7.55), dissolved oxygen (> 6 mg/L) and the conductivity (1 ± 0.3 ms/cm) were controlled during tests.

Tests with larvae were performed by placing 12 ml of each concentration of a tannin preparations in plastic Petri dishes containing between 10 and 15 larvae per dish. The mortality in each Petri dish was controlled after 24 h using a stereoscopic microscope (Figure 1). Larvae were considered dead if swimming or signs of internal activity were not detected (Figure 1).

Tests on adults were performed by selecting individuals by size (0.01 mm precision) the day before the start of the tests. Then, 12 individuals were randomly selected and placed in replicate in Petri dishes. Dishes were submerged in 250 ml dilution water. After 24 h, specimens that did not attach themselves to the Petri dishes with their byssal threads [28] were eliminated from the tests. Next, Petri dishes and attached specimens were submerged in 250 ml of different test concentrations to be tested for 168 h. The controls were prepared in a similar way. Mortality was determined after 168 h exposure. A mussel was considered dead when it does not respond to mechanical stimuli.

Standard toxicity tests of *Daphnia magna* and *Lactuca sativa* were used to evaluate any potential ecotoxic effects of the studied products. The acute toxicity tests of 24 and 48 hours exposure of *D. magna* neonates were performed by following the protocol of USEPA [29]; using tap water for solutions and control (conductivity 1,0 ms/cm; hardness 215 mg/L CO_3Ca; alcalinity 180 mg/L CO_3Ca, pH range 7,6 ± 0,2). Tests were carried out under controlled temperature (20 ± 2 ºC) and photoperiod (16:8 light:darkness) conditions, and the neo-nates were not fed during the assay. Germination/ elongation tests of roots of *L. sativa* were performed for 120 h of exposure to darkness using double distilled water as dilution water, according to the USEPA protocol [30], at 22 ± 2 ºC temperature. Tests for *D. magna* and *L. sativa*, were both carried out in triplicate using at least five concentrations and a control.

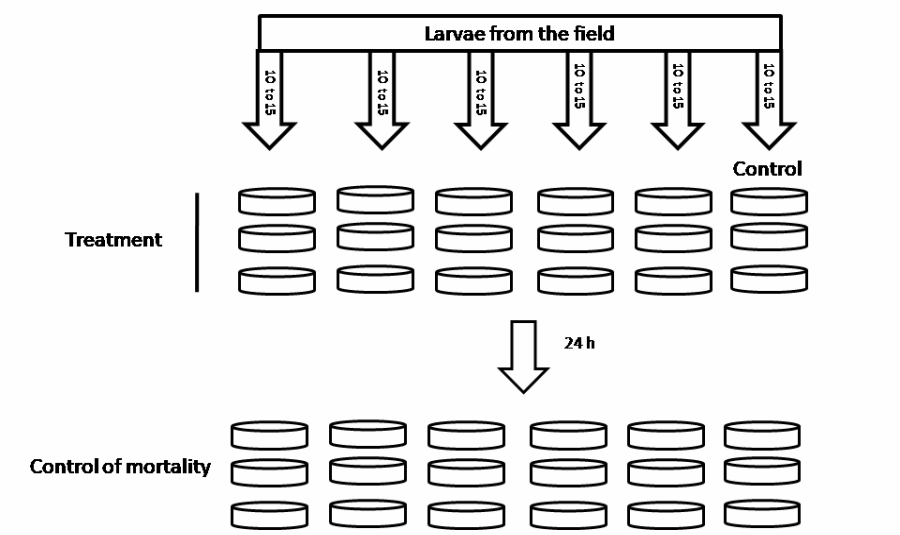

FIGURE 1 - Scheme of a larvae bioassay. The larvae are collected in the field and its number is checked under stereoscopic magnifying glasses. Then, three replicates of each concentration and a control are made. Twelve milliliters of each solution are added into plastic Petri dishes, along with 10 to 15 larvae. After 24 h, the mortality is checked. More details are provided in the text.

2.4. Analysis of the results

The LC_{50} (lethal concentration on 50% exposed organisms) was used as the measure of efficacy for *Limnoperna fortunei*. It was calculated using linear regression with the concentration previously transformed to the logarithm and the mortality to Probit units [31] by using the Probit program, version 1.5, of USEPA. The lower concentration that effectively produced 100% mortality of the exposed individuals was considered as LC_{100}.

In order to compare the toxicity of the three tannin extracts an analysis of variance (ANOVA) between extracts and between life and sizes of adults by application of a generalized linear model of homogeneous slopes [32] and considering the stages and sizes as covariables associated to the age of the individuals used.

In the case of *D. magna* all estimations of the LC_{XX} were undertaken with the Probit programme, considering LC_1 as NOEC (the highest concentration of the product at which no significant differences were found when compared to the control) and LC_{10} as LOEC (the lowest concentration of the product at which a significant difference was seen as regard to the control).

In the case of *L. sativa*, the IC_{50} (inhibitory concentration 50) was obtained by adjusting the results with a linear regression, with the concentrations previously transformed to logarithms, and using the percentage of inhibition as the dependent variable as relative to the control. The NOEC and LOEC were obtained using an *a posteriori* comparison of Dunnett before ANOVA.

3. RESULTS

Results obtained for each product and each stage of *L. fortunei* are summarized in Table 2. In the case of the larvae, a LC_{100} of 400 mg/L was determined for the three tested products. The LC_{100} for the adult stages was not estimated as no tests showed 100% mortality after 168 h exposure in any of the concentrations tested.

In the tests with adults it was seen that they stopped filtering while submerged in the tannin solutions, closing their valves. This information was not quantified to avoid manipulations during the tests. This behaviour was not observed in the controls where individuals continued with their normal filtering behaviour after being transferred.

TABLE 2 - Concentrations (mg/L) of LC_{50} and 95% confidence intervals of three tannin extracts tested on larvae and two sizes (13 and 19 mm) of adult *Limnoperna fortunei*.

	Larvae			Adults					
				13 mm			19 mm		
	LC_{50}	Lower limit	Upper limit	LC_{50}	Lower limit	Upper limit	LC_{50}	Lower limit	Upper limit
ECOTEC-UA®	160,21	94,06	235,31	983,27	763,36	1465,61	1273,73	983,27	2066,6
ECOTEC-L®	138,54	89,67	277,11	309,92	232,18	414,91	442,14	349,39	564,93
ECOTEC-MC®	138,53	96,7	182,95	160,1	75,45	283,4	557,71	428,83	818,96

TABLE 3 - LC_{50}, LOEC and NOEC of *Daphnia magna* for the tests of 24 and 48 h exposure. All concentrations are expressed in mg/L. NOEC is the highest concentration of the product at which no significant differences were found when compared to the control; LOEC is the lowest concentration of the product at which a significant difference was seen as regard to the control.

	24 h trials								
	LC_{50}	Lower limit	Upper limit	LOEC	Lower limit	Upper limit	NOEC	Lower limit	Upper limit
ECOTEC-UA®	896,21	823,75	994,18	584,48	508,6	644,17	412,51	323,5	479,92
ECOTEC-L®	635,76	547,42	701,14	409,54	284,01	491,18	286,15	163,06	374,91
ECOTEC-MC®	849,53	731,22	1081,05	462,62	389,17	525,78	281,87	197,26	344,7
	48 h trials								
	LC_{50}	Lower limit	Upper limit	LOEC	Lower limit	Upper limit	NOEC	Lower limit	Upper limit
ECOTEC-UA®	348,29	322,44	370,63	248,01	207,17	276,38	188,04	142,03	221,3
ECOTEC-Ñ®	363,65	329,78	385,82	290,00	224,85	322,41	241,14	162,26	282,44
ECOTEC-MC®	341,58	318,07	366,24	244,24	214,08	267,82	185,81	151,86	212,32

The LC_{50}, NOEC and LOEC for *D. magna* and the IC_{50}, NOEC and LOEC for *L. sativa* are shown in Tables 3 and 4, respectively. The IC_{50} for *L. sativa* were higher than the LC_{50} calculated for all stages of *L. fortunei* and overlap was only observed in the 95% confidence intervals for product ECOTEC-UA® (Tables 1 and 3). The LC_{50} for *D. magna* were higher than those estimated for the larval stages of *L. fortunei* and there is no overlap between their respective confidence intervals (Tables 1 and 2).

TABLE 4 - IC50, LOEC y NOEC for *Lactuca sativa*. All concentrations are expressed in mg/L. NOEC is the highest concentration of the product at which no significant differences were found when compared to the control; LOEC is the lowest concentration of the product at which a significant difference was seen as regard to the control.

	ECOTEC-UA®	ECOTEC-L®	ECOTEC-MC®
IC_{50}	1748,02	2005,83	2023,83
L_{inf}	1303,52	1555,61	1472,65
L_{sup}	2344,09	2586,33	2781,29
NOEC	750,00	500,00	750,00
LOEC	1000,00	750,00	1000,00

In the case of *L. sativa*, the NOEC were higher than the LC_{100} of the larvae for all three products, whereas in *D. magna* only product ECOTEC-UA® had a slightly higher NOEC, although it is not yet possible to confirm that these differences were significant.

The analysis of variance, using stages and sizes as covariables, the tested products as factor and the LC_{50} as dependent variables, indicates that there is no treatment effect ($p \leq 0,8893$) but there is a significant effect of the covariable ($p \leq 0,00986$). The same analysis shows that there is no interaction between the factor and the covariable ($p \leq 0,07386$).

4. DISCUSSION

The results achieved in this study indicate that tannin preparations have acute toxicity effects in *L. fortunei*. In addition larvae are more susceptible to the products than adults. This is in agreement with the literature on the vulnerability of early life stages of most organisms [33, 34].

When working with bivalve mollusks the biggest problem faced is their capacity to detect biocides, as they close their valves or reduce the siphoning activity [13, 33, 35], which makes it necessary to increase exposure times to obtain mortality. Tests on *L. fortunei* using sodium hypochlorite show 100% mortality at 1.2 mg/L in 576 h [36] and in 408 h with 1 mg/L at 25ºC [13]. Similar results were obtained with sodium hypochlorite for *D. polymorpha* (95% mortality after 552 h exposure at 1 mg/L) [34], *Mytilus edulis* (Linnaeus 1758) (480 h to reach 100% mortality with 1 mg/L) [37], and *Perna viridis* (Linnaeus 1758) (816 h to reach 100% mortality when exposed a continuous application of 1 mg/L residual chlorine) [38]. Although some molluscicides are effective in less time for both *L. fortunei* [13, 39] and *D. polymorpha* [28] they may be aggressive to other non-target species and they are often more costly.

Tests carried out on *D. magna* and *L. sativa* show higher LC_{50} than those for the larval stages but similar to those obtained for the adults. The comparison of the results for *D. magna* show that the lower confidence limits of LOEC at 24 h are higher than the LC_{50} of the larvae and they do not differ very much with the concentrations at which 100% mortality of the larvae (400 mg/L) occured. This evidence suggests that the evaluated tannins could be considered as biocide. The concentrations at which they would have the desired effect (100% mortality) with only one minimal dilution in an aquatic system would not show any acute effects on the two non- target species tested.

On the basis of this, use of biocides for control of the golden mussel should be concentrated on the larval stage. Studies of *D. polymorpha* show the vulnerability of the larval stages. Some studies show that the larvae are more vulnerable than the adults in five out of six products tested over 24 h [40]. Other studies working with cationic polymers, show that larvae are more sensitive than adults [34]. Similar conclusions are reached when working with *L. fortunei* exposed to quaternary polymers [39].

Some authors [39, 41] propose that biocides that do not cause mortality, but rather prevents settlement of larvae would be considered successful. Yebra et al [42] mentions that treatments without biocide capacity but highly anesthetic, settlement deterrent or with settlement inhibitory

properties should be considered in control strategies. Studies based on paints with quebracho tannins in seawater [26, 27] were developed following this logic, and they showed antifouling potential. Considering these and the present results, further studies with quebracho tannins and its derivates are recommended.

However, it should be noted that there is no general technique for controlling the *L. fortunei* on all man-made structures, on the contrary, a combination of treatments is necessary. Each water intake has to be considered separately by structure, given its complexity, and depends on the function of each one, so that the best sequence of control techniques can be established [7, 14, 16]. In this sequence, applying a biocide may be one of several treatments to be applied within the system.

Finally, studies in the field are necessary to determine the applicability of any control strategy. At present, it is common to consider that the aquatic invasions are difficult to control, often impossible [43, 44]. In fact, no method seems to stop the expansion of the golden mussel in the American continent and beyond [45, 46]. There is not even a local, regional or continental program to control or eradicate the problem; this is the same case with the zebra mussel in the U.S. [47]. Attempts to control those species in open waters are scarce and were made with potassium chloride for *D. polymorpha* [47] and chlorine for *L. fortunei* [48]. The impacts on non-target organisms of these attempts are not reported, but the deleterious effect of the chlorine is well known [17]. For example, eradication of the black-striped mussel, *Mytilopsis salei* (Recluz, 1849), of a 600 megalitre marina was made with about 200 tons of bleach, and killed all living organisms [49, 50]. In this context, to find an environmentally friendly biocide against fouling mussels is a management priority.

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CORRESPONDING AUTHOR

Patricio J. Pereyra
GIMIP División Zoología de Invertebrados
Facultad de Ciencias Naturales y Museo
Universidad Nacional de La Plata
Paseo del Bosque s/número
1900 La Plata
ARGENTINA

Phone/Fax: 054-0221-4257744
E-mail: p_pereyra@fcnym.unlp.edu.ar

FEB/ Vol 20/ No 6/ 2011 – pages 1432 - 1437