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Toxicity of Neem's oil, a Potential Biocide against the Invasive Mussel *Limnoperna fortunei* (Dunker 1857)

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ABSTRACT

The golden mussel *Limnoperna fortunei* (Dunker 1857) is one of the most distributed Nuisance Invasive Species (NIS) in South America, and a threat of great concern for the industry of the area. In this study, we carried out toxicity tests made with a Neem's oil solution with *L. fortunei* larvae and benthonic adults (7, 13 and 19 ± 1 mm). Tests with non-target species (*Daphnia magna*, *Lactuca sativa* and *Cnesterodon decemmculatus*) were also made with the aim to evaluate the potential toxicity of the Neem's solution in the environment. The LC_{100} of Neem's solution obtained for larvae was 500 µl/L, a value much higher than the one obtained for *D. magna* and *C. decemmaculatus*. Thus, we recommend that it should not be used in open waters. However, since the adults were killed in 72 h and the larvae in 24 h, this product can be used in closed systems, in man-made facilities.

Key words: biocide, impact, *Limnoperna fortune*, *i* Neem's oil.

INTRODUCTION

Nuisance Invasive Species (NIS) are a major worldwide environmental problem (Mack et al. 2000, McGeoch et al. 2010), chiefly in freshwater systems (Sala et al. 2000, Strayer 2010). Bivalve mollusks are considered an aggressive NIS, both for their ability to affect the structure of the environment and the availability of resources in invaded ecosystems (*i.e.* ecosystems engineers, *sensu* Strayer et al. 1999), and their ability to infest man-made structures (Darrigran 2002, Darrigran and Damborenea 2005).

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The golden mussel *Limnoperna fortunei* (Bivalvia: Mytilidae) is one of the NIS most distributed in South America (Boltovskoy et al. 2006) and since its accidental introduction in 1991 (Pastorino et al. 1993) it has invaded streams and rivers in the Paraná basin, in at least four countries: Argentina, Brazil, Paraguay and Uruguay (Brugnoli et al. 2005, Darrigran 2002, Darrigran and Damborenea 2011, de Oliveira et al. 2006).

Limnoperna fortunei infests all types of man-made facilities: treatment plants, irrigation channels, reservoirs, hydroelectric power plants and fisheries (Darrigran 2010, Pestana et al. 2008, Rolla and Mota 2010). The most common strategy

to fight this kind of trouble is the use of biocides (Mackie and Claudi 2010). The main advantage offered by this strategy is that it can be engineered to protect the entire facility, from the intake to the discharge, without altering the operability of the facility (Mackie and Claudi 2010).

Chlorine is the most used biocide, mainly because of its low cost, although its deleterious effects on the biota are well known (Mackie and Claudi 2010). Others treatments (e.g. ozone, sodium hydroxide) are starting to be used in man-made facilities in South America (Mackie and Claudi 2010). Now there is an increasing pressure to avoid the use of toxic substances against non-target organisms (e.g. IMO 2007), and the current tendency is to find environmentally friendly biocides (Qian et al. 2010, Yebra et al. 2004).

Indian's Neem tree (*Azadirachta indica* A. Juss) is one of the most studied trees in the world (Girish and Shankara Bhat 2008), due to its enormous potential as a source of pesticides, insecticides and organic agrochemicals (Brahmachari 2004). The oil from its seeds is used as fungicide and insecticide (Girish and Shankara Bhat 2008), and its medical use has been recently recommended by the World Health Organization (WHO 2007). Its principal active component is Azadichtin (Figure 1) (Schaaf et al. 2000). Azadichtin has low toxicity against non-target organisms and low persistence in the environment (Schaaf et al. 2000), both of which are desirable characteristics for a biocide.

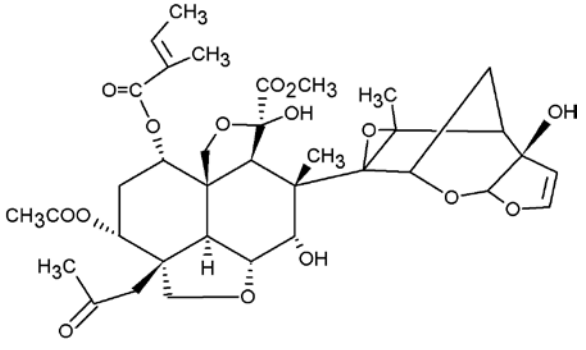

Figure 1 - Azaridachtin molecule.

Considering the problems caused by an infestation of *L. fortunei* in a man-made structure, and the potential of Neem's extracts, static bioassays have been proposed. With the aim to evaluate the acute toxicity of Neem's oil on *L. fortunei*, tests with three sizes of adults and the planktonic larvae were made. Also, tests with three non-target organisms, the cladocera *Daphnia magna*, the composite *Lactuca sativa* and the fish *Cnesterodon decemmaculatus*, were performed to evaluate the potential impact of Neem's oil in freshwater environments.

METHODS

Neem's solution consisted of a mix of 60% pure Neem's oil (Table I) and 40% Fatty Ethoxylated Alcohol of 9 mols as emulsifier (Table II). A generator was used to prepare the solution (Integración Química S.R.L. pers. comm.).

All living organisms of *L. fortunei* were captured in the Argentinean coasts of the Río de la Plata (34°49´S – 57°56´W).

Adult mussels were collected manually and immediately transported to the GIMIP's laboratory (UNLP). Mussels were acclimated for 15 days in 10-L aquaria (conductivity = 1 ± 0.3 ms/cm, temperature = 23 ± 2 °C, and pH = 7 ± 0.5). The water was completely replaced the first three days and then partially replaced (5 L) every two days until the end of the 15-day acclimation period. The mussels were fed with fish food (TetraMin®) daily, except for the last two days (Pereyra et al. 2011).

Larvae were collected by filtering 1000 L of water throughout a 45-µm mesh plankton net. The samples were immediately transported to the lab, where the existence of enough larvae for each assay was checked.

Neonates of *D. magna* were obtained from cultures maintained in the laboratory (CIMA – UNLP) under standard conditions (conductivity = 1.0 ms/cm; hardness = 215 mg/L de CO3Ca; alkalinity =180 mg/L de CO3Ca; pH range 7.6 ±

TABLE I
Neem's oil characteristics
(Integración Química S.R.L. pers. comm.).

	Physical and chemical properties
Physical state	Liquid
Appearence	Brown viscous liquid
Ph	7 ± 0.02
Boiling point	above 100 °C
Freezing point	14 °C
Auto Ignition temperature	above 200 °C
Flash point	above 170 °C – No fire Hazard
Decomposition temperature	None
Solubility in water	Emulsifies with water
Molecular formula	Not applicable
Molecular Weight	Not applicable
	Stability and reactivity
Chemical stability	Stable at ordinary condition of use and storage
Conditions to avoid	No restriction
Hazardous decomposition products	None
Hazardous polymerization	None
Primary eye irritation	Not irritant
Primary skin irritation	Not irritant
Skin sensitization	Non sensitizer

TABLE II
Characteristics of Fatty Ethoxilate Alcohol
(Integración Química S.R.L. pers. comm.).

Physical and chemical properties	
Physical estate	Pasty
Color	White
Boiling point	300°C
Fusion point	25 °C
Inflammation point	175 °C
Auto ignition point	500 °C
Solubility in water	Soluble
Ph	7.0
Stability and reactivity	
Conditions to avoid	Unknown
Hazardous decomposition products	None
Thermal decomposition	> 300 °C
Biodegradability	Biodegradable

0.2; temperature 20 ± 2 °C; photoperiod 16:8 light: darkness), and fed with chlorococcal algae.

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

Individuals of *C. decemmaculatus* were caught in streams of the zone, in fields with low or null utilization of agrochemicals. These individuals were transported to the laboratory (CIMA – UNLP) and held in 1200 L plastic pools (Pelopincho®), where they were daily fed with TetraMin® fish food. For the toxicity test, juveniles born in the laboratory were used.

Limnoperna fortunei tests were made with larvae and three sizes of adults (7, 13 and 19 ± 1 mm). Every test was made in triplicate, using at least five concentrations and one control. Dechlorinated tap water was used for the dilutions. Temperature (mean = 23 °C, min = 21.2 °C, max = 24.3 °C), photoperiod (16:8 light: darkness), pH (mean = 7.07, min = 6.19, max = 7.48) and conductivity (0.75 ± 0.3 ms/cm) were controlled.

Tests with adults were performed choosing individuals by size with a digital caliber (0.01 mm precision) 24 h before each assay. Twelve mussels were placed on plastic Petri dishes and held in plastic containers with 500 ml of dechlorinated tap water and artificial aeration. After 24 h, the plastic Petri dishes with the mussels attached by byssal threads were transferred to other plastic containers with 500 ml of Neem's solution. Mussels that had not attached to the Petri dish after the first 24 h were considered unapt for the assay and thus discarded (Pereyra et al. 2011). Mortality was checked after 72 h. Failure to respond to external tactile stimuli was used as the death criterion.

The assays with larvae were made with 12 ml of Neem's solution and adding 10 to 15 larvae in every plastic Petri dish. Mortality was checked after 24 h under a stereoscopic magnifying glass. A larva was considered dead if it was not swimming or if there were no signs of internal activity (by transparency) (Pereyra et al. 2011).

International standardized methods (USEPA 1996a, b, c) for all non-target species were used in these tests. All tests were made with at least five concentrations and one control.

For the tests with *D. magna*, individuals were exposed to the toxic for 48 h with dechlorinated tap water for solutions (conductivity = 1.05 ms/cm; hardness = 215 mg/L de CO_3Ca; alkalinity 180 mg/L de CO_3Ca, pH range 7.5 ± 0.2). The tests were carried under controlled conditions (temperature 20 ± 2 °C; photoperiod = 16:8 light: darkness), and without feeding the neonates. Ten neonates were placed in every test tube with 10 ml of solution each. Mortality was checked 48 hours later. A neonate was considered dead when it remained at the bottom of the test tube and showed no signs of activity.

For the tests with *L. sativa*, plants were exposed to the toxic for 120 h in darkness and controlled temperature (22 ± 2 °C). Double distilled water was used as dilution water. Twenty seeds were placed into plastic Petri dishes containing a sterilized filter paper with 3 ml of solution to be tested for every dish. Plastic Petri dishes were placed in darkness for 120 h, and germination and elongation of the roots were evaluated at the end of the test.

For the tests with *C. desemmaculatus*, individuals were exposed to the toxic for 96 h (USEPA 1996c). Five juveniles were held in plastic containers with 500 ml of solution each. Juveniles were kept without aeration or feeding. Solutions were replaced daily until completing 96 h of exposure to the toxic. A juvenile was considered dead when it remained at the bottom of the plastic container showing no signs of activity.

For *L. fortunei*, the LC_{50} were calculated using linear regression, previous transformation of the concentration with logarithm and the mortality to probit units (Finney 1978), using USEPA program Probit 1.5. The lowest concentration that effectively caused 100% mortality in exposed organisms was considered as LC_{100}.

For *D. magna* and *C. desemmaculatus*, all the estimations of the LC_{XX} were made with the probit program, considering the LC_1 as NOEC (the highest concentration of the product at which no significant differences as regards the control were seen), and the LC_{10} as LOEC (the lowest concentration of the product at which a significant difference as regards the control was seen).

For *L. sativa*, the IC_{50} (Inhibition concentration 50, i.e., the concentration at which 50% of inhibition as regards the control is observed) was obtained using linear regression with the logarithmic transformation of the concentrations, using the inhibition proportion concerning the control as the dependent variable. The NOEC and LOEC were calculated with *a posteriori* comparisons of Dunnet, previous ANOVA.

RESULTS and DISCUSSION

Table III shows the results obtained for *L. fortunei*, Table IV shows those for *D. magna* and *C. decemmaculatus*, and Table V shows those for *L. sativa*. The LC_{100} of *L. fortunei* larvae was 500 µl/L. The LC_{100} of adults could not be estimated because the concentrations tested did not achieve 100% mortality.

This results show i) Neem's oil has acute toxicity against *L. fortunei*, and ii) the larvae are more vulnerable to the toxic than the adults. This agrees with what was previously reported about the vulnerability of the initial stages of development (Maroñas and Damborenea 2006, Van Benschoten et al. 1995) and with previous assays made with other organic biocides against *L. fortunei* (Pereyra et al. 2011).

TABLE III
LC_{50} and confidence intervals (CI) for all stages of Limnoperna fortunei with Neem's oil solution. All concentrations are expressed in µl/L.

		CI	
	CL_{50}	Superior	Inferior
Larvae	8	315	2
7 ± 1 mm	241	315	171
13 ± 1 mm	249	827	101
19 ± 1 mm	122	153	98

TABLE IV
LC_{50}, NOEC, LOEC and respective confidence intervals of *Daphnia magna* y Cnesterodon decemmaculatus. All concentrations are expressed in µl/L.

Daphnia magna								
CL_{50}	Limit inferior	Limit superior	NOEC	Limit inferior	Limit superior	LOEC	Limit inferior	Limit superior
17	11.35	35.58	1.65	0.12	3.18	4.71	1.77	6.69
Cnesterodon decemmaculatus								
CL_{50}	Limit inferior	Limit superior	NOEC	Limit inferior	Limit superior	LOEC	Limit inferior	Limit superior
4.92	4.01	6.09	2.08	0.97	2.83	3.06	1.92	3.8

As mentioned in the introduction, chlorine is the most widespread biocide used in man-made facilities (Mackie and Claudi 2010). Chlorine shows 100% mortality at 576 h, with 1.2 mg/L (Morton et al. 1976), and at 408 h with 1 mg/L at 25 ºC (Cataldo et al. 2003). Similar results have been observed with other three macrofouler bivalves: *Dreissena polymorpha*, *Mytilus edulis* and *Perna viridis* (see discussion in Pereyra et al. 2011). Working with tannins of *Schinopsis balansae* (Englers.), we have previously obtained the LC_{50} of *L. fortunei*, for adult mussels of 13 and 19 ± 1mm, after 168 h of exposure to the toxic (Pereyra et al. 2011). In this study, although 100% mortality was not achieved (the maximum mortality reached was 80%, data not shown), a considerable reduction in the exposure time to the toxic was obtained.

Results show no overlap between the LC_{50} of *L. fortunei* (both stages) and *L. sativa* (Tables III and V), indicating that at these concentrations Neem's oil has no effect on *L. sativa*. There is overlapping between the LC_{50} of *L. fortunei* and those achieved with *D. magna* and *C. desemmaculatus* (Tables III and IV). In view of these results, we recommend not to use this product in open waters. Likewise, this product is recommended to be used in closed systems in man-made facilities.

It is important to note that the use of biocides to control macrofouling is one of the treatments to apply. The treatment to be used depends on the possibility of each section of the facilitie, the time of the year and the stages to control (Costa et al. 2008, Darrigran et al. 2007). Other techniques (e.g. ozone) are used to prevent macrofouling of bivalves, but are difficult to apply and expensive (Mackie and Claudi 2010). Using products like Neem's oil is a valid alternative in closed systems.

TABLE V
IC_{50}, and confidence intervals of Lactuca sativa. All concentrations are expressed in µl/L.

Neem's oil	
IC_{50}	3,020
L_{sup}	12,081
L_{inf}	750
NOEC	250
LOEC	500

Another emerging perspective is that combined techniques (e.g. Costa et al. 2011) can be applied to improve the control measures in man-made facilities.

Finally, the bioassays with biocides with *L. fortunei* carried out to date have been made in static conditions (e.g. Cataldo et al. 2003, Darrigran and Damborenea 2001, Pereyra et al. 2011). These kinds of assays have the advantage of their practicality and low impact, but the results should be tested in the industry. For example, Rolla and Mota (2010) have reported that the reduction in the spread of *L. fortunei* in the Paraíba River resulted, in part, from the elimination of the golden mussel from the transitory harbors with the use of chlorine. However, there are no reports on the effects of chlorine over the biota (Rolla and Mota 2010). This lack of communication between scientists

and the people involved (e.g. stakeholders, the company, the community) has been observed in other areas of NIS control (e.g. Donlan et al. 2003, Gardener et al. 2010, Shanley and López 2009), and represents another problem to be solved in order to achieve effective control programs of the golden mussel.

RESUMO

O Mexilhão dourado *Limnoperna fortunei* (Dunker 1857) é uma das espécies invasoras melhor distribuídas na América do Sul, sendo motivo de grande preocupação para a indústria local. Neste estudo, nós realizamos ensaios de toxicidade de soluções de Óleo de Neem em larvas e adultos bentônicos de *L. fortune* (7, 13 e 19 ± 1 mm). Com o objetivo de avaliar o potencial tóxico do Óleo de Neem no ambiente também foram realizados testes com organismos não alvo (*Daphnia magna*, *Lactuca sativa* e *Cnesterodon decemmculatus*). A LC_{100} da solução de Neem para larvas foi 500 µl/L, um valor muito superior ao obtido para *D. magna* e *C. decemmaculatus*. Desta forma, nossa recomendação é que este óleo não deve ser utilizado em ambientes naturais abertos. No entanto, uma vez que os adultos morreram em 72h e as larvas em 24h, este produto pode ser utilizado em sistemas fechados construídos pelo homem.

Palavras-chave: biodia, impacto, *Limnoperna fortune*, óleo de Neem.

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