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Acute toxicity of tributyltin to encapsulated embryos of a marine gastropod

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ABSTRACT

The marine gastropod *Buccinanops globulosus* is known to have high imposex incidence in areas moderately polluted by tributyltin (TBT). Acute toxicity was previously studied in adults but no information is known about embryonic intracapsular development. To estimate the potential effects of organotin pollution on the progeny of *B. globulosus*, acute toxicity tests were conducted on encapsulated and excapsulated pre-hatching embryos. The lethal median concentration estimated for 96 h (LC_{50} 96 h) in *B. globulosus* excapsulated embryos was 196.70 µg TBTCl L^{-1}, while in encapsulated embryos it was 2951.28 µg TBTCl L^{-1}. The LC_{50} 96 h was 15-fold higher for encapsulated embryos compared to excapsulated embryos, denoting egg capsule protection against pollutants from the external environment. Our results show that TBT pollution can have significant effects in molluscs other than the chronic effect of imposex.

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Buccinanops embryos; egg capsules; mortality; Patagonia; TBT pollution; toxicity assays

Introduction

In Argentina, organotin compounds, namely tributyltin (TBT), have been used for decades in antifouling paints for ships, even after the global banning in 2008 (IMO 2002). At least 11 marine gastropod species have been observed to be affected by imposex in Argentinean marine harbour areas, including three species of the endemic genus *Buccinanops* d'Orbigny, 1841 (Penchaszadeh et al. 2001; Bigatti et al. 2009; Averbuj and Penchaszadeh 2010). This toxic compound accumulates and persists in the sediments with a half-life in the range of years (de Mora et al. 1995; Sarradin et al. 1995; Okoro et al. 2011). Although its accumulation is reversible, sediments are sinks of TBT for long periods of time (Long et al. 1996). In areas highly polluted with TBT, sterile females and population declines have been observed (Oehlmann et al. 2007), and female sterility is irreversible at an individual level (Stroben et al. 1992). Studies on TBT toxicity are mostly focused on the chronic effects caused by long exposure in marine polluted environments (Antizar-Ladislao 2008).

Organotin pollution (TBT and derivatives) was detected at north Patagonia, Argentina in Nuevo Gulf (Bigatti et al. 2009). The highest concentrations of TBT reported in the harbour area of Nuevo Gulf were 174.81 ng TBT-Sn g^{-1} in sediments and 345.27 ng TBT-Sn g^{-1} in body tissues of the gastropod *Odontocymbiola magellanica* (Gmelin, 1791) (Del Brio et al. 2016). The marine gastropod *Buccinanops globulosus* (Kiener, 1834) showed a high incidence of imposex in moderately polluted areas with TBT concentrations of 0.4 ng TBT-Sn g^{-1} in sediments; thus it was classified as a good indicator due to its high sensitivity to low concentrations of TBT (Bigatti et al. 2009). In Argentina TBT measurements in polluted areas varied from 0.2 to 6500 ng TBT-Sn g^{-1} (Goldberg et al. 2004; Delucchi et al. 2007; Bigatti et al. 2009). These values are found in high maritime traffic harbours and are within the highest reported in the South American region; however, higher levels of TBT have been reported in Spain, Japan and the US, reaching up to 14,000 ng TBT-Sn g^{-1} (Braga de Castro et al. 2012).

Data on TBT concentrations in water are not abundant in Argentina. Nonetheless, sublethal effects on gastropods exposed to pollutants in maritime areas have been widely observed. Females of *B. globulosus* showed high incidence of imposex in sites with TBT pollution (Bigatti et al. 2009; Primost et al. 2015a). Recent investigations suggest a direct relationship between imposex development, together with changes to the reproductive output and shell shape variations of *B. globulosus* females living in a polluted area (Primost et al. 2015a, 2015b).

Buccinanops globulosus (Nassariidae) is an edible species that inhabits sandy-muddy bottoms and is part of an expanding artisanal fishery in north Patagonia (Narvarte 2006; Averbuj et al. 2014; Bigatti et al. 2015). Adult females spawn from October to March (Averbuj et al. 2014), producing a variable number of egg capsules that are attached to their own shells (Penchaszadeh 1971). Generally, one *B. globulosus*

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crawling juvenile, measuring between 3–7 mm, hatches from each egg capsule (Penchaszadeh 1971; Narvarte 2006; Averbuj et al. 2014).

A significantly lower frequency of spawning females was observed in a population inhabiting a polluted harbour area in *Buccinanops monilifer* (Kiener, 1834) (Averbuj and Penchaszadeh 2010). Recently, Primost et al. (2015c) determined the lethal dose LD_{50} of TBT in adult *B. globulosus* that showed low acute toxicity effects (high resistance) to TBTCl compared to doses required to induce imposex (chronic toxicity effect) in other nassariid species (Oberdörster et al. 1998; McClellan-Green et al. 2006). Primost et al. (2015a) suggested a relationship between imposex development and alterations in the reproductive output of *B. globulosus* females living in adverse environmental conditions (e.g., polluted harbour areas).

To estimate the possible effects of organotin pollution on the progeny of *B. globulosus*, acute toxicity tests were conducted on encapsulated and excapsulated (artificially removed from the egg capsule) pre-hatching embryos. The possible effects are discussed in relation to the actual organotin concentration levels in the Argentinian environment. To our knowledge, this work represents the first study on the effect of lethal concentrations of organotin on marine gastropod encapsulated embryonic stages.

Materials and methods

Buccinanops globulosus females carrying egg capsules were collected during March 2015 when embryos were still encapsulated, in late stages of development and abundant (Averbuj et al. 2014). *Buccinanops globulosus* females with encapsulated embryos in pre-hatching stages together with sea water were collected at Cerro Avanzado beach (42°49′ S, 64°52′ W), an area 16 km away from Puerto Madryn city with very low imposex incidence and low maritime traffic, where TBT levels are below the limit of detection (Del Brio et al. 2016). In this area *B. globulosus* are abundant and sporadic catches are carried out for human consumption.

Gravid females were collected by SCUBA diving (up to 6 m deep), placed in glass aquaria with filtered and UV-irradiated sea water, and acclimated for 1 week at a temperature of 12 ± 1 °C and salinity of 35 PSU, with a 12 h:12 h light:dark photoperiod. Internal circulation inside each aquarium and constant aeration were provided to ensure survival of organisms in laboratory conditions, but without recirculation among aquaria. Embryos were not fed during the experiment.

Later, egg capsules (Figure 1) were detached from the females' shells, counted and randomly divided into two groups: encapsulated and excapsulated embryos. Spawn were placed under a stereoscopic microscope and a small cut was made with dissecting scissors to the flexible wall of the egg capsules to facilitate the removal of the embryos. To determine acute toxicity by median lethal concentration (LC_{50} 96 h) assay, embryos of each group (encapsulated and excapsulated) were submerged in 500 ml glass containers with different concentrations of TBT in sea water for 96 hours. Conditions of temperature, salinity and photoperiod were checked at 8-hour intervals throughout the experiment to ensure that they remained the same as for adult acclimation. The tests were carried out with 20 excapsulated embryos exposed in the experiments in each container. Encapsulated embryos were separated into groups of approximately 20 and carefully placed in the glass containers with the same treatment as the released embryos to avoid triggering of hatching by manipulation. Encapsulated embryos that hatched during the experiment were not included in the analysis.

TBT concentrations in sea water were selected following previously published reports, with a maximum concentration five-fold higher than the maximum sea water TBT measurement registered in the field (USEPA 2003; Antizar-Ladislao 2008), and prepared from a stock solution of TBT chloride (Sigma Aldrich) in absolute ethanol (960 µg of TBTCl per μL^{-1}). Exposure to TBT was performed by six nominal concentrations (1, 50, 100, 250, 500 and 2000 µg TBTCl L^{-1}) diluted in sea water, and two controls (sea water only and sea water with 0.05% of absolute ethanol, considering the highest TBT dilution used).

Embryos were monitored daily. Using a stereoscopic microscope, the total number of dead individuals at each concentration was recorded 96 hours after the beginning of the experiment. Encapsulated embryos were released from the egg capsules and all embryos were placed in petri dishes with sea water before assessing for mortality rate. Embryos were considered dead if they showed no mobility or muscular reaction after repeated pricking. Lethal median concentration LC_{50} 96 h and confidence intervals (95%) were calculated by XLStat 2012-XLSTAT-Dose software separately for encapsulated and excapsulated embryos. The Probit method (Finney 1947) with 100 iterations and 0.000001 of convergence was used.

Results

No gastropod mortality was observed in either of the two controls during the experiment. No mortality was registered in excapsulated embryos of *Buccinanops globulosus* exposed to 1 µg TBTCl L^{-1}, while mortality increased to 5% for 50 µg TBTCl L^{-1}, 10% for 100 µg TBTCl L^{-1} and 55% for 250 µg TBTCl L^{-1}. In water with concentrations of 500 and 2000 µg TBTCl L^{-1} all embryos died. For encapsulated embryos, no mortality was observed at concentrations of 1 to 250 ng TBTCl.ml^{-1}, while 21.43% and 34.62% mortality

Figure 1. Encapsulated pre-hatching embryo of *Buccinanops globulosus*. Scale bar = 700µm.

occurred for the 500 and 2000 ng TBTCl.ml^{-1} concentrations, respectively. The maximum exposure concentration was insufficient to produce 100% mortality of encapsulated embryos.

The lethal median concentration calculated for 96 h (LC_{50} 96 h) in excapsulated *B. globulosus* embryos was 196.70 µg TBTCl L^{-1} with 154.54 and 253. µg TBTCl L^{-1} as 95% confidence intervals, while in encapsulated embryos it was 2951.28 µg TBTCl L^{-1} with 1585.46 and 14043.85 µg TBTCl L^{-1} as 95% confidence intervals (Table 1).

Discussion

There are few reports of studies on lethal concentrations (LC_{50}) of TBT in marine gastropods (Roberts 1987; Laughlin et al. 1989; Horiguchi et al. 1998). The methods used include concentrations applied in the water or by injection into the gastropod body at varying times (12 h, 24 h, 48 h or 96 h experiments) and the use of organisms with contrasting characteristics (rocky or sandy habitats, wide latitudinal/temperature range and phylogenetically diverse taxa). To test acute toxicity of TBT organotins in gastropod embryos, Horiguchi et al. (1998) worked with three rocky habitat species (two abalones and a neogastropod of the genus *Thais*) that develop as free larvae, and tested them at 24 h and 48 h. Meanwhile, Untersee (2007) worked with encapsulated/excapsulated embryos of the neogastropod *Crepidula fornicata*, testing for sublethal toxicity effects of copper. The variety of methods and conditions makes a straightforward comparison of the results impossible.

Table 1. LC_{50} 96 h in encapsulated and excapsulated embryos of *Buccinanops globulosus*.

	Encapsulated embryos		Excapsulated embryos	
Concentration TBT tested (µg TBTCl L^{-1})	Individuals per treatment	Dead in each treatment	Individuals per treatment	Dead in each treatment
1	20	0	20	0
50	30	0	20	1
100	19	0	20	2
250	17	0	20	11
500	28	6	20	20
2000	26	9	20	20
LC_{50} 96 h	2951.28 [1585.46;14043.85 µg TBTCl L^{-1} as 95% CI]		196.70 [154.54; 253.48 µg TBTCl L^{-1} as 95% CI]	

Our results indicate that *Buccinanops globulosus* embryos have a relatively high tolerance to acute toxicity (TBT LC_{50}), when compared to the larvae of the Pacific oyster *Crassostrea gigas* (LC_{50} = 1.56 µg/L) (USEPA 2003). This appears to contrast with the high sensitivity of *B. globulosus* to chronic exposure to TBT, even at low concentrations (Bigatti et al. 2009). Unfortunately, *B. globulosus* adult LC_{50} was obtained by injection of TBTCl, thus it is not comparable with this study (Primost et al. 2015c). However, those results also showed a high resistance to TBT compared to other gastropods exposed to TBT by the same method (USEPA 2003).

The LC_{50} 96 h estimated for encapsulated embryos was c. 3000 µg TBTCl L^{-1} with a mortality of c. 35%, being 15-fold higher than the LC_{50} 96 h estimated for excapsulated embryos. This shows a significant increase in the LC_{50} 96 h for encapsulated embryos, compared to excapsulated embryos, suggesting that the egg capsule offers significant protection in polluted environments. This result apparently contradicts previous reports on embryos of *Ilyanassa obsoleta* exposed to No. 2 fuel oil, showing a reduced susceptibility of encapsulated embryos to the oil stress that may reflect the importance of toxicant uptake by ingestion (as opposed to diffusion) rather than protection afforded by encapsulation (Pechenik and Miller 1983).

The protective effect of encapsulation does not imply the absence of TBT in intracapsular embryos spawned in polluted environments, as TBT could be introduced directly into the egg capsule by the female when spawning (Goldberg et al. 2004). These authors suggested that the egg capsule of *Adelomelon brasiliana* is permeable to TBT, but the concentrations observed inside the capsule at early stages of development were higher than that determined in the water of the most contaminated study areas. The degree of permeability of the egg capsule to TBT is uncertain for this species. TBT was said to be principally accumulated in gonads (Oehlmann et al. 1992; Del Brio et al. 2016).

Therefore, an input of TBT directly from the gonads to the intracapsular content is probable in marine gastropods exposed to organotins.

Protective effects of the egg capsule against desiccation, mechanical damage, predation or UV exposure are well known (Rawlings 1994, 1999); but little is known about the protective effect of encapsulation against pollution (Untersee 2007). Several studies have shown that some invertebrate egg capsules' walls are permeable to water, small solute/salts and small organic molecules regardless of the wall thickness (Pechenik 1983; Rawlings 1999); it would be desirable to investigate whether there is a selective permeability that excludes molecules of large size and could offer some protection against TBT or other organic pollutants (Untersee 2007).

This study is the first report of acute toxicity to TBT in marine gastropod encapsulated embryos. It represents a valuable reference for TBT pollution effects in the biota, other than the widely-studied chronic effect of imposex. The criteria to protect saltwater aquatic life from chronic toxic effects and from acute toxic effects of TBT are 0.0074 µg L^{-1} and 0.42 µg L^{-1}, respectively (USEPA 2003). Acute toxicity effects on *B. globulosus* embryos as judged by the lethal dose estimated in this study occur only at much larger values. Although 100% mortality was not reached at any concentration in encapsulated embryos, the maximum concentration used in this study is five-fold higher than the maximum sea water TBT measurement registered worldwide in the field (Antizar-Ladislao 2008), suggesting a high resistance to TBT compared to other gastropods exposed by the same method.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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