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Tributyltin and copper effects on encystment and *in vitro* excystment of *Parorchis acanthus* larvae

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Abstract

Effects of tributyltin (TBT) and copper (Cu) on cercariae and metacercariae of the trematode *Parorchis acanthus* (Digenea: Philophthalmidae) were investigated. Cercariae released by the dogwhelk, *Nucella lapillus* were maintained in natural seawater (SW) or solutions of TBT or Cu ranging from 0.001–100 $\mu g\, l^{-1}$ and 1–6 $mg\, l^{-1}$ respectively before they encysted. Over 79% of the cercariae encysted in control and test solutions. Low concentrations of TBT reduced encystment success more than low concentrations of Cu. The percentage of cercariae that formed cysts in the highest concentrations of both pollutants was higher than in the controls, perhaps representing an 'emergency response' to the pollutants. Before being induced to excyst *in vitro*, metacercariae were left in the heavy metal solutions for 3 weeks. Metacercariae exposed as cercariae to TBT and Cu achieved lower percentage excystment success than those that had encysted in SW. Cyst walls provided greater protection against Cu than TBT. It was concluded that TBT and Cu had a detrimental effect on the larval stages of *P. acanthus* at the higher concentrations used but the cyst wall afforded an element of protection if formed in unpolluted seawater before the larval stages were subjected to the pollutants.

Introduction

Parorchis acanthus is a digenean trematode parasite, the adults of which are found in seabirds, especially gulls (*Larus* spp.). In Europe, the common dogwhelk (*Nucella lapillus*) is the intermediate host for the larval stage of *P. acanthus*. *Nucella lapillus* is a widespread and generally abundant member of most rocky shore communities throughout the British Isles and it is widely distributed on both sides of the Atlantic. Cercariae of *P. acanthus* emerge from infected dogwhelks and, after a brief period of free swimming, they encyst on solid objects in the nearby locality (Rees, 1966). Those fortunate enough to encyst on a potential food item (such as crab or mollusc shell) may be taken up by definitive hosts and thereby complete the life cycle.

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Nucella lapillus has been extensively studied as a potential biomonitor for the marine environment. Having direct development and being relatively immobile it is an ideal indicator species. It is highly sensitive to tributyltin (TBT) and possibly other heavy metals such as copper (Alziue, 1998). Tributyltin has been used extensively as the active biocide in anti-fouling paints that are used on boat hulls. Environmental scientists began addressing the issue of TBT in the early 1980s when it was shown that its presence in certain coastal areas was responsible for shell calcification anomalies in farmed oysters and for severe sexual disorders leading to sterility (imposex) in certain gastropod species (Bryan *et al.*, 1987). In fact, *N. lapillus* was shown to have become locally extinct from many regions where concentrations of small crafts were moored (Smith, 1981). On the basis of these findings, worldwide concern was raised about the use of TBT in such coastal areas. Small boats were believed to be the main source of contamination and in 1982 the French government prohibited TBT-based paints on vessels less than 25 m in length. Similar regulations were enforced in the UK in 1987. As a consequence of these restrictions, consumers were forced to return to the use of traditional formulae based on copper compounds such as cuprous oxides, copper thyocyanate or metallic copper as the principle biocide (Voulvoulis *et al.*, 2002). 'TBT free' coatings containing copper as the active biocide are usually combined with an organic booster biocide (e.g. Diuron, Dichlofluanid, Irgarol 1051) to improve the efficacy of the formulation and have been used on boats less than 25 m for the last 13 years (Thomas *et al.*, 1998). Recent findings indicate that imposex should no longer be regarded as a TBT-specific effect on gastropods. Other studies have shown that imposex appears to be associated with the presence of boats and that copper or even generalized environmental stress induce imposex (Alziue, 1998).

Parasites are attracting increasing interest as potential indicators of environmental quality because of the variety of ways in which they respond to anthropogenic pollution (Sures *et al.*, 1999). Changes in the environment may affect larval digeneans at many stages of their complex life cycles. Even though it has been assumed that such parasites are vulnerable to toxicants during their free living phases, little experimental work on the effects of marine environmental contaminants upon the viability of these stages has been carried out to date. Importantly, the speed by which the effect of pollution events can be identified using parasites has only recently been addressed (Cross *et al.*, 2001; Morley *et al.*, 2001).

The present study was designed to investigate the effects of TBT and copper on the encystment and *in vitro* excystment success of *P. acanthus* larvae. Although marine pollutants can gain access to trematode larvae either indirectly (while developing inside their molluscan intermediate hosts), or directly (while free-living), this study will deal only with the effects of direct contact between the pollutants and the parasites. It will determine if TBT and copper affect the ability of *P. acanthus* cercariae to encyst, and if their metacercarial cysts act as a barrier to TBT and copper. Cercarial susceptibility to these pollutants and any protection afforded by metacercarial cysts would have implications for parasite community structure in polluted environments and also affect their potential adoption as rapid indicators for biomonitoring.

Materials and methods

Test solutions

Stock solutions of copper containing 100 mg l^{-1} of metal ions were prepared by dissolving copper (II) hydroxide ($Cu(OH)_2$) (Aldrich Chemicals) in distilled water. Two stock solutions of TBT acetate (TBTA) (Fluka Chemicals) were prepared using acetone as a solvent. Test solutions of 1, 2, 3, 4, 5 and 6 mg l^{-1} of copper were made up, the chosen concentrations were within the ranges used by (Evans, 1982) and (Cross *et al.*, 2001). The concentrations chosen 0.001, 0.01, 0.1, 1.0, 10 and 100 μg l^{-1} of TBT were also made up in seawater. The wide range of TBT concentrations was chosen because TBT in the natural environment can fluctuate from minute traces to over 400 μg l^{-1} in or near marinas.

Control solutions

The two controls used throughout this investigation were natural sea water (SW) and sea water containing 5 μg l^{-1} of acetone (SW/AC). The amount of acetone used in the SW/AC control was equivalent to that required to dissolve the TBT acetate. Controls were compared by a G test using Statview 4.0 to indicate if any 'acetone effect' occurred.

Sample site

Specimens of *N. lapillus* were collected at low tides at Portavogie, Co. Down, Northern Ireland. The site chosen was 2 km from a fishing harbour on the east coast of the Ards Peninsula. The collection site was a relatively sheltered sandy beach containing both large and small scattered rocks on which gulls are known to congregate.

Infected *N. lapillus* were identified by shaking them individually in freshly aerated seawater at 80 rpm for 1 h in an illuminated orbital incubator (Gallenkamp) and checking for cercarial releases. Rea & Irwin (1991) developed this method for inducing *Cryptocotyle lingua* cercariae to release from *Littorina littorea*. Infected *N. lapillus* were segregated and maintained at 4°C in a refrigerator.

Cercarial release

Nucella lapillus were allowed to warm up to room temperature before experimental work began. Cercariae were induced to release as described above from individual dogwhelks in natural seawater. After an initial release for 30 min, infected *N. lapillus* were placed in clean containers of seawater and induced to release again for a further 30 min. The initial release was discarded to ensure all cercariae were the same age. Each *N. lapillus* was then removed from each container and the water volume reduced by carefully pipetting extra water from near the surface. This ensured that no cercariae were lost, as *P. acanthus* cercariae are known to swim to the bottom of their container after release (Rees, 1967).

Cercarial encystment

Using a pipette, 0.5 ml of seawater containing cercariae was placed in clean sterile 24-well culture plates (Whatman) to allow observation of encystment. Each well already contained 2.5 ml of test or control solutions at 15°C. Each experiment was repeated six times from six different *N. lapillus* releases. Cercariae were allowed to encyst in control solutions (SW or SW/AC) or the test solutions (0.001, 0.01, 0.1, 1.0, 10 and 100 μg l^{-1} TBT and 1, 2, 3, 4, 5 and 6 mg l^{-1} Cu) at 15°C in a constant temperature room. Every 24 h, cercariae that had encysted or died were counted. Any dead cercariae were removed. As Rees (1967) established that fully formed cysts are normally found after 2–4 h in optimum

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conditions, all cercariae that had not encysted after 72 h were noted and disregarded. A logistic regression was used to confirm overall treatment effects, followed by a one-way ANOVA of % encystment. Dunnett's post-hoc test was used to compare treatments with controls using JMP (SAS Institute, 1995). A logistic regression is insensitive to differences in the number of cercariae per treatment; 20–62 cercariae were present in the 0.5 ml aliquots.

Excystment of metacercariae in vitro

Excystment success was investigated following exposure of metacercarial cysts (in 24-well culture plates) to the TBT and Cu solutions in one of two ways.

1. Cercariae were allowed to encyst in natural seawater, before being maintained in the test solutions for 3 weeks.
2. Cercariae were allowed to swim freely in the test solutions before encysting. They were then maintained for 3 weeks in the solutions. The metal concentrations and controls were the same as those used in the encystment study. Three sets of replicates, each containing 20 cysts, were used for each concentration.

Cysts were gently scraped from the wells of the culture plates and rinsed in Hanks Balanced Salts Solution (HBSS) (Sigma Chemicals). They were then incubated for 30 min in 1% acid pepsin (Sigma Chemicals) (pH 2) at 42°C before being rinsed again in HBSS. Excystment was carried out for 2 h at 42°C using an excystment method devised by Fried & Roth (1974). The cysts were examined every 15 min and excysted metacercariae counted and removed. All results were analysed by a logistic regression and one-way ANOVA using the methods employed for the encystment results.

Results

Control solutions

For each replicate there was no significant difference between the proportion of cercariae encysting in SW and in the SW/AC control (G tests, $P > 0.05$). However, when data from all encystment studies were amalgamated a just significant ($P = 0.03$) effect was shown with the larger sample size ($N = 1041$). In view of this small effect, the SW/AC control was used in the analysis of TBT data and the SW control for copper data.

Cercarial encystment

A logistic regression showed significant effects of TBT and Cu on encystment success ($P < 0.0001$ in both cases). A one-way ANOVA on % encysted produced similar results. For TBT, $F_{6,14} = 27.96$, $P < 0.00001$; for Cu $F_{6,14} = 31.42$, $P < 0.0001$ and allowed post-hoc comparison with controls (fig. 1).

Over 79% cercariae encysted in controls and all concentrations of TBT and Cu. As the concentration of both metal concentrations increased, the % cercariae encysted initially decreased (fig. 1), but at the higher concentrations of 10 $\mu g\,l^{-1}$ and 100 $\mu g\,l^{-1}$ TBT and

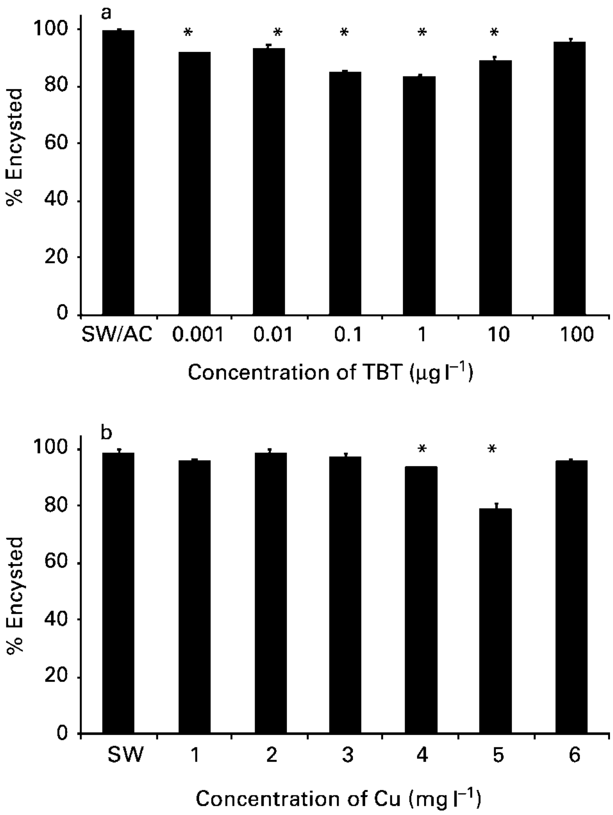

Fig. 1. Effects of (a) tributyltin (TBT) and (b) copper (Cu) concentrations on the encystment success of *Parorchis acanthus*. SW/AC, seawater + acetone control; SW, seawater control; *, concentrations significantly different from controls

6 $mg\,l^{-1}$ copper the % encysted increased. All concentrations of TBT except 100 $\mu g\,l^{-1}$ were significantly different from the SW/AC control (Dunnett's comparison), whereas 4 and 5 $mg\,l^{-1}$ copper were significantly different from the control. Low concentrations of TBT therefore had a greater effect on encystment success than the low concentrations of Cu.

Excystment in vitro

Excystment achieved using metacercariae encysted in natural seawater and then maintained in test solutions

A logistic regression showed a significant effect of TBT ($P < 0.0001$) but not Cu ($P = 0.2615$) on excystment success. A one-way ANOVA of % excysted, however, showed a just significant Cu effect ($F_{6,14} = 3.25$, $P = 0.0324$) as well as a significant TBT effect ($F_{6,14} = 58.73$, $P < 0.0001$).

The % of metacercariae excysted decreased with increasing TBT concentration (fig. 2a), and marginally with increasing Cu concentration (fig. 2b). Only the highest concentrations of TBT and Cu (100 $\mu g\,l^{-1}$ and 6 $mg\,l^{-1}$ respectively) had significantly reduced excystment compared to controls. Although the % excystment achieved in all copper concentrations was relatively constant (between 81% and 93%), a wider range (90%–35%) was observed for TBT.

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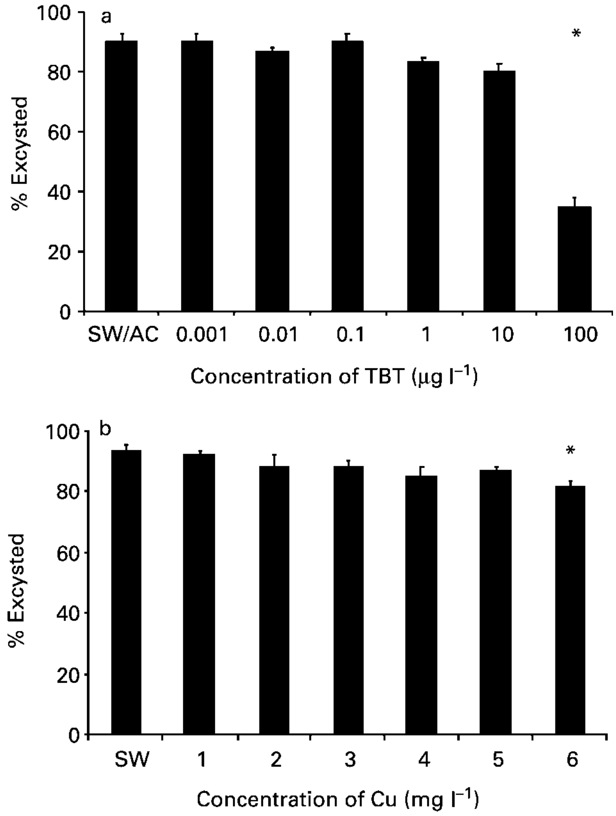

Fig. 2. The excystment success of metacercarial cysts of *Parorchis acanthus* maintained in (a) tributyltin (TBT) or (b) copper (Cu) solutions for 3 weeks after encystment in natural seawater. SW/AC, seawater + acetone control; SW, seawater control; *, concentrations significantly different from controls.

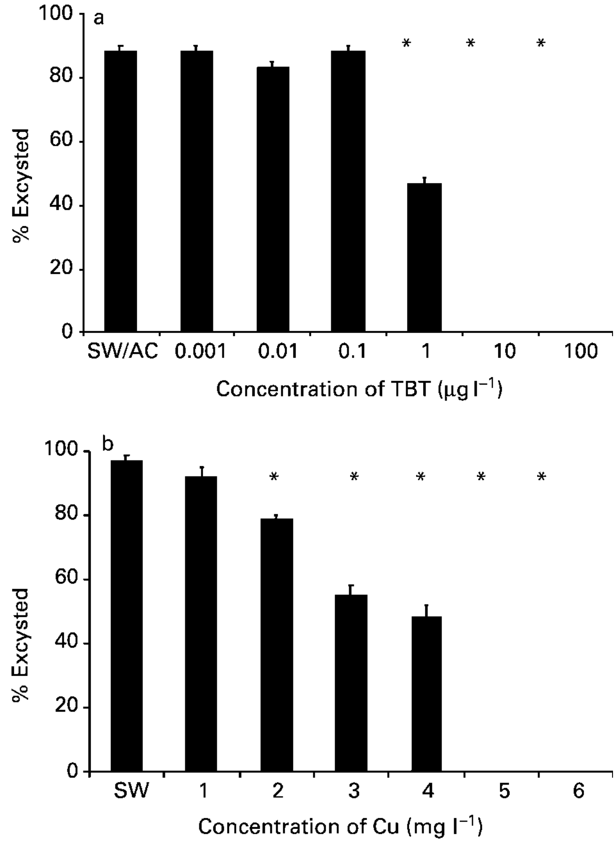

Fig. 3. The excystment success of metacercarial cysts of *Parorchis acanthus* maintained in (a) tributyltin (TBT) or (b) copper (Cu) solutions for 3 weeks after encystment in the metal solutions. SW/AC, seawater + acetone control; SW, seawater control; *, concentrations significantly different from controls.

Excystment achieved using metacercariae encysted and maintained in test solutions

A logistic regression showed highly significant effects of both TBT and Cu on excystment ($P < 0.001$ in both cases). A one-way ANOVA on % excysted also showed highly significant effects of TBT ($F_{6,14} = 860.27$, $P < 0.0001$) and Cu ($F_{6,14} = 312.97$, $P < 0.0001$) and allowed comparison with controls (fig. 3).

The % of metacercariae excysted decreased with increasing concentrations of both TBT and Cu. No excystment occurred at the higher concentrations of TBT and Cu (10 and 100 μg l^{-1} TBT and 5 and 6 mg l^{-1} Cu respectively). Only 1, 10 and 100 μg l^{-1} TBT had significantly reduced excystment success compared to controls (fig. 3a), whereas, with copper, a significant decrease in % excysted occurred at all concentrations greater than 1 mg l^{-1} Cu (fig. 3b).

These experiments showed that metacercariae which were exposed as cercariae to TBT or Cu achieved lower % excystment success compared to those that encysted in SW and were subsequently exposed to metal pollution only while inside their cysts (fig. 4). Excystment success was unaffected by low TBT concentrations but decreased dramatically at higher concentrations of TBT especially in metacercariae encysted in TBT solutions (fig. 4a). Metacercariae encysted in SW/AC showed a decreased excystment only at the highest concentration of TBT used (fig. 4a). For Cu, excystment success differed markedly between cercariae encysted in SW and in the copper solutions. Only metacercariae encysted in the lowest Cu concentration did not show decreased excystment. Results indicated that the cysts provided more effective protection against Cu than against TBT.

Discussion

It is not surprising that increasing levels of TBT and Cu in seawater should have a progressively detrimental effect on the ability of *P. acanthus* cercariae to encyst. It was Rees (1937) who showed that slight changes in salinity and temperature delayed encystment of these organisms. Morley *et al.* (2001) found that increased levels of cadmium and zinc decreased encystment success in *P. acanthus* in the same way. Copper at 4, 5 and 10 mg l^{-1} had previously been shown to progressively inhibit encystment of the cercariae of *Notocotyle attenuatus*, a species that releases and encysts in fresh water (Evans, 1982). A similar effect was observed by Graczyk & Shiff (1994) and Morley *et al.* (2002) who showed that

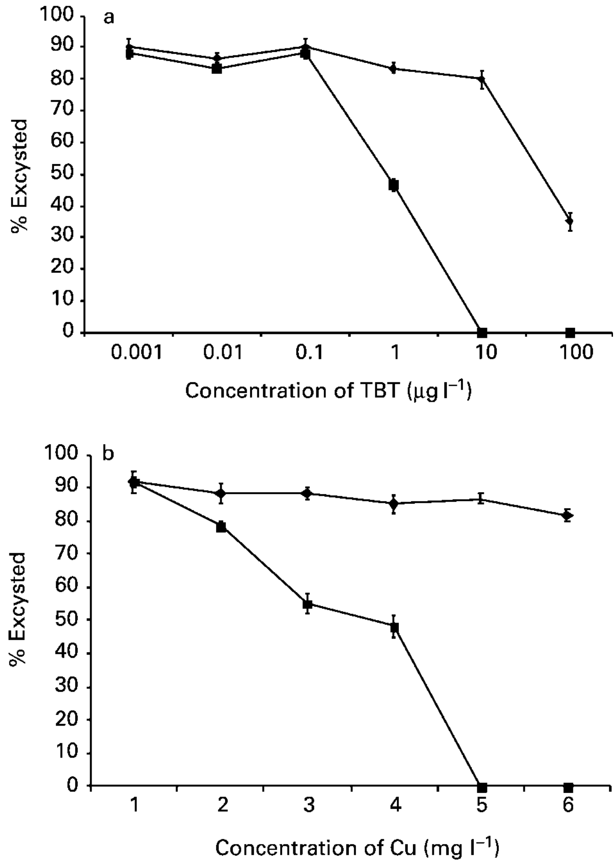

Fig. 4. Comparison of the excystment success of *Parorchis acanthus* metacercariae exposed to (a) tributyltin (TBT) and (b) copper (Cu) solutions before and after encystment (■) and those encysted in natural seawater and exposed only as encysted metacercariae (◆).

N. attenuatus cercariae were less likely to form cyst associations under stress from mechanical agitation of water and increased levels of cadmium and zinc respectively. The present experiment has produced a novel result in that the percentage of cercariae that formed cysts at the highest levels of metals adopted (10 and 100 $\mu g\,l^{-1}$ TBT and 6 $mg\,l^{-1}$ Cu) was actually greater than in the less concentrated solutions. Perhaps this phenomenon could be likened to an 'emergency response' in that, under such adverse conditions, if the cercariae rapidly enclose themselves within a protective sheath they thereby reduce the time that they are in direct contact with the pollutant. How such a response might have evolved is open to speculation, but it is likely that cercariae often find themselves exposed to various environmental factors, such as agitated water, high or low temperature, or low salinity as well as anthropogenically derived pollutants. An ability to rapidly produce a barrier against such adverse conditions could provide a selective advantage.

The fact that *P. acanthus* metacercariae that formed their cysts in unpolluted seawater were not significantly affected by concentrations up to 10 $\mu g\,l^{-1}$ TBT and 5 $mg\,l^{-1}$ Cu is very important. It clearly shows that the metacercarial cyst wall provides protection against both TBT and Cu at and below these concentrations. According to Rees (1966) the cyst wall of *P. acanthus* consists of two main layers, the outer and the inner cyst walls. The outer cyst wall is secreted first, followed shortly afterwards by the inner cyst wall. Although no tanning mechanism occurs in the cyst wall of *P. acanthus* (Rees, 1966), it is a complex and efficient structure made up of linked glycopolysaccharides and lipids. The cyst wall clearly provides physical protection for encapsulated metacercariae and the present work has shown that it also has a role as a chemical barrier. This may constitute a survival factor for *P. acanthus* because, in the event of a relatively brief episode of heavy metal pollution, all newly released cercariae would be vulnerable. However, all the already encysted metacercariae would be protected and therefore survive to sustain the endangered population.

The results presented in this paper show that metacercarial cysts laid down in concentrations of TBT ranging from 1 to 100 $\mu g\,l^{-1}$ and 2 to 6 $mg\,l^{-1}$ Cu provided significantly less protection than those laid down in unpolluted seawater. It could be assumed that the formation of the cyst walls may have been adversely affected by the pollutants. A similar effect on *P. acanthus* metacercariae was attributed to Cd, Zn and Cd/Zn mixtures by Morley *et al.* (2001). Work by Cable & Schutte (1973) identified the presence of keratin-like 'granules' during *P. acanthus* cyst formation. Moreover, Evans (1982) provided evidence that metals inhibited the complete extrusion and unrolling of keratin-like 'granules' from the cystogenous glands of *N. attenuatus*. These 'granules' are a necessary constituent part of this protective cyst wall. If the cystogenous glands of *P. acanthus* are affected in a similar manner by TBT and Cu, it is not surprising that the effectiveness of the cyst is compromised. On the other hand, the period spent by cercariae in contact with the TBT and Cu before their encystment could have resulted in reduced viability in a way that is unrelated to cyst formation. In either case, those larval stages that encysted as an 'emergency response' to the higher concentrations of pollutants did not retain the levels of viability exhibited in the lower concentrations. This would indicate that the 'emergency response' was largely unsuccessful, at least under the experimental conditions adopted here. Further experimentation is required to ascertain its efficacy for shorter-term exposures to pollutants.

Tributyltin and copper were chosen for this study because they are known to cause imposex in *N. lapillus*, the intermediate host of *P. acanthus.* It was clear that TBT had a greater detrimental effect on the encystment and excystment success of *P. acanthus* than Cu. In each condition tested, significant decreases in encystment and excystment success were produced by TBT well over 1000 times more dilute that the copper solution producing similar reductions. It would appear, therefore, that legislation bringing about a reduction in the use of TBT as an anti-foulant and the subsequent reintroduction of copper-based anti-foulants may not only be beneficial to the *N. lapillus* population but even more so to the digenean *P. acanthus* which utilizes this gastropod as an intermediate host. If the legislation is effective, the parasite will have access to greater numbers of intermediate hosts in areas where they had become locally extinct. In addition, the cercariae and metacercariae will only be subjected to the less toxic of the two antifoulants. For example, a low concentration ($1\,\mu g\,l^{-1}$) of TBT would cause an excystment failure of about 16%. Of the successful 84%, continued exposure to and excystment in $1\,\mu g\,l^{-1}$ TBT would result in only 47% excystment, i.e. an overall transmission success of 39%. In contrast, exposure to $1\,mg\,l^{-1}$ Cu would produce 95% encystment, 92% excystment and an overall transmission success of 87%, instead of 91% in unpolluted water. This could have a significant effect on the prevalence and intensity of *P. acanthus* in the definitive host, the gull.

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