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Chronic toxicity of water tributyltin (TBT) and copper to spat of the bivalve *Scrobicularia plana*: ecological implications

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ABSTRACT: Two 30 d long static toxicity tests were performed on small spat (2 to 3 mm in length) of the estuarine bivalve *Scrobicularia plana* (da Costa): the effects of tributyltin (TBT) and copper on the survival and burying activity in sand of juveniles were monitored in 1991, and growth and burying activity as affected by TBT were investigated on a sample of a different cohort in 1992. Results showed that an LC_{50} (concentration killing 50% of the population considered) could be set for TBT at <1.3 µg Sn l^{-1} (as analysed), while exposure to up to 80 µg Cu l^{-1} did not result in increased mortalities with respect to the controls. Every dose of TBT tested in 1991 (nominal 0.5, 1, 2 and 4 µg Sn l^{-1}) impaired the burying activity of spat significantly from Day 6, and Cu concentrations at 20 µg l^{-1} and above also increased the burying time of juveniles by the end of the experiment; the no-observed-effect concentration (NOEC) for Cu was the lowest dose tested (i.e. 10 µg l^{-1}). In 1992 the effect of 4 lower TBT concentrations (nominal 50, 125, 250 and 500 ng Sn l^{-1}) on the juvenile burying activity was somewhat inconsistent; however, burying time of individuals exposed to 500 ng Sn l^{-1} was always higher than that estimated to be characteristic of spat at initiation of the test. In addition, juvenile growth (weight gain) was significantly reduced by all TBT concentrations (i.e. lowest range of 12 to 43 ng Sn l^{-1}, as analysed). It is concluded that TBT has probably caused the disappearance of some *S. plana* populations in the UK; similarly, organotin may have also caused the decline in *S. plana* abundance in populations from elsewhere in Europe.

KEY WORDS: Static toxicity test · Cu · TBT · Bivalve juvenile · *Scrobicularia plana*

INTRODUCTION

Marine environmental pollution by tributyltin (TBT) from antifouling paints has been the subject of much scientific literature over the last 10 yr. Evidence relating unprecedentedly low concentrations of TBT with deleterious effects on non-target organisms (particularly molluscs; see Bryan & Gibbs 1991) led to its partial ban in countries all over the world; this promoted a return to the usage of copper as the biocide agent in formulations. Probably the best-known and documented pathological condition specifically induced by TBT is 'imposex' in neogastropods (see review by Gibbs et al. 1991) and shell thickening in oysters (see for instance Alzieu et al. 1986). However, such conspicuous bioindications of TBT contamination are the exception rather than the rule, and it is feared that coastal populations of non-commercial and less-noticeable species may have suffered undetected damage.

The tellinid *Scrobicularia plana* (da Costa) is an infaunal bivalve commonly inhabiting the intertidal soft bottoms of Northeast Atlantic estuaries, from the Norwegian Sea into the Mediterranean and south to Senegal (Tebble 1966). *S. plana* is principally a deposit-feeder (Hughes 1969, Zwarts 1986), and it has documented potential as a biomonitor of metallic contamination in sediments (Bryan et al. 1985). Its usage as such in the UK for more than a decade has led to the

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observation that *S. plana* populations have virtually disappeared in many estuarine sites within embayments such as Poole Harbour and Southampton Water; environments at these areas have been impacted by substantial levels of TBT contamination, both in the water (up to 600 ng Sn l^{-1}) and sediments (up to 0.5 µg Sn g^{-1} dry wt). An inverse correlation between the concentration of TBT and abundance of *S. plana* was suggested (Langston et al. 1987, 1990). On the other hand, *S. plana* was selected as one of the 'key species' by the Intertidal Sediment Working Group within the European programme COST 647 because of its ubiquity, local abundance and importance in the estuarine trophic chain (Keegan 1986). The compilation of COST data pertaining to several locations from Germany to France over periods of up to 18 yr has revealed decreasing densities of the clam which, in some areas, are ascribed to factors such as hard winters and sediment instability (Essink et al. 1991). In addition, 'Indications were found for a gradual decline or even disappearance of the populations of *S. plana* in the northern part of the German and Danish Wadden Sea, the cause of which is unknown' (Essink et al. 1991). Other than this review, references reporting a similar decline of *S. plana* can be found scattered in the literature (Jensen 1992, Zwarts et al. 1992).

Previous laboratory experiments have demonstrated acute toxicity to adult *Scrobicularia plana* at relatively high levels of TBT (Beaumont et al. 1989, Langston & Burt 1991) in spite of TBTs exceptional field bioavailability with respect to that of inorganic tin (Langston et al. 1990). These authors postulated that the observed lack of recruitment into clam populations might result via induction of some reproductive failure and/or through TBT toxicity to more sensitive early life stages. While studies on *S. plana* embryos and larvae have shown that environmentally realistic levels of TBT prevent a major proportion of these planktonic phases from undergoing normal development (Ruiz 1993), it is particularly relevant to address the pattern of size frequency histograms of *S. plana* populations in healthy estuaries, with uninterrupted modes reflecting successive recruitments, and in sites affected by TBT pollution, showing a perturbed, discontinued structure (see Langston et al. 1990). Thus, although some settlement of spat may occur even at moderately contaminated sites (possibly representing imports from less polluted areas), recruits do not reach maturity and they are presumed to die out as toxic burdens are accumulated in tissues. Similar short-lasting recruitment, whether resulting from primary (i.e. pediveligers metamorphosing *in situ*) or secondary (i.e. colonizing postlarvae) settlement, has also been observed during non-winter periods in several of the estuaries indicated by Essink et al. (1991) as lacking previous *S. plana* populations.

Siphon size is one of the principal factors determining the burying depth of benthic bivalves (Zwarts & Wanink 1989) and, therefore, recently metamorphosed spat of *Scrobicularia plana* (<1 mm; see Frenkiel & Mouëza 1979) are confined to the top few cm layer of the sediment for at least several months. Over that period, juveniles are exposed to a number of natural risks (cold winters, predators) to which they are particularly sensitive because of their shallow burial; this also results in accidental emergence caused by mechanical disturbances such as wave action in stormy weather (Hughes 1970) and current scour (Palmer & Gust 1985). Such events promote a prompt reburial as a behavioural response to reduce obvious exposure to predators and also to avoid being washed away from the selected substratum. Consequently, there is a very high mortality among juveniles, a significant percentage of which is due to predation by flatfish, shrimp, crabs (see several references in Zwarts & Wanink 1989) and birds (Hughes 1970). Their most effective means of protection is to increase burial depth and, as a result, quick growth to increase siphon mass seems to take priority over reproduction in benthic bivalves (Zwarts & Wanink 1989). Thus, in addition to acute toxicity, the ecological significance of sublethal effects of pollutants such as reduction of the normal growth rate of juveniles is also obvious. On the other hand, the applicability of behavioural measures in environmental stress assessment has long been recognized (Olla et al. 1980), and the relevance of impairment or curtailment of burying activity induced by pollution for bivalve avoidance of crab predation has been explicitly proved (Pearson et al. 1981). The objective of this work was to determine the effects of dissolved TBT and Cu on survival, growth and burying activity of small *S. plana* juveniles; the effects of sediment TBT are dealt with in a companion paper (Ruiz et al. 1994, this issue).

MATERIAL AND METHODS

1991 experiment: high TBT and Cu concentrations. ***Procedure:*** Surficial sediment from an intertidal mudflat in the Torridge estuary (North Devon, England, Ordnance Survey Grid Reference SS453308) was sieved *in situ* through a 1 mm pore stainless steel mesh and brought to the laboratory in February 1991. About 900 juveniles of *Scrobicularia plana* were sorted out and sized to the nearest 166 µm using a stereo microscope and ocular graticule calibrated against a micrometer slide. This process took 3 d, during which time the clams were held at the experimental temperature (15 ± 1°C in a constant temperature room illuminated 12 h d^{-1}) in sea water of 24 ± 2‰ salinity. Only those

800 juvenile clams from 1.67 to 3.50 mm in length (mean 2.33 ± 0.43) were used, and their size distribution is given in Table 1. As differences of a few mm between juveniles this size may result in increased variability (either in terms of growth and/or burying activity), 20 experimental groups (40 specimens each) were made up to have a mean length ranging from 2.32 to 2.34 mm (SD = 0.40 to 0.50). Since the external morphology of the juvenile of *S. plana* resembles some other cohabitant clams in estuaries, notably *Abra tenuis* (Montagu), and can lead to error (Gibbs 1984), some positive identification of the clams used was deemed necessary. Although no specimen was sacrificed for this purpose, every juvenile that died in the course of the experiment was examined and species identity confirmed.

Cylindrical Pyrex bowls (9 cm diameter) were used as containers in the experimental microcosm; they had been soaked for several hours in detergent, later in acid (~1 N HCl) and finally rinsed twice in distilled water. The bottom of each vessel was covered with a layer (~7 mm thick) of 75 g of acid-washed sand (40 to 100 mesh, BDH Co.) into which juvenile clams could promptly bury themselves without constituting any possible nutritional source. Filtered (0.45 µm) sea water (24 ± 2‰, hereafter referred to as FSW) was poured at 200 ml per bowl and, after levelling up the layer of sand with some hand-tapping, a set of 40 clams was gently introduced; the flagellate *Isochrysis galbana* (Parke) was added as food to achieve a concentration in bowls of 15 cells μl^{-1}. Four TBT doses (0.5, 1, 2 and 4 µg Sn l^{-1}) and 4 Cu doses (10, 20, 40 and 80 µg l^{-1}) were then made up in vessels and tested together with water (WC) and ethanol (OC) controls (see subsection 'Toxicants' in this section); all treatments were run in duplicate bowls containing sets of clams chosen at random.

The experiment was designed as a 30 d static renewal test. Every other day the entire contents of each bowl were sieved through a tea strainer (1 mm pore) in which clams were retained; they were carefully observed under a microscope and dead individuals (i.e. showing wide-opened valves and not reacting to gentle probing) removed for their later identification and measurement. After decanting the 2 d old water, fresh FSW was added to the original bowls and the layer of sand rearranged. Finally, juveniles were introduced with care so that all of them were deposited on the sand surface simultaneously, food was added by pipette and the toxicant concentrations were made up; vessels were then left undisturbed for the next 48 h. A record of the number of clams found on the surface of the sand layer before each renewal was kept. As a burrowing assay conducted immediately after renewal of each bowl on Days 6, 12, 18, 24 and 30, the number of clams failing to be totally buried was registered over a 1 h period, at 5 min intervals for the first 40 min and every 10 min thereafter. McLachlan & Young (1982) and Donn & Els (1990) measured burying time of adults of other bivalve species as the time from initiation of digging by the foot until complete burial or until all burrowing activity stopped, and Phelps (1989) chose achievement of vertical position prior to digging as the behavioural end point because the clam species she was working on does not bury entirely. In the present case, time from deposition of juveniles on top of the sand until their complete burial was monitored for 1 h because this lapse of total or partial exposure is the period of maximum risk for clams; in addition, all burying individuals (some others were observed to draw the shell erect but failed to progress inwards and fell laterally) dug their whole shells beneath the sand surface. Depth of burial was not measured. After the Day 30 burying tests, every clam was sieved out again; surviving spat (replicates pooled) were blotted dry for 2.5 h, weighed and frozen. Samples of sand (pooled per treatment) were also frozen for subsequent chemical analyses.

Toxicants: Stock solutions at 100, 200, 400 and 800 µg Sn ml^{-1} in ethanol were prepared from standard TBT (bis tri-n-butyltin oxide, 97%, Ventron GmbH), and spiking of 200 ml FSW bowls with 1 µl of these stocks rendered nominal concentrations of 0.5, 1, 2 and 4 µg Sn l^{-1}, respectively. A volume of 1 µl ethanol was added to the OC (ethanol control) bowls so that they were at the same ethanol concentration reached

Table 1. *Scrobicularia plana*. Distribution of the 800 juveniles used in the 1991 experiment into the size classes defined; and size class composition of every experimental set of 40 juveniles, year 1992, and initial weight of individuals as estimated from pooling a number (n) of spare clams

	Size class (mm)											
	1.67	1.83	2	2.17	2.33	2.5	2.67	2.83	3	3.17	3.33	3.5
1991 Distribution	13	107	154	144	109	74	62	42	43	19	17	16
1992 Size class composition		5	12	12	6	5						
Initial weight (mg)		0.291	0.326	0.410	0.500	0.817						
n		11	23	10	3	6						

in TBT vessels (i.e. 5 µl EtOH l^{-1}). Small volumes (up to 16 µl) of standard cupric nitrate (Spectrosol, BDH) were added with a micropipette or a microsyringe to render the desired Cu levels (10, 20, 40 and 80 µg l^{-1}) in experimental bowls.

Given the characteristics of the experiment and the tendency of hydrophobic chemicals such as TBT to be adsorbed onto the walls of the container, therefore diminishing the concentration in solution, it was deemed necessary to have an estimation of the range of toxicant levels the juveniles were actually exposed to during the 48 h between renewals. Thus, on Day 20 of the experiment, 48 h old experimental TBT solutions were pooled per treatment in acid-soaked bottles, acidified (5 ml concentrated HCl l^{-1}) and kept in the dark until analysed; 0 h volumes were freshly made up (FSW + stock TBT + algae) and stored accordingly. Although this method ignores some possible dose to dose variability, it is assumed that analyses of solutions used on Day 20 are a good estimation of conditions throughout the test. TBT solutions (~400 ml) were extracted and analysed as detailed in Bryan et al. (1986). In summary, test samples and 24 ± 2‰ sea water plus standard additions were extracted with 5 ml of hexane by hand-shaking for 4 min; tin was measured in a Perkin-Elmer 603 AA coupled with 76B graphite furnace, rendering a detection limit of <1 ng Sn l^{-1} ($[TBT^+] \approx 2.5[Sn]$). Cu levels were directly determined on a few ml of acidified sample in a Varian SpectrAA-300 Zeeman graphite furnace. Concentrations of Cu and tin in sand were determined following the standard procedure described for sediments in Bryan et al. (1985 and 1986, respectively).

1992 experiment: low TBT concentrations. ***Procedure:*** In February 1992 new material within surficial sediment retained by a 1 mm pore sieve at the same Torridge site was brought to the laboratory. The day after, ~1200 juvenile *Scrobicularia plana* were sorted out and placed in several holding bowls with acid-washed sand; 3 h later 87 clams had not buried and were discarded. One day later another 120 juveniles found on the surface of the sand were also rejected; the rest were sized to the nearest 166 µm as above (see subsection 'Procedure' in the previous section) and 800 of those juveniles from 1.83 to 2.50 mm in length were distributed in 20 bowls so that each set of 40 clams had identical size class composition (mean 2.14 ± 0.20 mm; see Table 1). Of the 20 sets, 8 were used in the sediment toxicity bioassay described in the companion paper, 10 were used in this experiment and 2 were utilised to estimate initial values of burrowing activity and weight. For this purpose, the 2 initial clam sets were deposited on sand in individual bowls and observed for 1 h as described above (see subsection 'Procedure' in the previous section); sets of clams were then dug out, blotted dry for 2.5 h, weighed and frozen. Spare clams 1.83 to 2.50 mm in length other than the 800 used in toxicity tests were pooled per size class and similarly weighed to estimate dry weight of individuals of a certain size (Table 1). An additional 100 clams 2.67 to 3.17 mm long were not used in the trial but sacrificed for identification purposes. The 10 sets of 40 clams considered here were the subject of an experiment similar to that of 1991; this experiment was also run at 15 ± 1°C. Each group of clams was deposited on a sand layer ~7 mm thick contained in a bowl to which 200 ml FSW (24 ± 2‰) and algae *Isochrysis galbana* at 15 cells $µl^{-1}$ had been added. This time duplicated treatments consisted of 4 lower TBT doses (50, 125, 250 and 500 ng Sn l^{-1}) as well as the ethanol or solvent control (OC). Experimental solutions were renewed every second day and dead clams separated, measured and identified. Effects of the lower TBT doses were expected to be more subtle and the response of clams less marked than the previous year; therefore, 1 h burrowing activity was only observed on Days 6, 18 and 30. After the Day 30 burying assay, the juveniles were sieved out, blotted dry for 2.5 h and individual sets weighed and frozen.

Toxicant: Stock solutions at 10, 25, 50 and 100 µg Sn ml^{-1} in ethanol were prepared from standard TBT, and spiking of bowls with 1 µl of these stocks rendered nominal concentrations of 50, 125, 250 and 500 ng Sn l^{-1}, respectively. A volume of 1 µl ethanol was added to the OC vessels so that every bowl had the same solvent concentration (i.e. 5 µl EtOH l^{-1}). On Day 26 of the experiment, 48 h old solutions were kept, 0 h solutions made up and ~400 ml volumes extracted and later analysed as described above.

RESULTS

1991 experiment: high TBT and Cu concentrations

Effects on survival

Of a total of 163 clams found dead in the course of the experiment, up to 15 were accidentally killed by experimental handling, particularly on Days 0 and 30; since all of the 163 were positively identified as juveniles of *Scrobicularia plana*, it is assumed that all clams used belonged to this species. The number of survivors as assessed in each bowl every second day was expressed as a percentage of the original 40, and the mean result for every duplicated TBT and control treatment is plotted in Fig. 1. When transformed data were analysed by ANOVA and subsequent Student-Newman-Keuls test (SNK, $\alpha = 0.05$; Table 2), it was shown that survival of juveniles was significantly

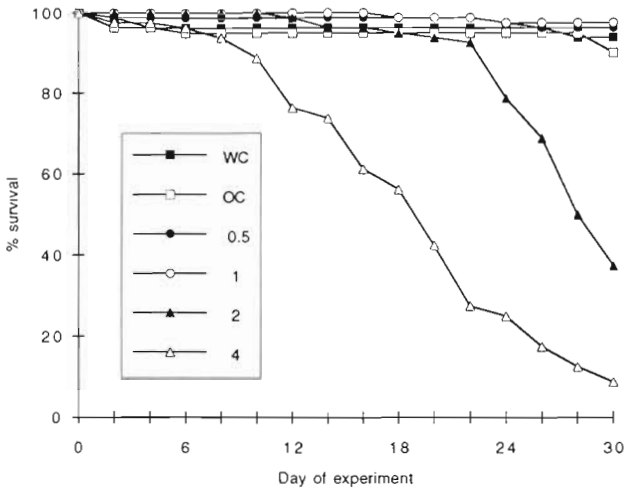

Fig. 1. *Scrobicularia plana*. Mean percentage survival of juveniles after exposure to TBT (nominal 0.5, 1, 2 and 4 µg Sn l^{-1}) and water (WC) and ethanol (OC) control treatments during the 1991 experiment

reduced by Day 12 in bowls exposed to the highest TBT dose (4 µg Sn l^{-1}), and by Day 24 also in bowls at 2 µg Sn l^{-1}. At the end of the experiment, survivors in the 2 highest TBT doses amounted only to ~10% and ~40% of initial numbers, respectively. No other treatment caused any mortality significantly different from that in controls (i.e. ~10%).

Effects on burying activity

Behaviour of spat once in bowls was uniform and the great majority of them readily accepted the sand as a burying substratum; only a few of them found it unacceptable and crawled up the walls of the container to reach the water surface and drifted. However, this tendency was only observed during the first few days, and after a week every clam either buried or stayed on the sand surface.

Table 2. *Scrobicularia plana*. ANOVA of percentage survival of juveniles after exposure to TBT doses (nominal 0.5, 1, 2 and 4 µg Sn l^{-1}) and water (WC) and ethanol (OC) control treatments in the 1991 experiment as measured on Days 12, 24 and 30. Variances were shown to be homogeneous by Cochran's test: Days 12 and 30, $C < C_{95}$ (6,1); Day 24, $C < C_{99}$ (6,1). $^{\bullet}p < 0.05$, $^{\bullet\bullet}p < 0.01$, $^{\bullet\bullet\bullet}p < 0.001$

Source	df	MS	*F*	p
Day 12				
Dose	5	160.8	19.3	0.0012$^{\bullet\bullet}$
Error	6	8.33		
SNK: 1 = 2 = 0.5 = WC = OC > 4				
Day 24				
Dose	5	1426	60	0.0001$^{\bullet\bullet\bullet}$
Error	6	23.96		
SNK: 0.5 = 1 = WC = OC > 2 > 4				
Day 30				
Dose	5	2886	60.9	0.0001$^{\bullet\bullet\bullet}$
Error	6	47.4		
SNK: 1 = 0.5 = WC = OC > 2 > 4				

The number of juveniles buried in every bowl as observed during 1 h every sixth day of the experiment was transformed and expressed as a percentage of the total participating in a particular assay (i.e. total surviving to that moment); the results (mean of duplicates) are plotted in Fig. 2 (values for ethanol control and TBT treatments) and Fig. 3 (water control and Cu doses). Clams in both control treatments maintained a consistent and relatively quick (100% dug in by 35 to 40 min) burying performance throughout the duration of the experiment. On the contrary, spat in every toxicant treatment (except 10 µg Cu l^{-1}) deviated from control activity in all (TBT case) or some of the 5 burying trials carried out every sixth day (Cu experiment). This is reflected in a reduction of the slope (i.e. burying speed) of the curve defined by the data points, and also in a decrease of the asymptote reached by the 1 h curve (i.e. number achieving complete burial), from 100% in the control and some toxicant treatments down to ~12% on Day 30 for clams exposed to 80 µg Cu l^{-1}. Furthermore, the numbers still unburied 2 d after the burrowing trial of Days 6, 12, 18 and 24 (as noted in the record kept of clams found on surface before renewal every second day) were mostly equal to or greater than the number left unburied after 1 h, suggesting that those clams which did not achieve complete burial in 1 h could not dig themselves in during the following 48 h; this would argue for a permanent inability to bury in those circumstances. However, this possibility cannot be confirmed since individuals on the surface 2 d after 1 h observations may include some clams which were buried at some stage but dug themselves out later on; a monitoring technique such as continuous video recording would have been required to ascertain juveniles remaining permanently on the surface.

Further data transformation was then required to study results obtained in 1 h assays. Although experiments relating time with percentage response usually resort to probit-like treatment of data (see for instance Abel 1991), present numbers would not render meaningful results because the percentage response crucial for probit (i.e. 50%) was in many cases either reached by the time of the first observation (5 min) or not achieved at all during the whole experimental period considered (1 h). The average burying time for each individual observation was then calculated as follows:

$$\frac{\sum_{t=0}^{70}\left(n_{t'} - n_{t''}\right)\left(\frac{t'' + t'}{2}\right)}{n_0}$$

where t is time (min), n is the number of clams not totally buried at a given time (all juveniles for $t = 0$), and t'' is the time point immediately after t'; for this transformation individuals not buried by 60 min are necessarily assumed to be buried by a hypothetical time of 70 min.

Fig. 4 plots the mean burying time (min) of juveniles in all assays as estimated by the above transformation for clams in TBT and control treatments; when data were subject to ANOVA (Table 3), differences were shown to be significant in all 5 trials considered: Day 6 (all treatments), Days 12 and 18 (4 µg Sn l^{-1} not included because survival was already affected) and Days 24 and 30 (2 µg Sn l^{-1} also excluded for same reason). SNK test ($\alpha = 0.05$) included both controls in the same group every time; also, it always considered that the results for all TBT doses were different from each other and separated from the control group. Lack of independence of samples (same spat throughout) precludes use of ANOVA to test effects of one particular treatment over time; however, there is no significant difference between the burying time of water controls on Day 6 and that of ethanol controls on Day 30, and vice versa. Although there is no estimation of initial burying time of clam sets, it can be assumed that it was similar for every set and it was not substantially different than that maintained by control groups for the duration of the experiment (5 to 6 min overall). It can surely be concluded that exposure to every TBT dose considered resulted in a progressive and significant increase of the time taken by juveniles to rebury as early as Day 6.

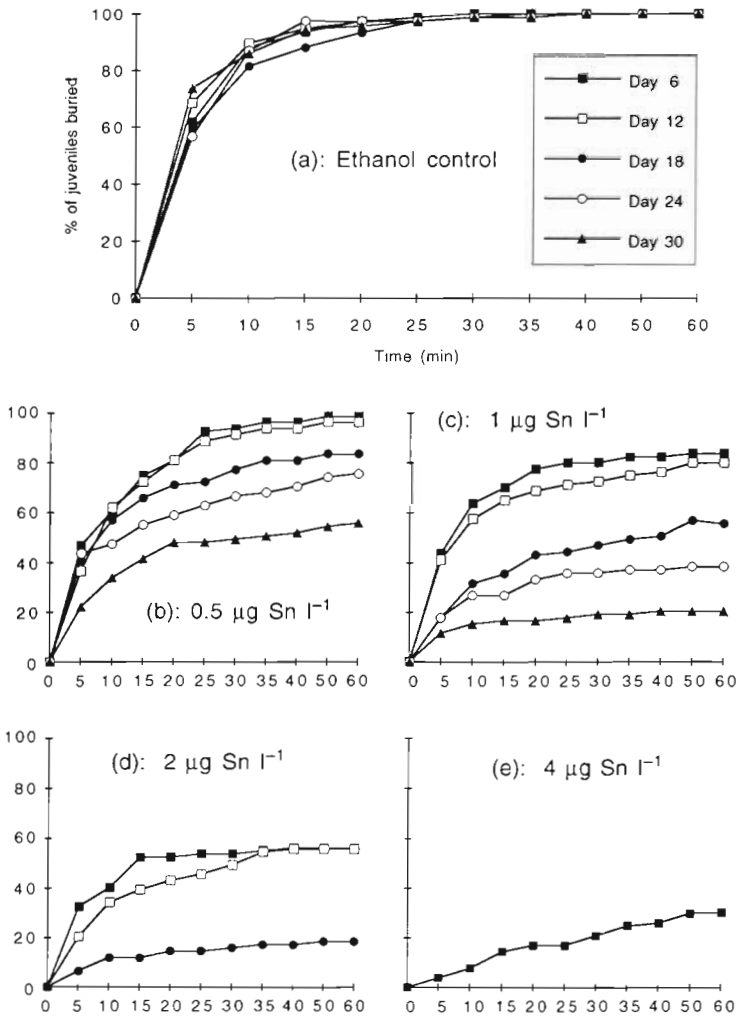

Fig. 2. *Scrobicularia plana*. Mean reburying activity every sixth day (1 h observation) displayed by juveniles after exposure to (a) ethanol control and (b to e) TBT (nominal 0.5, 1, 2 and 4 µg Sn l^{-1}) treatments during the 1991 experiment, expressed as (no. buried/clams surviving to that time) × 100. Symbols in (b to e) as in (a). No data are plotted for 2 and 4 µg Sn l^{-1} after Days 18 and 6, respectively, because survival was significantly affected

Fig. 5 plots the estimated burying time of juveniles in Cu and water control treatments, and Table 4 compiles results of ANOVA and SNK tests performed on transformed data. No differences between treatments are displayed for Day 6; however, differences are shown to be significant ($p < 0.05$) in every trial thereafter, and by Day 30 burying time of clams in all Cu doses except 10 µg l^{-1} was longer than the time taken by control clams. Effect of Cu was also progressive over time and its severity became more acute with increasing levels of exposure.

Although no attempt was made to measure depth of burial, the sand surface of each bowl was closely observed at the time of counting unburied individuals before renewal every second day; while this could not detect the exact position of clams within the control bowls (except if there were signs of activity such as siphonal movement), location of individuals in most of the toxicant bowls was often possible because of the distinct coloration of shells showing through the thin covering layer of sand. This is a clear indication that many individuals in the toxicant treatments did not achieve a burial as deep as the control juveniles.

TBT and Cu concentrations in solution and sand

Results of analyses of TBT solutions performed on test volumes freshly made up and those used on Day 20 of the experiment are

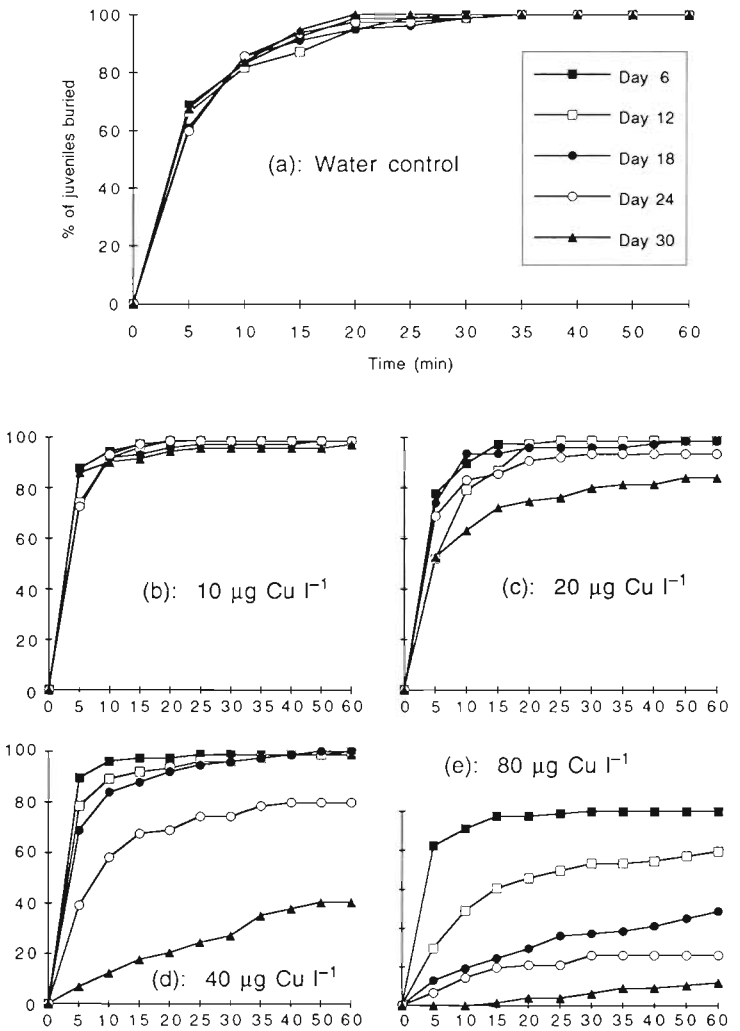

Fig. 3. *Scrobicularia plana*. Mean reburying activity every sixth day (1 h observation) displayed by juveniles after exposure to (a) water control and (b to e) copper (10, 20, 40 and 80 µg l^{-1}) treatments during the 1991 experiment, expressed as (no. buried/clams surviving to that time) × 100. Symbols in (b to e) as in (a)

plotted in Fig. 6. While actual concentrations yielded by preparation procedure amounted up to around 80% of nominal values, TBT levels after 48 h in experimental conditions decreased to 40, 53, 70 and 67% of initial values, respectively, for concentrations of 0.5, 1, 2 and 4 µg Sn l^{-1}; TBT in control solutions was not detectable. The marked drop between initial and final values is due to TBT adsorption characteristics and also to the presence in bowls of microalgae (15 *Isochrysis galbana* cells μl^{-1}) which are known to bind appreciable amounts of organotin (Laughlin et al. 1988). It could then be calculated that the range of TBT concentrations the juveniles were actually exposed to is 0.145–0.365, 0.463–0.867, 1.281–1.827 and 2.511–3.752 µg Sn l^{-1} for the nominal concentrations of 0.5, 1, 2 and 4 µg Sn l^{-1}, respectively. Levels of TBT in solution resulted in no detectable concentration of organotin in sand from any treatment. In view of the results of analyses carried out on initial and final Cu solutions (Fig. 7), levels of metal during the 48 h between renewals were consistent and close to nominal figures; deviations from this general rule may be due to interaction of spikes with Cu present at detectable concentrations in supply water. Levels of Cu as analysed in sand exposed for 30 d to solution concentrations were 0.4, 0.7, 0.6, 0.8 and 1.3 ppm dry weight, respectively for water controls and 10, 20, 40 and 80 µg Cu l^{-1} treatments.

1992 experiment: low TBT concentrations

Effects on burying activity

Only 4 of the 400 clams considered died during the course of the experiment; all of them, together with the 100 specimens sacrificed beforehand, were positively identified as juveniles of *Scrobicularia plana*. The reburying activity of each lot of clams (initial sets and experimental treatments) during 1 h assays conducted on Days 6, 18 and 30 is plotted in Fig. 8 after transformation of data as percentage juveniles buried. No decrease of the asymptote reached by the resulting curves (i.e. percentage buried in 1 h) is prominent, but reductions over time of slopes (i.e. burying speed) with respect to that of initial sets become apparent; this is more marked with increasing TBT doses, but it also seems to happen in ethanol control on Day 18. Using the formula described in the previous section, original data were transformed and the average burying time calculated for each individual observation; results are plotted in Fig. 9. Considering the mean burying time of initial sets as an acceptable and independent estimation of the burying time of every group before exposure, data were analysed by ANOVA and post hoc SNK test ($\alpha = 0.05$; Table 5). Significant differences were displayed in every assay (Days 6, 18 and 30) between mean burying times in the treatment of 500 ng Sn l^{-1} and those in initial sets, but never between these and ethanol control values; lower TBT doses were also shown to have resulted in burying times higher than initial on Day 18, but not on Days 6 and 30. Indications of shallower burial of juveniles as described above were also observed in bowls treated with 250 and 500 ng Sn l^{-1} from Day 20 onwards.

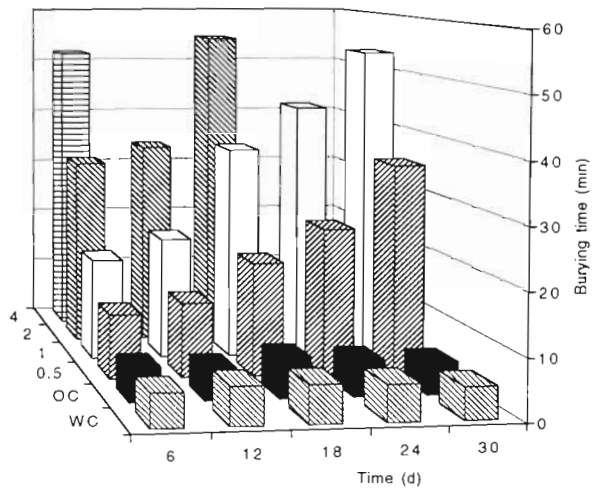

Fig. 4. *Scrobicularia plana*. Mean burying time of juveniles every sixth day after exposure to TBT (nominal 0.5, 1, 2 and 4 µg Sn l^{-1}) and water (WC) and ethanol (OC) control treatments during the 1991 experiment. No data are plotted for 2 and 4 µg Sn l^{-1} after Days 18 and 6, respectively, because survival was significantly affected

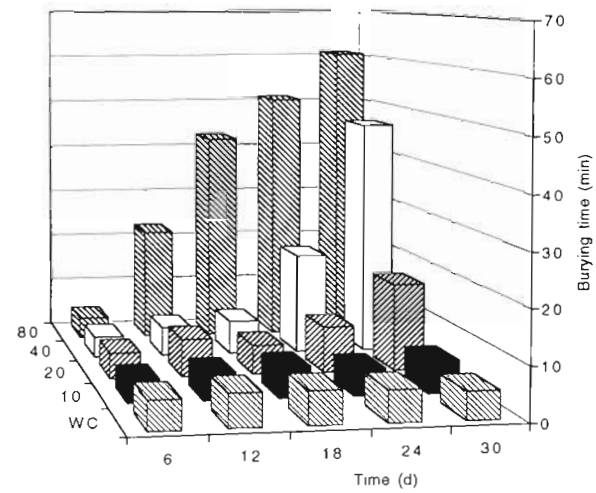

Fig. 5. *Scrobicularia plana*. Mean burying time of juveniles every sixth day after exposure to copper (10, 20, 40 and 80 µg l^{-1}) and water control (WC) treatments during the 1991 experiment

Effects on growth

The net weight gain of experimental sets where no mortality occurred (n = 40) was calculated as the blotted dry weight (mg) of groups on Day 30 minus the mean weight of initial sets; weight gain of sets with final n < 40 was corrected by subtracting from the mean initial weight the individual weight of specimens from the same size class as the dead clams (as measured on 1.83 to 2.5 mm spare clams; see Table 1). The net weight gained by juveniles in each individual bowl

Table 3. *Scrobicularia plana*. ANOVA of burying time of juveniles after exposure to TBT doses (nominal 0.5, 1, 2 and 4 µg Sn l^{-1}) and water (WC) and ethanol (OC) control treatments in the 1991 experiment as measured on Days 6, 12, 18, 24 and 30. All variances were shown to be homogeneous by Cochran's test: Day 6, $C < C_{95}$ (6,1); Days 12 and 18, $C < C_{95}$ (5,1); Days 24 and 30, $C < C_{95}$ (4,1). Significant values as in Table 2

Source	df	MS	*F*	p
Day 6				
Dose	5	672	548	0.0001***
Error	6	1.23		
SNK: 4 > 2 > 1 > 0.5 > OC = WC				
Day 12				
Dose	4	312	82	0.0001***
Error	5	3.79		
SNK: 2 > 1 > 0.5 > WC = OC				
Day 18				
Dose	4	893	113	0.0001***
Error	5	7.9		
SNK: 2 > 1 > 0.5 > OC = WC				
Day 24				
Dose	3	659	443	0.0001***
Error	4	1.49		
SNK: 1 > 0.5 > WC = OC				
Day 30				
Dose	3	1135	173	0.0001***
Error	4	6.55		
SNK: 1 > 0.5 > OC = WC				

Table 4. *Scrobicularia plana*. ANOVA of burying time of juveniles after exposure to copper doses (10, 20, 40 and 80 µg l^{-1}) and water control (WC) treatments in the 1991 experiment as measured on Days 6, 12, 18, 24 and 30. All variances were shown to be homogeneous by Cochran's test at C_{95} (5,1) except Day 12: $C < C_{99}$ (5,1). ns: not significant; other significance values as in Table 2

Source	df	MS	*F*	p
Day 6				
Dose	4	0.71	0.8	0.5844 ns
Error	5	0.92		
Day 12				
Dose	4	112	8.7	0.018*
Error	5	13		
SNK: 80 > 20= WC = 40 = 10				
Day 18				
Dose	4	537	271	0.0001***
Error	5	1.98		
SNK: 80 > 40= WC = 10 = 20				
Day 24				
Dose	4	749	64.3	0.0002***
Error	5	11.7		
SNK: 80 > 40 > 20 = WC = 10				
Day 30				
Dose	4	1277	245	0.0001***
Error	5	5.21		
SNK: 80 > 40 > 20 > 10 = WC				

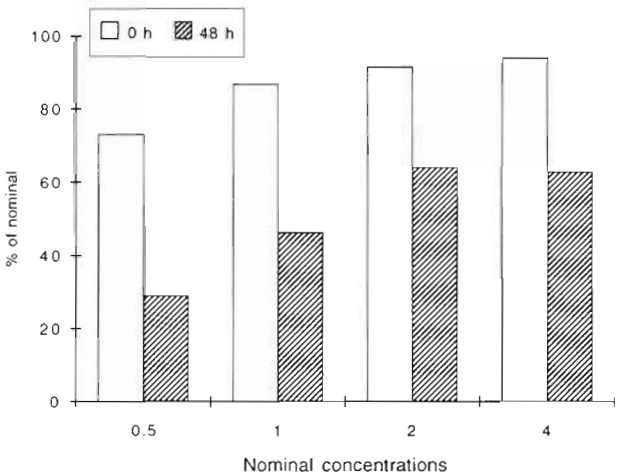

Fig. 6. Initial (0 h) and final (48 h) tin concentrations in TBT treatments (nominal 0.5, 1, 2 and 4 µg Sn l^{-1}) as analysed on Day 20 of the 1991 experiment. TBT in controls was not detectable

was then transformed as a percentage of the mean weight of initial sets, and the average results for all treatments are plotted in Fig. 10. When data were analysed by ANOVA (Table 6), juveniles exposed to every dose of TBT were shown to have gained significantly ($p < 0.05$) less weight than clams in ethanol control sets.

TBT concentrations in solution

The actual concentrations yielded by direct spiking of experimental volumes with stock TBT amounted on Day 26 of experiment to 87 to 115 % of nominal values; however, levels detected in 48 h solutions dropped to 27, 23, 23 and 29 % of initial figures, respectively, for nominal treatments of 50, 125, 250 and 500 ng Sn l^{-1}. This remarkable drop is due again to the adsorption characteristics of TBT and the presence of microalgae in solutions; while reduction of TBT levels due to the former cause should be comparable to that which also occurred the previous year (same type of bowls), the action of the latter was apparently more acute during this experiment on lower concentrations of TBT. The range of TBT levels (as analysed) the juveniles were actually exposed to could then be estimated as 12–43, 27–122, 65–289 and 134–459 ng Sn l^{-1} for the nominal concentrations of 50, 125, 250 and 500 ng Sn l^{-1}, respectively.

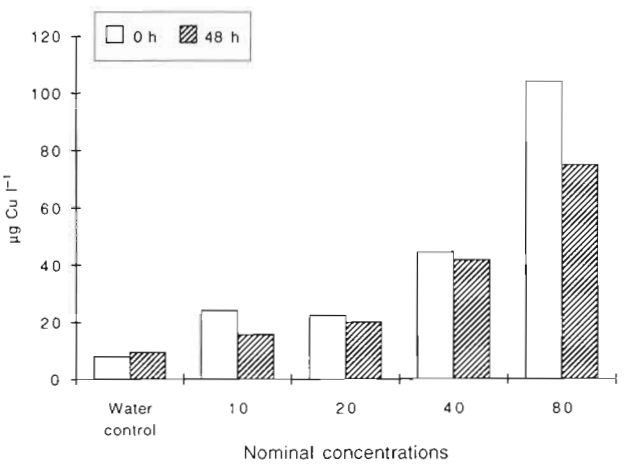

Fig. 7. Initial (0 h) and final (48 h) copper concentrations in treatments (nominal control and 10, 20, 40 and 80 µg Cu l^{-1}) as analysed on Day 20 of the 1991 experiment

DISCUSSION

Survival

According to data in Fig. 1, a 30 d LC_{50} (concentration killing 50 % of the population considered) for *Scrobicularia plana* ~2.3 mm long could be set for TBT at < 1.3 µg Sn l^{-1} (lower end of the actual range detected in the nominal concentration of 2 µg Sn l^{-1}). This suggests that spat of *S. plana* may be more resistant to organotin than those of mussels *Mytilus edulis* (L.) (14 d $LC_{100} \approx 1$ µg Sn l^{-1} or 2.6 µg l^{-1} TBT oxide as analysed) subjected to a flow-through, 45 d long test (Thain & Waldock 1985). Even though proper comparisons between the work of Thain & Waldock and the present study cannot be established because of methodological differences, the hypothesis of *S. plana* being more resistant to organotin than mussels is reinforced by the fact that initial weight of their experimental spat was 3 orders of magnitude higher than that of *S. plana* juveniles used here; the latter also applies to the work of Paul & Davies (1986) on juvenile scallops which, unfortunately, does not provide data on levels of TBT exposure. Alternatively, since mussels are filter-feeders (as opposed to *S. plana*, mainly a deposit-feeder), it could also be that their better filtration efficiency resulted in raised TBT uptake from tainted algal foodstuff and, hence, increased acute toxicity. Since the organotin concentrations shown to be lethal to *S. plana* are rather high, it is thought that only those clam populations established in areas adjacent to some boating centres may have suffered the acute effects of TBT.

Growth

Although both shell length and weight gain may be monitored to assess growth of bivalves, only the latter was used here due to simplicity and accuracy of proce-

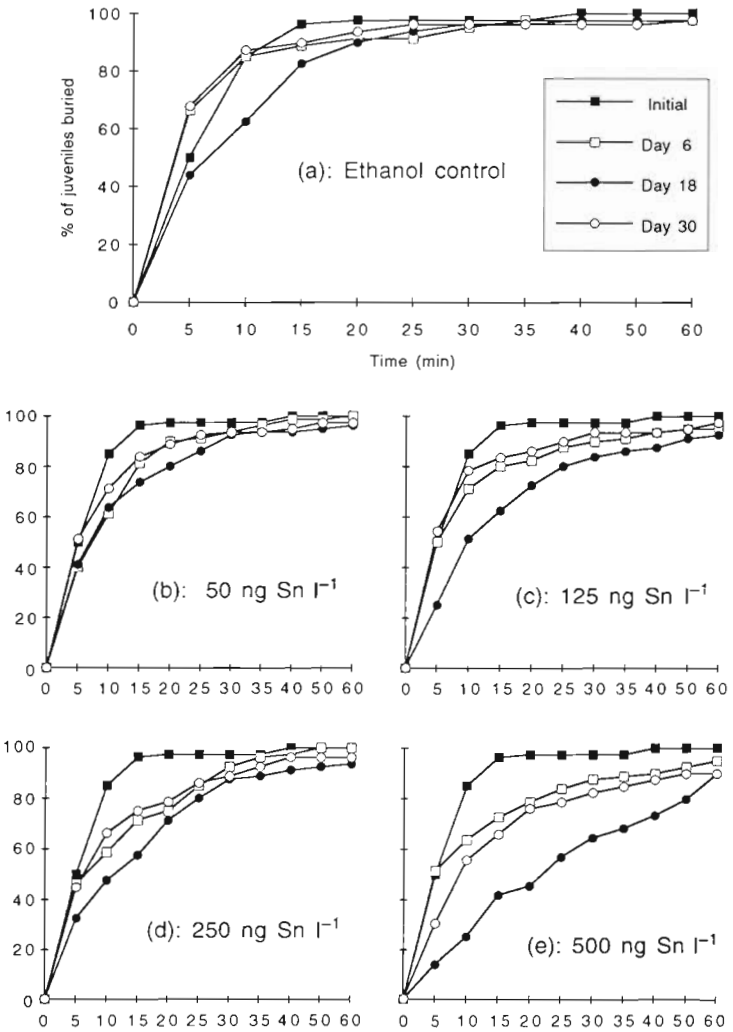

Fig. 8. *Scrobicularia plana*. Mean reburying activity (1 h observation) displayed by juveniles after exposure to (a) ethanol control and (b to e) TBT (nominal 50, 125, 250 and 500 ng Sn l^{-1}) treatments during the 1992 experiment, expressed as (no. buried/clams surviving to that time) × 100. Symbols in (b to e) as in (a)

dures; nevertheless, shells were measured initially to guarantee uniform distribution of size classes among experimental groups. As data from the 1992 experiment show (Fig. 10, Table 6), treatment of *Scrobicularia plana* juveniles for 30 d with nominal concentrations from 50 to 500 ng Sn l^{-1} (from a range of 12–43 to one of 134–459 ng Sn l^{-1}, as analysed) resulted in weight gain (up to 35% of initial) being significantly reduced with respect to controls (~60% of initial). Decreased growth rates induced by low concentrations of TBT have also been reported for juveniles of *Mytilus edulis* (as shell length; Strømgren & Bongard 1987), *Crassostrea gigas* Thunberg (as shell length; Lawler & Aldrich 1987) and *Ostrea edulis* (L.) (as weight; Thain & Waldock 1985). In this latter work, weight increase of control *O. edulis* (2 to 3 mm) after 20 d amounted up to 200%, whilst *S. plana* control individuals (mean 2.14 mm long) in the present study only gained 60% of initial weight after 30 d; apart from species-specificity of toxicants, this may be also due to composition of algal diet and, again, differences in the filtration efficiency of the suspension-feeder oyster and the deposit-feeder clam, as further suggested by the results of a bioassay in which similarly sized juveniles held for 36 d in a control sediment gained up to 200% of initial weight (see companion paper, Ruiz et al. 1994). Although available data are not conclusive, a dose of 80 µg Cu l^{-1} would also have impaired normal weight gain of *S. plana* spat.

Burying activity

The act of burial is the result of a series of events (see Trueman 1983) which demands considerable mechanical energy cost, particularly in bivalves where the entry into the sediment is achieved after drawing the shell vertical (Trueman & Brown 1992). The energy required for burying into artificial coarser sand may have been higher than the cost needed to dig into the naturally finer deposits usually selected as a settling substratum by juveniles of *Scrobicularia plana*; however, the unaltered burying time of control individuals through both 1991 and 1992 tests proves that small-juveniles (2 to 3 mm long) were fit enough to complete successfully every second day trial after enforced surfacing resulting from the sea water renewal procedure. This was expected from individuals inhabiting the less stable layers of the sediment.

Levels of organotin in sand at the end of the high TBT experiment (1991) were undetectable; this permits us to ascribe increased burying time of exposed clams to gradual debilitation induced by the dissolved toxicant, whether directly uptaken from solution and/or via ingestion of tainted algae. On the contrary, levels of Cu in sand (up to 1 µg g^{-1} dry wt) may have activated some behavioural avoidance as a factor contributing with debilitation to the enhanced burying time of clams; however, these Cu levels are much lower that those in the surficial sediments from which the clams were collected (~25 ppm Cu; see companion paper), and the possibility that 0.6 ppm Cu (difference between sand in bowls exposed to the highest and lowest copper dose) added to observed burying times seems unlikely. The time course

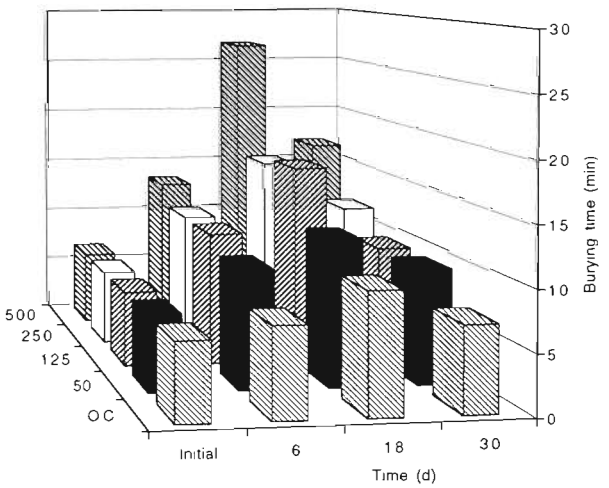

Fig. 9. *Scrobicularia plana*. Mean burying time of juveniles after exposure to TBT (nominal 50, 125, 250 and 500 ng Sn l^{-1}) and ethanol control (OC) treatments during the 1992 experiment

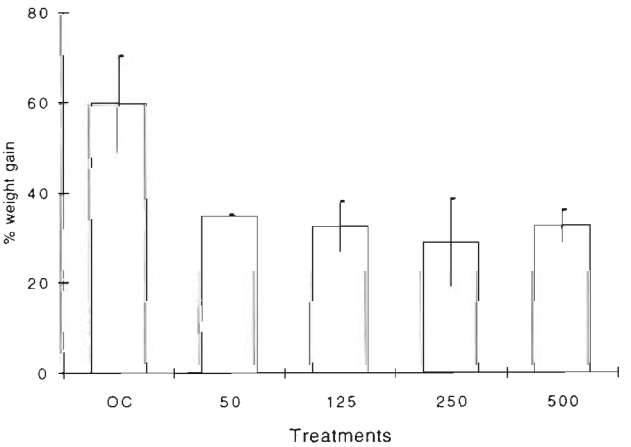

Fig. 10. *Scrobicularia plana*. Growth of juveniles (mean ± SD) after exposure to ethanol control (OC) and TBT (nominal 50, 125, 250 and 500 ng Sn l^{-1}) treatments for 30 d during the 1992 experiment, expressed as percentage weight gained (corrected for mortalities) in relation to weight of initial sets

of clam debilitation can be readily deduced from Fig. 5 (copper test): while burying time of juveniles exposed to 80 µg Cu l^{-1} was not affected by Day 6, it was significantly higher than that of controls for every trial thereafter; similarly, exposure to 40 and 20 µg Cu l^{-1} took 24 and 30 d, respectively, to induce significant deviations from control burying activity. Progression of prolonged burying time is also clear in Fig. 4 (high TBT experiment, 1991), where effects of every dose of organotin were significant from the first assay (Day 6) and the effects were considerable by the Day 2 of the experiment in nominal 2 and 4 µg Sn l^{-1} if attention is paid to the record kept of numbers found on the surface before renewal.

Table 5. *Scrobicularia plana*. ANOVA of burying time of juveniles after exposure to TBT doses (nominal 50, 125, 250 and 500 ng Sn l^{-1}) and ethanol control (OC) treatments in the 1992 experiment as measured on Days 6, 18 and 30. SNK: treatments not underlined by the same line are significantly different at $\alpha = 0.05$. All variances were shown to be homogeneous by Cochran's test at C_{95} (6,1). Significance values as in Table 2

Source	df	MS	*F*	p
Day 6				
Dose	5	13.3	5.3	0.0332•
Error	6	2.51		
SNK: 500 250 125 50 OC Initial				
Day 18				
Dose	5	99.6	32.5	0.0003•••
Error	6	3.07		
SNK: 500 125 250 50 OC Initial				
Day 30				
Dose	5	28.1	5.7	0.0284•
Error	6	4.96		
SNK: 500 250 125 50 OC Initial				

On the contrary, the effects of lower TBT levels on juvenile burying time were not gradual in the 1992 experiment (Fig. 9), and burying times peaked on Day 18 for every treatment including the control. Differences between burying time of day 18 and those of other days within the same treatment were more marked in TBT-exposed clams and, as a consequence, some of their burying times were significantly different from the baseline defined by the initial sets only on Day 18; however, differences between control burying time and initial burying time are not significant for any assay (including Day 18), while juveniles exposed to 500 ng Sn l^{-1} always buried significantly slower than initial sets (Table 5). This is consistent with results of the previous year for the same dose, and variations could therefore be attributed to subtle differences of the degree of sublethal TBT stress on cohorts of consecutive years. Although failure to bury is the main indication of reduced fitness, some index integrating this with the depth of burial achieved could have been useful in explaining variability of the degree of affection should it have been used. Since curtailed burying

Table 6. *Scrobicularia plana*. ANOVA of percentage weight gained by juveniles (in relation to weight of initial sets) after exposure to TBT doses (nominal 50, 125, 250 and 500 ng Sn l^{-1}) and ethanol control (OC) treatments for 30 d in the 1992 experiment. Variances were shown to be homogeneous by Cochran's test at C_{95} (5,1). Significance as in Table 2

Source	df	MS	*F*	p
Dose	4	314	6.27	0.0347•
Error	5	50.1		
SNK: OC > 50 = 500 = 125 = 250				

performance was shown by bivalves which had actually gained weight with respect to initial sets, whether observed effects resulted from TBT-induced narcotization of clams (as oil was suggested to induce in polychaetes; Olla et al. 1984) is a contentious question.

CONCLUSIONS

It has been shown that juvenile *Scrobicularia plana* (~2.2 mm long) held for a month in solutions with TBT at a lowest range of 12 to 43 ng Sn l^{-1} (as analysed) displayed a growth rate significantly reduced with respect to that of controls, and that exposure to TBT levels within the range 134 to 459 ng Sn l^{-1} (and possibly less) or Cu at ~20 µg l^{-1} also impaired the normal burying performance of spat in sand; noxious effects of dissolved butyltin will be worsened by concurrent uptake of sediment-bound TBT through active deposit-feeding (see companion paper, Ruiz et al. 1994). The ecological implications of these findings are clear: diminished growth rate will result in prolonging the stay of recruits in shallower sediment layers and delaying their incorporation to the reproductive stock of the population. Extended juvenile life will also increase chances of definitive loss through dispersal, disease and particularly predation since, in agreement with the optimal foraging theory, crabs consume more small than large bivalves (Walne & Dean 1972, Pearson et al. 1981) as a result of increased vulnerability and reduced handling times (Boulding 1984); the probability of crab predation will be increased further by restrictions on burying speed and depth (Pearson et al. 1981, Blundon & Kennedy 1982, Haddon et al. 1987). While the lowest-observed-effect concentration (LOEC) for Cu (20 µg l^{-1}) is rarely found in estuarine waters (Mance et al. 1984, Bryan & Langston 1992), relatively low to moderate TBT levels (i.e. >10 to <500 ng Sn l^{-1}) which are sublethally stressful to juveniles of *S. plana* in laboratory experiments were commonly and persistently detected in subsurface waters of UK estuaries during the 1980s (Waldock et al. 1987, Waite et al. 1991). More specifically, these TBT levels were of concern for years at field sites where spat were observed to disappear without constituting permanent recruitment into declining populations of the clam (Langston et al. 1987, 1990). It is therefore concluded that TBT has probably caused this demise but, as in most environmental studies, possible contribution to observed field effects of other pollutants displaying similar gradients of contamination, synergism and antagonism of TBT with other natural or anthropogenic factors, and deleterious effects of lower TBT concentrations on these and/or other ecologically relevant parameters, cannot be fully discarded. This hypothesis is not so directly applicable to explain the disappearance of *S. plana* populations from other North Atlantic estuaries (Essink et al. 1991) due to lack of simultaneous studies on TBT concentrations. However, independent chemical monitoring of coastal environments throughout Europe has detected during the mid and late 1980s levels of TBT within the range now proved to be noxious to the early stages of *S. plana* (see for instance Quevauviller & Donard 1990) and, interestingly enough, in some of these areas (e.g. the Dutch Eastern Scheldt) the native population of the specific TBT-biomonitor *Nucella lapillus* (L.) (see Gibbs et al. 1987) has been concurrently exterminated by organotin pollution (Ritsema et al. 1991). Thus, ecotoxicity of TBT is thought to be the most plausible available theory to account for the unexplained decline of *S. plana* throughout Northern Europe and, particularly, for the non-winter mortality of juveniles in affected populations.

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