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THE EFFECTS OF TRIBUTYLTIN (TBT) ACCUMULATION ON ADULT DOG-WHELKS, *NUCELLA LAPILLUS*: LONG-TERM FIELD AND LABORATORY EXPERIMENTS

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(Figs. 1–11)

Following treatment of the shells of adult *Nucella lapillus* with different marine paints under field conditions, the development of 'imposex' (the growth of a penis and vas deferens in females) was promoted by three TBT-based antifouling paints but not by a tin-free copper-based paint nor by yacht enamels.

When adult animals were exposed to a range of sea-water leachates from TBT copolymer antifouling paint the initial rate of tin accumulation was approximately proportional to the ambient concentration over the range <1–107 ng/l of tin in the TBT fraction. In experiments of between 8 and 14 months duration, concentration factors (dry tissue/water) for tin (as TBT) reached about 10^5 at sea water concentrations of 1·5–18·7 ng/l. At 107 ng/l, however, the factor fell to about 3×10^4 owing probably to the accelerated degradation of TBT to DBT by the tissues. The bioaccumulation data from the laboratory experiments compare favourably with field data from populations around south-west England.

At all experimental sea water concentrations, the bioaccumulation of tin by adult females was accompanied by increased development of imposex. However, the sterilisation of adult females through blockage of the oviduct by the vas deferens (as observed in declining field populations) occurred only after at least 12 months exposure to 107 ng/l of tin as TBT: it also occurred after more than 18 months in some adults transplanted to a rocky shore exposed to a mean concentration of about 28 ng/l. Growing juveniles, on the other hand, are far more sensitive and may be sterilized at concentrations of only a few ng/l before reaching maturity.

The depuration of tin was followed for up to 9 months and, depending on the conditions, half-times for loss of the TBT fraction from the tissues ranged between about 50 days and more than 100 days. There was no evidence that loss of tin leads to any remission of imposex.

INTRODUCTION

Populations of the gastropod, *Nucella lapillus* (L.), normally one of the commonest of rocky-shore macroinvertebrates, have recently suffered from declining numbers at many sites along the south coast of England. Bryan *et al.* (1986) showed that these declining populations exhibited a high degree of imposex (the induction of a penis and vas deferens in females) and that imposex was almost certainly caused by tributyltin (TBT) compounds leached from ships' antifouling paints. Evidence implicating TBT compounds in the development of imposex included: (1) a good relationship between the degree of imposex and the proximity of affected populations to harbours and marinas; (2) in Plymouth Sound, the degree of imposex increased dramatically between its discovery in

1969 (Blaber, 1970) and 1985, thus coinciding with the introduction and increasing usage of TBT-based paints; (3) tissue concentrations of tin as TBT increased consistently with the degree of imposex; (4) animals transplanted from a 'clean' area to a harbour site absorbed TBT and developed imposex; (5) preliminary experiments showed that imposex was induced by exposure to 20 ng/l of tin as TBT leached from a TBT-based paint; (6) TBT is implicated in the induction of imposex in other stenoglossan gastropods including *Nassarius obsoletus* (Say) (Smith, 1981) and *Ocenebra erinacea* (L.) (Féral & Gall, 1982).

Further work by Gibbs & Bryan (1986) demonstrated that in populations having moderate to high degrees of imposex, the development of vas deferens tissue in females can occlude the oviduct, thus preventing the release of egg capsules. The build-up of unlaid egg capsules in these sterile females may also lead to their premature death. Thus declining populations usually contain an abnormally low percentage of females, a large proportion of which are sterile.

The objectives of the long-term experiments described in the present paper were (1) to study the time course of tin accumulation and the induction of imposex in adult dog-whelks exposed to realistic concentrations of dissolved tin leached from antifouling paint; (2) to discover whether the development of imposex is reversible; (3) to provide conclusive evidence linking TBT-based antifouling paints to imposex and the decline of *N. lapillus* populations around the United Kingdom.

MATERIALS AND METHODS

Tidal tank experiments

Four tanks of 200 l capacity were continuously supplied with sea water at a rate of about 0·6 l/min: to produce a tidal effect they were pumped out every 12 h and took 5–6 h to refill. *N. lapillus* from Crooklets Beach, Bude, were placed in the tanks in plastic laundry baskets containing barnacle-covered rocks from the same area. The rocks were replaced at intervals to ensure a constant supply of food. The water supplied to each of the three experimental tanks was contaminated by flowing it through a mixing chamber in which the end of a rod treated with TBT-based antifouling paint (International 'Cruiser' copolymer) was immersed. Concentrations of tin in the water were controlled by changing the depth of the rod in the mixing chamber.

Measurement of imposex and sexual development

In both males and females the length of the penis was measured to the nearest 0·1 mm and expressed as the relative penis size (RPS) index, $(\text{female length})^3/(\text{male length})^3 \times 100$, also termed the 'degree of imposex' by Bryan *et al.* (1986). The condition of the vas deferens in females was assessed using the vas deferens sequence (VDS) index described by Gibbs *et al.* (1987).

The degree of sexual development was placed in one of four categories based on the appearance and size of the gonad and capsule gland in females and on the size of the gonad and visibility of the seminiferous tubules in males (cf. Feare, 1970).

Analysis of tin

In *N. lapillus* tissues, tributyltin (TBT) and dibutyltin (DBT) fractions were separated from monobutyltin and inorganic tin by extracting an acidified homogenate with hexane. The extract was then analysed for tin by graphite furnace atomic absorption before and after the dibutyltin (DBT) fraction was removed by shaking with sodium hydroxide solution (Ward *et al.* 1981; Bryan *et al.* 1986). The method is not necessarily specific for TBT or DBT species and other compounds might be present; however, tin added to homogenates as dimethyltin dibromide, trimethyltin chloride, butyltin trichloride or stannous chloride was not extracted significantly.

Water samples of 0·5–1 l were acidified with 5 ml of hydrochloric acid and extracted with 5 or 10 ml of hexane for about 10 mins. After shaking with one-third of its volume of 1 N sodium hydroxide the extract was analysed for TBT tin by graphite furnace atomic absorption. Standardisation was achieved by spiking a replicate sample with a suitable amount of tributyltin oxide dissolved in ethanol and blanks were prepared from pre-extracted sea water. Detection limits below 1 ng/l of tin were reached by reducing the volumes of weighed aliquots of hexane extracts in a desiccator under reduced pressure prior to analysis.

Throughout this work, concentrations in the TBT or DBT fractions extracted from tissues or waters are expressed as $\mu g/g$ dry weight or ng/l of tin (since this is what was measured) rather than as concentrations of TBT or DBT (which would be about 2·5 times higher). Tissue concentrations may be converted to a wet weight basis by dividing by 3·3 for males or 3·0 for females.

RESULTS

Field experiments with marine paints

In May 1985, 300 *N. lapillus* were collected at Widemouth Bay (N. Cornwall) and, after drying, the shell spires of 150 were painted with a free-association antifouling paint (International, 'TBT Antifouling', white) the remainder being treated with a TBT copolymer paint (International, 'Cruiser', self-polishing, white). After a few hours, the animals were replaced on the shore. A month later, 150 additional animals were painted with yacht enamel (International, 'Super High Gloss', Oxford 105) and replaced more than 100 m distant from the

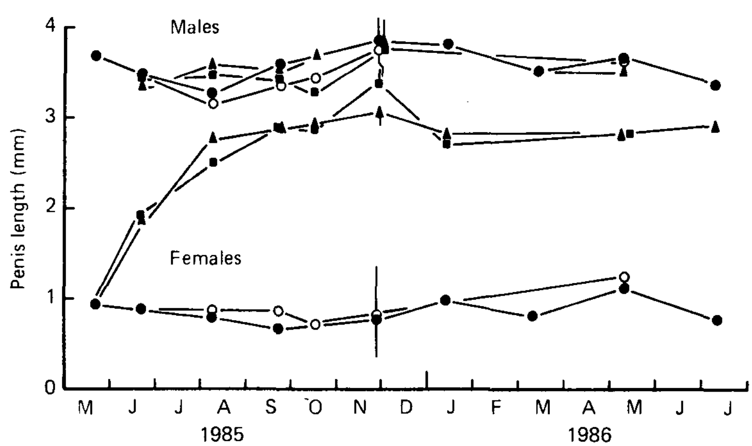

Fig. 1. *N. lapillus*. Effect on penis lengths of males and females (Widemouth population) of coating shell spires with various marine paints. Symbols: ▲, TBT-based free-association paint; ■, TBT-based copolymer; ○, yacht enamel; ●, untreated controls. Examples of standard deviation of penis lengths are shown by vertical lines.

antifouled animals. Fig. 1 shows that penis length in antifouled females increased to approach that of the males, whereas penis size in the enamelled females was not significantly different from that of unpainted controls. Those controls were collected more than 100 m from the antifouled animals, since unpainted animals collected within a few metres of antifouled animals were also affected. There is no obvious effect of antifouling paints on penis length in males (Fig. 1). Of the

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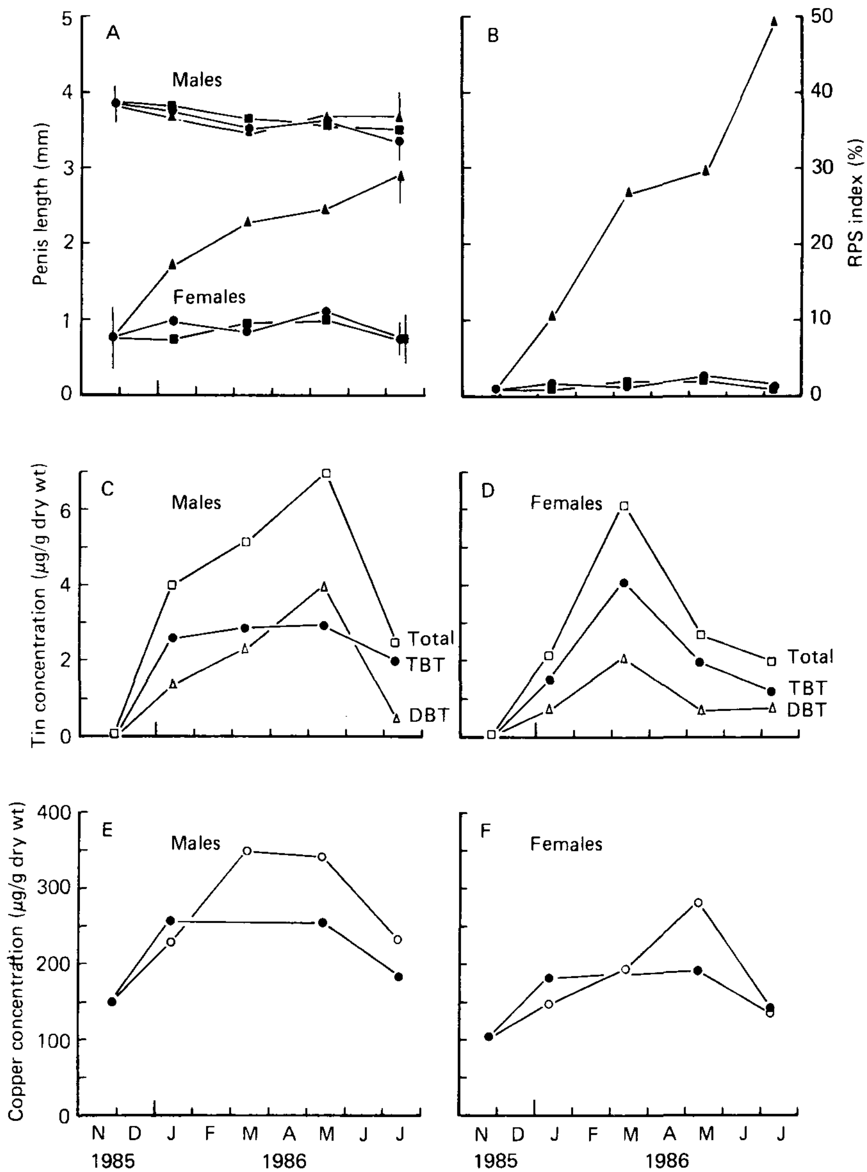

Fig. 2. *N. lapillus*. Effect on penis lengths and tin and copper tissue levels of males and females (Widemouth population) of coating shell spires with TBT-based and copper-based antifouling paints. (A) Penis lengths of males and females coated with: ▲, TBT paint; ■, copper paint; ●, untreated controls. Examples of standard deviations given by vertical lines. (B) RPS indices (female penis length^3/male penis length^3 × 100), symbols as for (A). (C, D) Concentrations of tin in tissues of TBT-treated males and females; ●, TBT fraction; △, DBT fraction, □, TBT+DBT fractions. (E, F) Concentrations of copper in males and females coated with ○, copper-based paint; ●, left untreated. Concentrations are based on samples pooled from 6 individuals.

animals treated with free-association, copolymer and enamel paints, 86, 88 and 72% respectively were recovered, and this suggests that mortalities caused by the antifouling paints were probably negligible.

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A second experiment compared the influence of a copolymer TBT antifouling paint (Blakes, 'Tiger' copolymer, red) with that of a copper-based paint (Blakes, 'Tiger' tin-free, red). The results show that the TBT-based paint induces penis development in females whereas the copper-based paint does not (Fig. 2A, B). Concentrations of total hexane-extractable tin in the unpainted and copper-treated animals remained at around 0·1–0·2 μg/g dry weight of which about half was in the TBT fraction. Much higher levels are found in the TBT-treated animals (Fig. 2C, D), the mean contribution of TBT tin to the totals being 61 ± 16 % in the males and 68 ± 5 % in the females. Since the availability of dietary DBT to the TBT-treated animals is presumably low, the presence in the tissues of 30–40 % of the total extractable tin as DBT tin suggests that TBT, leached from the paint and then absorbed from solution by the tissues, is degraded to DBT (Lee, 1986).

The results in Figs. 2E, F suggest that animals exposed to the copper-based paint absorb some of the metal. However, far higher tissue concentrations have been observed in populations from copper-contaminated sites in Cornwall (Bryan *et al.* 1986).

Uptake of tin as butyltin species

Preliminary experiment

Mature *N. lapillus* from Porth Joke in north Cornwall (degree of imposex or RPS index ~ 0·1 %) were placed, 38 to a tank, in 4 tanks containing 45 l of laboratory circulation water. Tributyltin oxide dissolved in ethanol was added to two of the tanks to give a nominal concentration of 1000 ng/l of tin and ethanol only was added to the control tanks. Food in the form of barnacle-covered rocks was placed in one experimental and one control tank and in all tanks the *N. lapillus* were retained by netting. The water was changed 5 times per week. Measurements of hexane-extractable tin made before and after each change gave a mean concentration of 760 ng/l in the experimental tanks compared with less than 2 ng/l in the control tanks. The mean water temperature was about 16·5 °C. At intervals, 4 animals were sampled from each tank and, after measuring and sexing, analysed individually for hexane-extractable tin (TBT + DBT fractions) against standards of tributyltin oxide.

Since no influence of sex or feeding (it is doubtful there was much feeding) on the uptake of hexane-extractable tin was observed, the results from all animals were combined. Fig. 3 shows that after 52 days a mean concentration of about 8·5 μg/g dry weight of tin was reached. The calculated equilibrium concentration of 10 μg/g of tin represents a concentration factor of 13160 on a dry tissue/water basis. There appeared to be little uptake by the shell, the concentration of hexane-extractable tin being 0·011 ± 0·008 μg/g ($n = 4$) after 14 days.

Only one control animal (1·3 %) died during the experiment but 12 experimental animals (15·8 %) died between 17 and 29 days and had a mean hexane-extractable tin concentration of 4·17 ± 1·07 μg/g dry weight. Between 29 days and the end of the experiment there were no mortalities, suggesting perhaps that the animals had become in some way acclimatized.

Measurements of penis lengths in females beyond 30 days of exposure gave mean values of 0·48±0·54 mm ($n = 10$) in the controls and 1·12±1·14 mm ($n = 11$) in the experimental animals, the difference being statistically insignificant. Included in these means are 5 control and 4 experimental females lacking

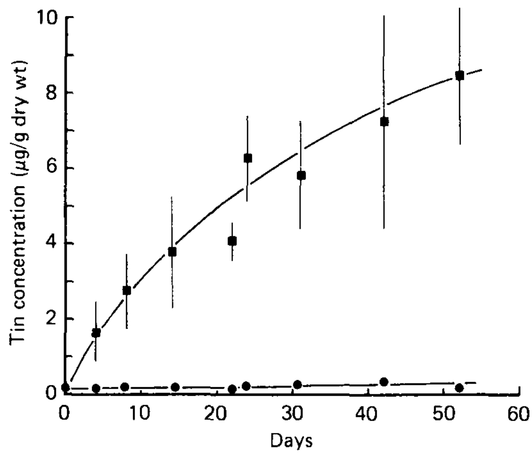

Fig. 3. *N. lapillus*. Absorption of hexane-extractable tin by animals exposed to water having: ●, no added tin; ■, a mean concentration of 760 ng/l tin added as TBTO; equation of curve is tin level = $10(1-e^{-0.0346\,\text{days}})$. Vertical lines show standard deviations. Mean temperature 16·5 °C.

a measurable penis. However, penis lengths in 4 of the experimental females (1·9, 2·1, 2·8, 2·9 mm) appreciably exceeded the highest value (1·5 mm) found either in the control group or in 37 other females sampled from the collection site during 1985. A possible explanation of the results is that penis growth was stimulated in females already possessing a small penis, but that insufficient time was available for visible development in animals lacking a penis.

The level of tin (760 ng/l) in the tributyl form employed in this experiment is typical of some marinas (Waldock & Thain, 1983; Cleary & Stebbing, 1985). Since this concentration appears to be lethal to some animals, and is likely to suppress growth, subsequent experiments were carried out at lower concentrations.

Tidal tank experiments

(*a*) *Accumulation of hexane-extractable tin*

In the three experimental tanks, the mean concentrations of tin leached into the water from rods treated with TBT copolymer paint were 3·4±0·8 ng/l ($n = 32$), 18·7±5·1 ng/l ($n = 78$) and 107±30 ng/l ($n = 58$). Tin could not be detected in the control tank water (< 1 ng/l) during the 1985–6 winter, but later measurements (Fig. 5B) show that concentrations in the TBT fraction rose from about 0·4 ng/l in February and March 1986 to around 2 ng/l in the summer, declining to 0·6 ng/l at the end of the experiments in December. Presumably due to losses within the laboratory seawater system, concentrations in the control tank were only about one-tenth of those in Plymouth Sound from which the water is pumped. Relatively unaffected adult animals from Crooklets Beach, Bude, were used in the experiments and were fed with barnacles from the same area. The dietary contribution to tin uptake is likely to be lower than under field conditions, since the barnacles were probably eaten before they had equilibrated with the water. In October 1985 the first control and 18·7 ng/l experiments commenced, followed in December by the 107 ng/l experiment, and in March 1986 by the second control and 3·4 ng/l experiments: the experiments ended in December 1986. Water temperatures varied from 9 °C in February to 19 °C in July and August.

Figs. 4 and 5 illustrate changes in the concentrations of tin in the TBT, DBT and total (TBT + DBT) tissue fractions in the experimental and control animals. In the first control (Fig. 4A, B) the total extractable tin level increases by roughly 2·5 times and is highest when concentrations of TBT tin (and probably DBT tin) in the water are greatest (Fig. 5B). The relative contribution of the TBT fraction to the total varies, the mean values being $64 \pm 8\%$ in males and $72 \pm 13\%$ in females. Animals from the 18·7 ng/l experiment accumulate tin to levels which are approximately 10 times higher than in the first control experiment (Fig. 4C, D). The contribution of TBT tin to the total is also greater, with mean values of $75 \pm 6\%$ in males and $80 \pm 10\%$ in females. Concentrations of tin accumulated in the 107 ng/l experiment are only about double those achieved at 18·7 ng/l (Fig. 4E, F). This may be indicative of saturation of the tissues, although the presence of a much lower proportion of extractable tin in the TBT fraction ($54 \pm 10\%$ in males and $52 \pm 13\%$ in females) may also reflect the induction of a process to counteract the accumulation of TBT by degrading it to DBT, and perhaps monobutyltin (Lee, 1986). It is conceivable that the degradation of the TBT tin occurs in the barnacles which are consumed, but would appear more likely that it is metabolised by *N. lapillus* (see also p. 529).

In both the 18·7 and 107 ng/l experiments there are significant differences between the patterns of tin absorption in males and females since, in the latter, there is a secondary period of accumulation toward the end of the experiment (Fig. 4D, F). These changes may be related to the sexual condition of the females (and males) which was high initially, fell to a minimum in the summer and was increasing in December at the end of the experiment. Tributyltin compounds are lipophilic and their secondary accumulation in females may relate to rising body lipid levels prior to egg laying (Stickle, 1975).

In the second control experiment, higher concentrations of extractable tin are achieved than in the first experiment (Fig. 5A, B). This may be related to the condition of the two sets of animals. For example, weight losses may lead to apparently higher tissue concentrations: the average wet weight of the second group of control animals declined by more than 40% during the experiment

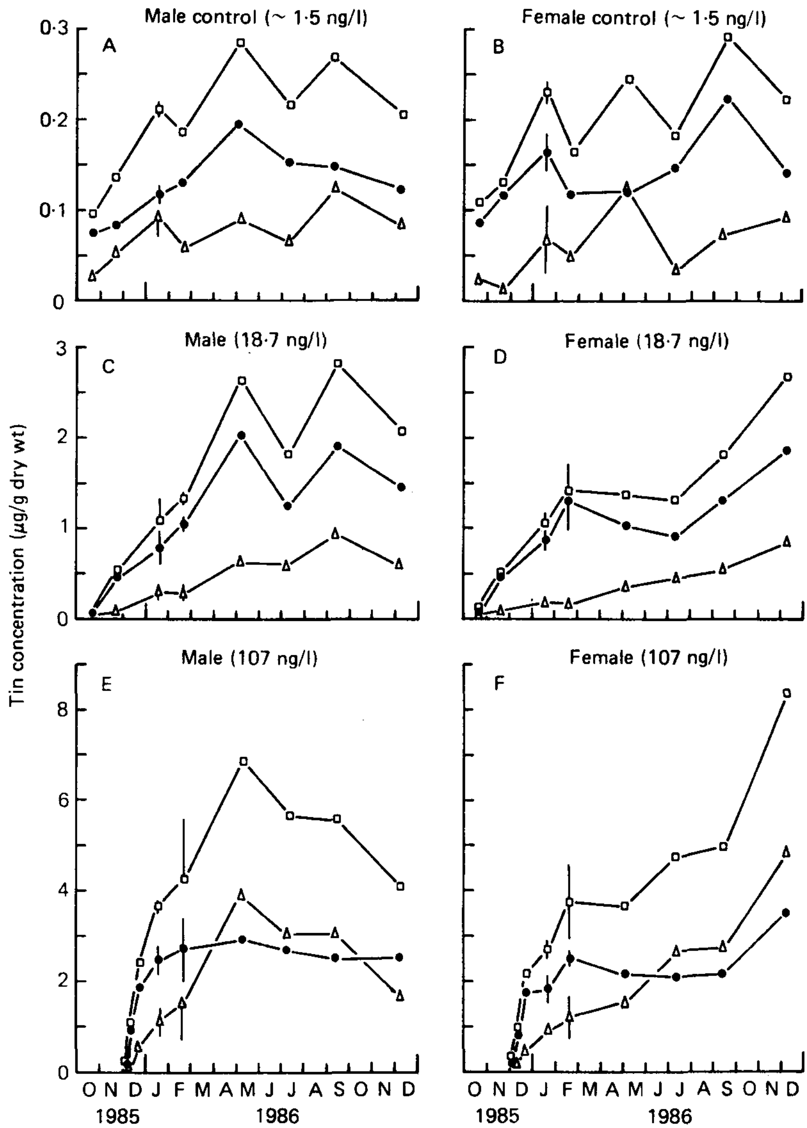

Fig. 4. *N. lapillus*. Absorption of tin from seawater leachates of TBT-based antifouling paint. (A, B) Tin levels in tissues of control animals (~ 1·5 ng/l in water); ●, TBT fraction; △, DBT fraction; □, TBT+DBT fractions. (C–F) Tin levels in animals exposed to mean concentrations of 18·7 and 107 ng/l; symbols as above. Concentrations based on samples pooled from 5–6 animals. Standard deviations of between-batch replicates are shown.

compared with maximum weight losses, observed during July in the first control, of 26% in males and 13% in females. Concentrations of TBT tin achieved by animals in the 3·4 ng/l experiment are approximately double those from the second control experiment (Fig. 5). The contribution of TBT tin to the total extractable concentration increases from control levels of 50±13% in males and 49±15% in females to 67±9% and 63±8% respectively in the 3·4 ng/l experiment. Possibly because both groups were exposed to the same control levels of dissolved DBT, tissue concentrations of DBT tin remain relatively similar.

The salient data from these experiments are summarised in Table 1. The initial rates of uptake of TBT tin are approximately proportional to concentrations in

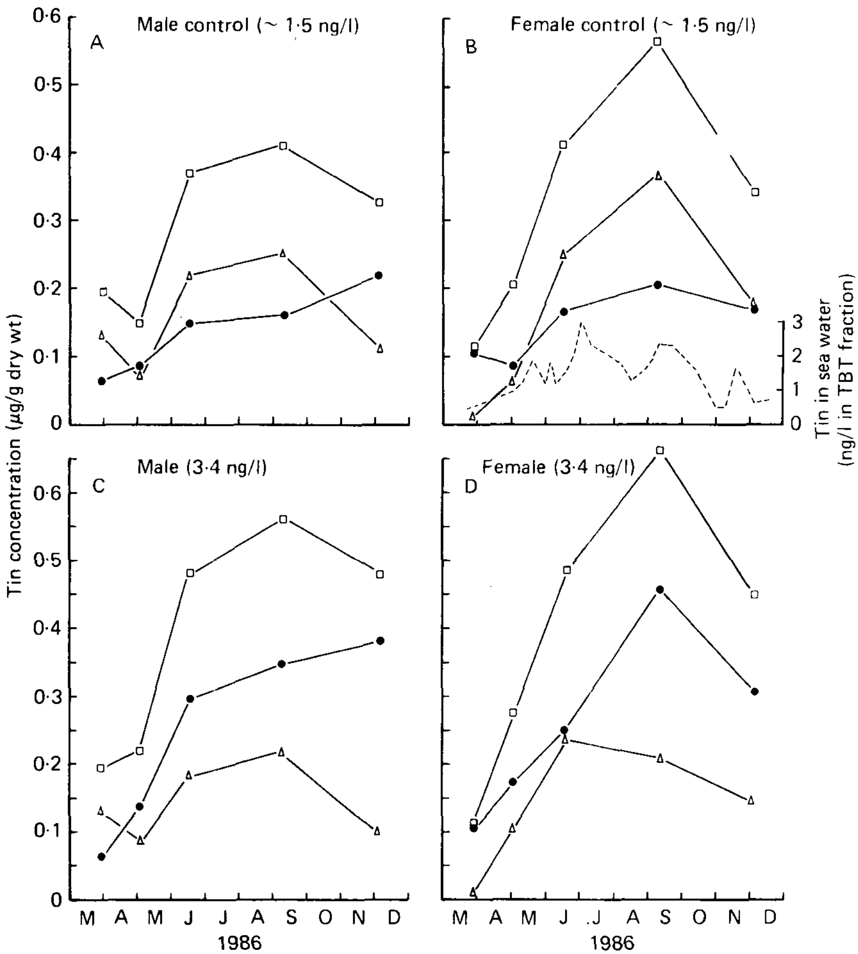

Fig. 5. *N. lapillus*. Absorption of tin from sea-water leachates of TBT-based antifouling paints. (A, B) Tin levels in tissues of control animals: ●, TBT fraction; △, DBT fraction; □, TBT+DBT fractions; broken line shows the levels in water. (C, D) Tin levels in animals exposed to a mean concentration of 3·4 ng/l; symbols as above. Concentrations based on samples pooled from 5–6 animals.

the water. However, the final concentrations achieved by animals exposed to 107 ng/l are much lower than might be predicted, the concentration factors being about 3 times lower than at 18·7 ng/l. The more efficient degradation of TBT tin to DBT tin in animals from the higher concentration may partially explain this phenomenon.

Table 1. N. lapillus. *Summary of data from tidal-tank and field-transplant experiments*

Experiment	Duration of experiment (days)	Tin in TBT fraction of sea water (ng/l)	Initial tin uptake rates by TBT fraction and (TBT+DBT fraction) (ng/g dry wt/day)[a]		Final tin level in TBT tissue fraction and (TBT+DBT fraction) (ng/g/dry wt)		Final TBT concentration factor (dry tissue/water)[b]		Relative penis size (RPS) index (%)	Vas deferens sequence (VDS) index (% females at each stage)					
			Male	Female	Male	Female	Male	Female		*n*	1	2	3	4	5
Tidal-tank experiments															
Crooklets Beach population	—	< 0·5	—	—	64–90 (97–196)	57–102 (108–158)	—	—	2–6·5	89	2·2	24·7	50·6	22·5	0
'Control' sea water 1	408	~ 1.5	0·53[c] (1·16)	1·05[c] (1·00)	122 (204)	140 (224)	83000[e]	95 200[e]	10·0	7	0	0	28·6	71·4	0
'Control' sea water 2	249	~ 1·5	1·10[d] (2·33)	0·75[d]. (3·83)	216 (325)	167 (340)	147000[e]	114000[e]	14·2	6	0	0	33·3	66·7	0
Sea-water leachate (low)	249	3·4	3·0 (3·73)	1·86 (4·71)	381 (478)	305 (446)	112000	89700	43·8	9	0	0	11·1	88·9	0
Sea-water leachate (medium)	408	18·7	13·0 (14·4)	11·8 (12·9)	1475 (2050)	1864 (2689)	77900	99700	56·4	8	0	0	0	100	0
Sea-water leachate (high)	366	107	114 (121)	100 (121)	2436 (4056)	3498 (8344)	22800	32700	63·3	8	0	0	0	87·5	12·5
Field transplant experiment															
St Agnes population	—	< 0·5	—	—	44 (70)	50 (67)	—	—	0·27	61	0	11·5	72·1	16·4	0
St Agnes→ Plymouth[f]	529–634	27·8	—	—	1250 (2405)	1390 (2417)	45000	50000	55·4	15	0	0	0	86·7	13·3

[a] Rates from slopes of linear regressions through first few points. [b] To convert to wet wt basis divide by 3·3 for males and 3·0 for females. [c] Initially exposed to about 0·8 ng/l. [d] Exposed to about 1·2 ng/l. [e] Exposed to about 1·5ng/l. [f] Based on mean values from last 2 samples.

(*b*) *Changes in imposex during tin accumulation*

During the first control experiment (TBT tin ~ 1·5 ng/l), penis length increased significantly ($P < 0{\cdot}05$) in females (Fig. 6A) and the RPS index of imposex (♀ penis length^3/♂ penis length$^3 \times 100$) rose from 2% to 10%. In the second control experiment the overall increase in female penis length was not significant (Fig. 6B), but at a concentration of 3·4 ng/l female penis length was significantly

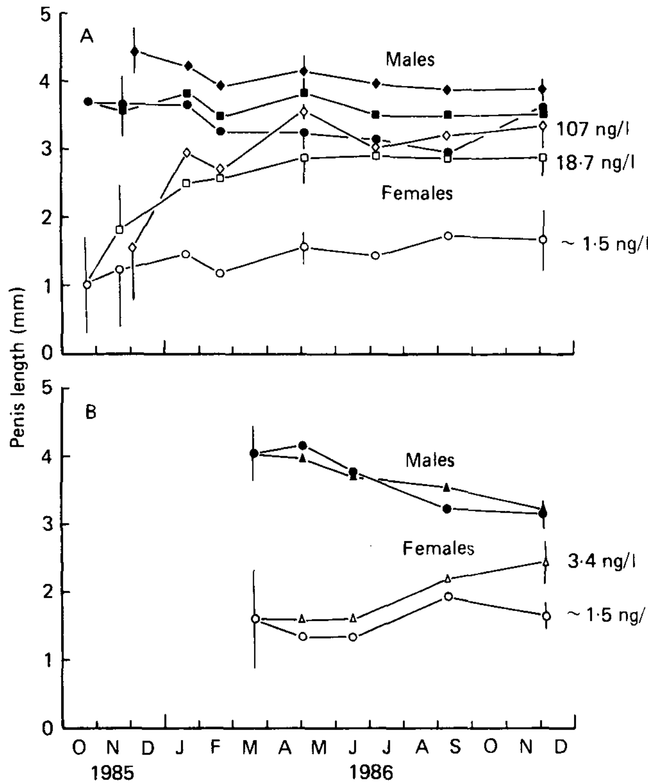

Fig. 6. *N. lapillus*. (A, B) Penis lengths of males (closed symbols) and females (open symbols) from experiments in Figs. 4 and 5. Examples of standard deviations are shown by vertical lines.

increased ($P < 0{\cdot}01$). In these two groups there was a significant reduction in male penis length when, as referred to in the previous section, sexual condition and body weight declined in both sexes. Partially because of the reduction in male penis length, the RPS index of imposex increased from 6·5 to 14·2% in the second control and 6·5 to 43·8% in the 3·4 ng/l experiment. Female penis size also increased very significantly in the 18·7 and 107 ng/l experiments and the RPS indices of imposex rose from 2 to 56·4 and 63·3% respectively (Table 1).

The development of a female penis appears to be good evidence of the exposure of *N. lapillus* to TBT tin leached from antifouling paint, but is less useful as an index of the competence of a population to reproduce. For this purpose the sequential development of the vas deferens and penis was used as an index (VDS index, Gibbs *et al.* 1987). Briefly, this index divides imposex in females into 6 stages: stage 0 females have no visible male characters; stage 1 exhibits the first signs of a vas deferens; stage 2 the first signs of penis development; stage 3 has a small penis but the vas deferens is still incomplete; in stage 4 the penis is larger and the vas deferens is complete; in stage 5 the genital papilla is blocked by overgrowth of vas deferens tissue and the female is sterile; at stage 6 aborted egg capsules are found in the capsule gland. The staging of females at the end of the experiments is compared with that of the original population in Table 1. Whereas originally stage 3 females were dominant, stage 4 became dominant in the control tank (~ 1·5 ng/l tin) and still more dominant in the 3·4 ng/l experiment. At tin concentrations of 18·7 and 107 ng/l all females achieved late stage 4, apart from one example of stage 5 (sterile female) reached at 107 ng/l.

Overall, the results show that while optimum penis development in the adult female can be achieved in 6–12 months (Fig. 6), sterilization of the female (stage 5) takes at least a year at 107 ng/l of tin as TBT.

(*c*) *Transplant experiment* (*uptake*)

Mature *N. lapillus* were transplanted from St Agnes, one of the least affected sites, to a rocky shore at Queen Anne's Battery, Plymouth, near the entrance to Sutton Harbour. Of 315 animals transplanted, 53% were ultimately recovered. The results from 1985 have already been reported (Bryan *et al.* 1986) but Fig. 7 includes data from 1986, during which a new marina was constructed within 200 m of the site and was partially occupied during the last few months of the experiment. As might be expected, total extractable tissue tin concentrations which had appeared to level out toward the end of 1985 increased during 1986. Although, concentrations of TBT tin in the tissues declined during the winter of 1985–6, when concentrations in the water were low, they rose again in 1986 in response to the seasonal increase in dissolved concentrations (Fig. 7A). In the tissues, the proportion of tin in the TBT fraction during 1985 was within the range 60–68%: in 1986, however, a lower range of 42–62% was observed. It is not known whether this apparent change reflects higher levels of DBT tin in the water or barnacle diet, or whether it results from an increasing capacity for degrading TBT. The tissue concentration factors for TBT tin reached in this experiment are compared with the results of the tank experiments in Table 1.

In female transplants, penis length reached a maximum after 10–11 months exposure and showed no further increase during 1986 (Fig. 7B). However, changes in vas deferens development (VDS index) continued throughout the experiment. The majority of transplanted animals were originally at stage 3 (small penis, incomplete vas deferens) whereas the 15 females collected in September and December 1986 included 13 at late stage 4 (large penis and complete vas deferens) and 2 at stage 5 (large penis and occlusion of the oviduct by vas deferens tissue). This suggests that a minimum of 18 months is necessary for adult females exposed to a mean dissolved TBT tin concentration of 28 ng/l to reach stage 5 at which the animal is sterile. This compares with a minimum of 12 months in the 107 ng/l tidal tank experiment (see above).

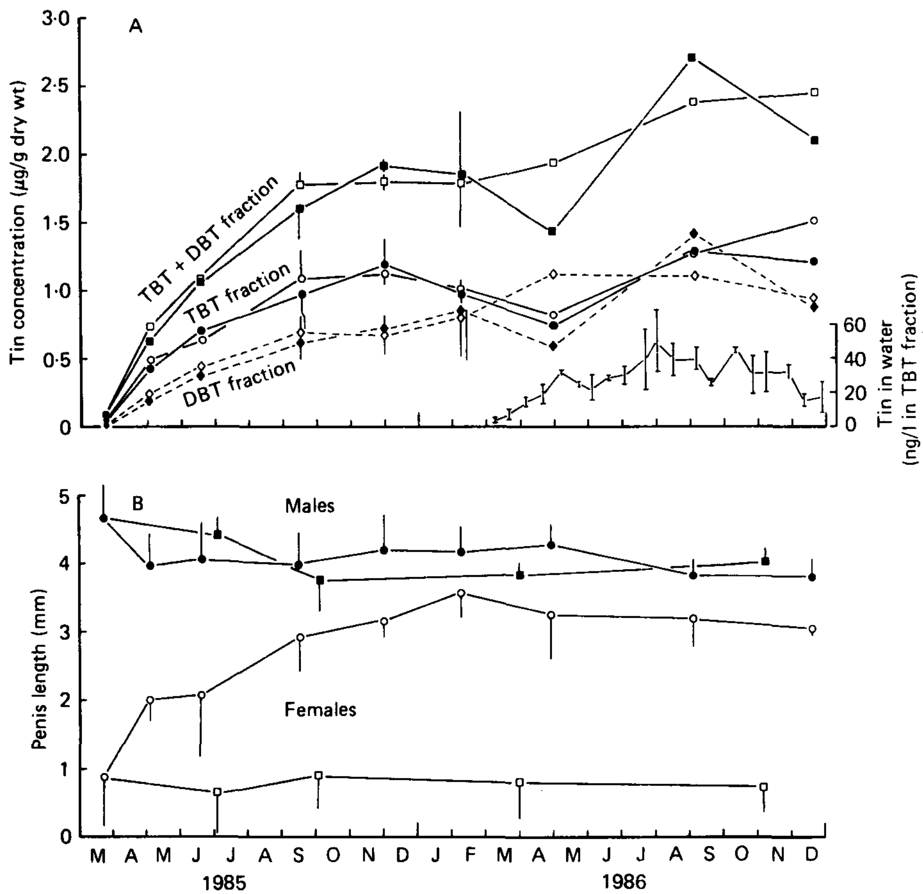

Fig. 7. *N. lapillus*. Effect of transplantation from a 'clean' locality (St Agnes) to a contaminated site (Plymouth marina). (A) Tissue tin concentrations in males (closed symbols) and females (open symbols); ● ○, TBT fractions; ◆ ◇, DBT fractions; ■ □, TBT + DBT fractions. Results are based on pooled samples. Standard deviations of between-batch replicates are shown. Water concentrations were measured at high and low tide and line is plotted through means. (B) Penis lengths of males (closed symbols) and females (open symbols), transplanted (circles) and St Agnes controls (squares).

The loss of organic tin under different conditions

(*a*) *Pulse experiments*

Three groups each of 200 animals from Crooklets Beach, Bude, were marked with yacht enamel and then exposed to 3 different sets of conditions in tidal tanks at 10–12 °C. The first group was exposed for 2 weeks to a dissolved concentration of about 107 ng/l of TBT tin leached from antifouling paint and a second group was exposed to control tank water (~ 0·4 ng/l TBT tin) for one week and to 107 ng/l TBT tin for a second week. The third group was kept in the control tank for 2 weeks. A sample of each group was retained for analysis and the remainder returned to Crooklets Beach. At intervals the treated animals were recovered, together with untreated animals from the same site. The resultant

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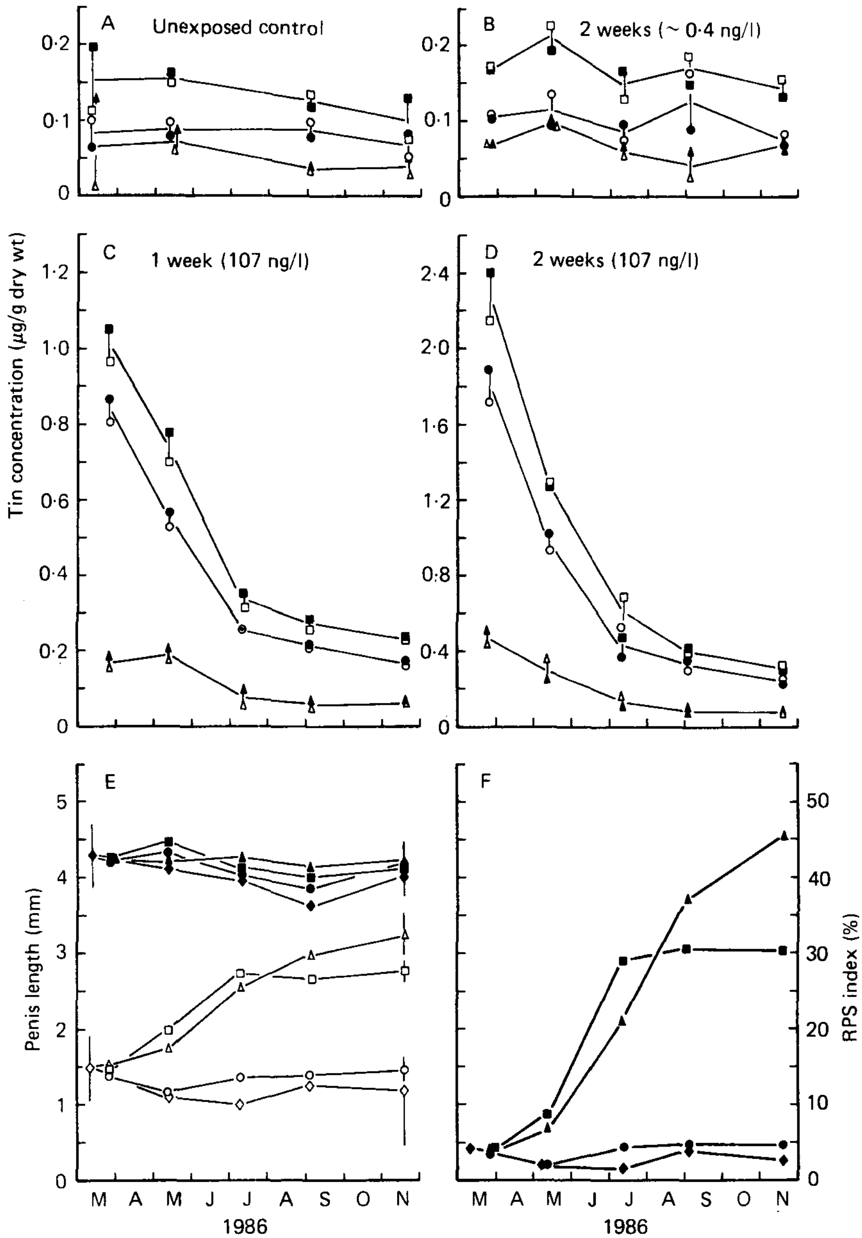

Fig. 8. *N. lapillus*. Effect of 'pulse' exposure to TBT contamination. Three groups of animals were exposed to TBT contaminated water for 2 weeks and then returned to the site of collection (see text). (A) Tin levels in tissues of unexposed control males (closed symbols) and females (open symbols); ● ○, TBT fractions; ▲ △, DBT fractions; ■ □, TBT+DBT fractions. (B) Tin levels in animals exposed to control water (~ 0·4 ng/l tin as TBT) for 2 weeks; symbols as above. Tin levels in animals exposed for (C) 1 week and (D) 2 weeks to 107 ng/l of tin as TBT; symbols as above. (E) Penis lengths in males (closed symbols) and females (open symbols) in ◆ ◇, unexposed controls; ● ○, control water for 2 weeks; ■ □, 107 ng/l tin for 1 week; ▲ △, 107 ng/l tin for 2 weeks; Examples of standard deviations shown by vertical lines. (F) RPS indices (♀ penis length^3/♂ penis length^3 × 100) in the four groups: ◆, unexposed control; ●, 2 weeks in control water; ■, 1 week at 107 ng/l; ▲, 2 weeks at 107 ng/l.

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changes in tissue tin concentrations, penis size and in the degree of imposex (RPS index) are illustrated in Fig. 8.

Concentrations of extractable tin in the exposed control animals are rather higher than those in unexposed native Crooklets animals (Fig. 8A, B). More than 50% of the tissue tin is accounted for by that in the TBT fraction (unexposed males 53±15%; females 73±13% ($n = 4$): exposed control males 55±5%;

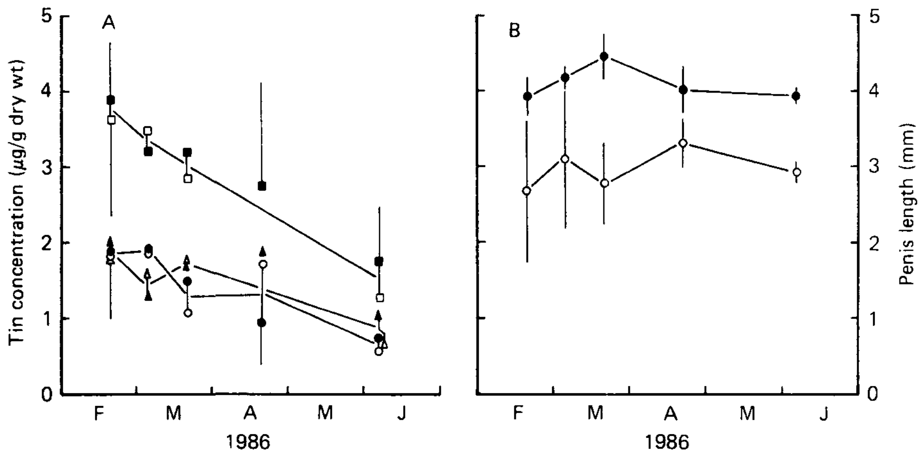

Fig. 9. *N. lapillus*. Effect of loss of tin to laboratory sea water from animals previously exposed for 121 days to 107 ng/l of tin as TBT. (A) Tissue tin levels in males (closed symbols) and females (open symbols); ● ○, TBT fractions; ▲ △, DBT fractions; ■ □, TBT+DBT fractions. Concentrations are means of individual analyses and examples of standard deviations are shown. (B) Penis lengths in ●, males; ○, females with standard deviations.

females 64±14% ($n = 5$)). In addition, mean penis lengths and the RPS index of imposex in the unexposed native animals are consistently but not significantly lower than those in the exposed controls (Fig. 8E). Animals exposed to 107 ng/l of TBT tin for 2 weeks contain about twice as much tin as those exposed for 1 week (Fig. 8C, D). The loss of excess TBT tin (experimental level minus control) from the more contaminated group has a half-time in both sexes of about 48 days over the first 4 months, falling to about 120 days thereafter. By comparison the half-time for the uptake of TBT tin is 10–11 days (Fig. 4E, F). Over the whole (2-week) pulse experiment the proportion of TBT tin in the tissues did not change appreciably, being 79±4% in males and 77±3% in females ($n = 5$). Results for the less contaminated (1 week) group are very similar, the half-time for loss being initially about 58 days and the proportions of TBT tin in the tissues 75±4% in males and 79±6% in females ($n = 5$). Both 1- and 2-week exposures to 107 ng/l of TBT tin result in appreciable penis development in females and consequently a marked rise in the RPS index of imposex (Fig. 8F). In terms of the stages of imposex (VDS index) the controls were largely stage 3 whereas the experimental animals had all reached stage 4. No detectable effect was observed on male penis size.

(b) Laboratory loss experiment

After an exposure of 121 days in the 107 ng/l experiment (see p. 531) more than 40 animals were transferred to flowing laboratory sea water and supplied with small mussels for food. During the experiment the TBT tin level in the laboratory water rose from about 0·5 to 1·5 ng/l and the water temperature

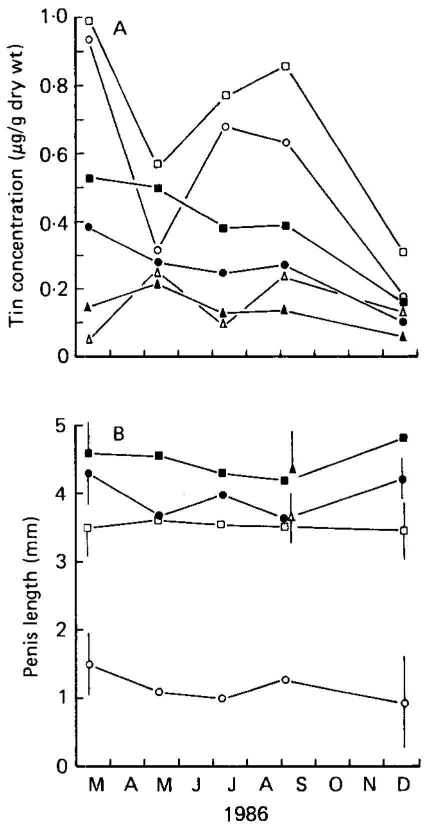

Fig. 10. *N. lapillus*. Effect of loss of tin from contaminated animals (Torcross population) transplanted to a clean locality (Crooklets Beach). (A) Tissue tin levels in pooled samples of males (closed symbols) and females (open symbols); ● ○, TBT fractions; ▲ △, DBT fractions; ■ □, TBT+DBT fractions. (B) Penis lengths of Torcross transplants (■, males; □, females); Torcross controls (▲, males; △, females) and Crooklets controls (●, males; ○, females). Examples of standard deviations are shown by vertical lines.

increased from 9 to 15 °C. The loss of tin from the tissues is illustrated in Fig. 9. Although the results are rather variable, the half-time for the loss of excess TBT tin is 50–60 days, and therefore of the same order as the values of 48 and 58 days observed in the pulse experiments described above. The proportion of TBT tin in the tissues was on average about 48 % of the total in males and females, a much lower proportion than in the pulse experiments. There is no evidence that the female penis is resorbed in response to falling levels of organotin, although the presence of 0·5–1·5 ng/l of TBT tin in the laboratory water might have been enough to prevent tissue levels from falling sufficiently to induce penis resorption (Fig. 9B).

(*c*) *Transplant experiment* (*loss*)

About 400 animals from Torcross (S. Devon) having a fairly high degree of imposex (RPS index of imposex 44%), were marked with enamel paint and transplanted to Crooklets Beach, Bude. Samples of 20–30 individuals were recovered at intervals over the next 9 months, but only 4 were found on the final visit. The experiment (Fig. 10) was carried out in parallel with the pulse experiments (Fig. 8) and the two sets of results are directly comparable. Unlike the pulse experiments the results for the Torcross animals are erratic, particularly for the females. However, the concentrations of TBT tin reached in both sexes after 9 months of loss are approaching those of the native animals (Fig. 10A) and this would be accounted for by an overall half-time for loss of about 100 days. Over the experimental period the contribution of TBT tin to the total extracted from the tissues of the Torcross animals was $65 \pm 7\%$ in males and $73 \pm 18\%$ in females ($n = 5$), values somewhat lower than in the pulsed animals.

Despite the loss of tin from the tissues there is no evidence for penis resorption in females (Fig. 10B).

DISCUSSION

Field experiments, in which the shell spires of adult *N. lapillus* from a 'clean' area were coated with marine paints and the animals then replaced on the same shore, show conclusively that the TBT-based paints induce a high degree of imposex whereas tin-free paints do not. In addition, females exposed to water containing tributyltin oxide at a mean concentration (as tin) of 760 ng/l showed evidence of increased penis lengths, despite the fact that there were also some mortalities.

Tidal tank experiments, more closely simulating natural conditions, show that the initial rates at which *N. lapillus* absorbs tin as TBT from sea-water leachates of TBT-based antifouling paint are approximately proportional to the ambient concentrations of tin over the range <1–107 ng/l. Over the lower range of 1·5–18·7 ng/l, the levels ultimately achieved by the tissues largely retain this proportionality, the concentration factor being about 10^5 (dry tissue/water). The tissue concentrations of TBT tin achieved experimentally also come within the compass of field data collected from sites in south-west England in 1985–6 (Fig. 11A, B). A closer fit is observed if the water concentrations are related to the total (TBT+DBT) tissue tin levels (Fig. 11C, D). This may be fortuitous but could imply that, although TBT and DBT levels in the water under field conditions are often of the same magnitude (Valkirs *et al.* 1986), TBT is the form preferentially absorbed and its degradation within the animal is a major source of tissue DBT. Under the conditions employed in the uptake experiments, levels of DBT in the water were presumably low, but at the lower concentrations of

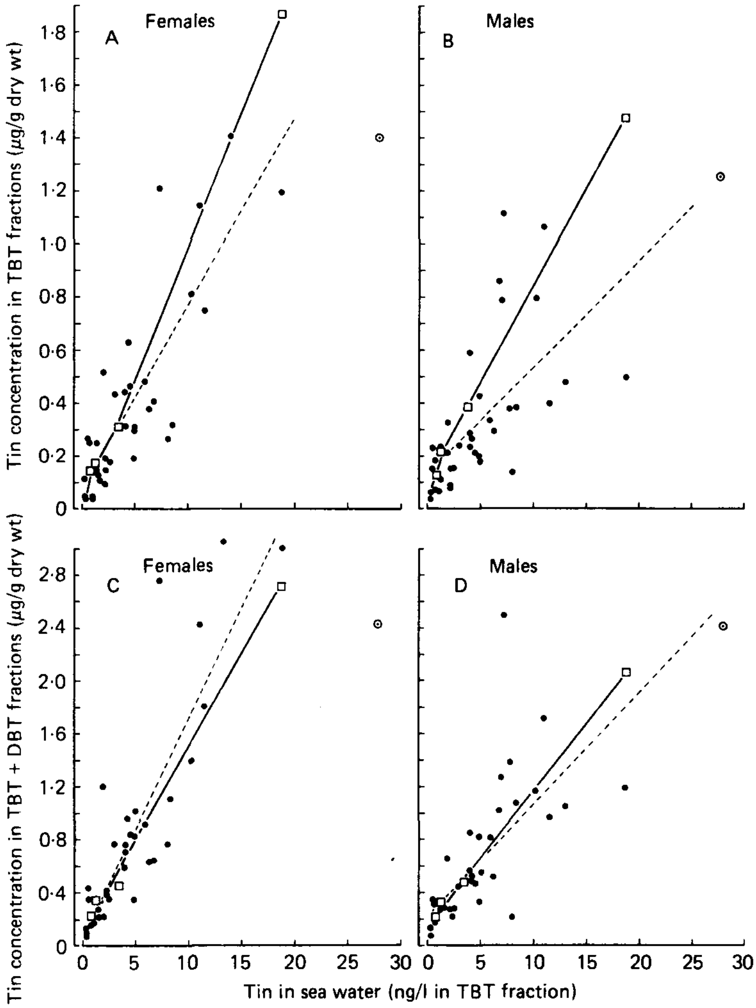

Fig. 11. *N. lapillus*. Relationship between tin concentrations in tissues and sea water. (A, B) TBT fraction in females and males vs TBT fraction in sea water. (C, D) TBT + DBT fractions in females and males vs TBT fraction in sea water. □—□, tidal-tank experiments (Table 1); ⊙, field transplant experiment (Table 1); ●, field data from S.W. England. Regressions of field data (broken lines) are:

(A) tin in female = 0·0696 tin in water + 0·076 ($r = 0·826$);
(B) tin in male = 0·0395 tin in water + 0·143 ($r = 0·594$);
(C) tin in female = 0·1660 tin in water + 0·081 ($r = 0·865$);
(D) tin in male = 0·0839 tin in water + 0·232 ($r = 0·680$).

TBT tin (1·5–18·7 ng/l) doubt about the origin of tissue DBT remains since this could have originated from the degradation of TBT in the barnacle diet. However, in *N. lapillus* exposed to a mean of 107 ng/l of TBT tin the contribution of the DBT fraction to the tissue tin level increased markedly and sometimes exceeded that of TBT (Fig. 4E, F). This suggests that TBT was being degraded by the animal, possibly via the mixed function oxidase system (Lee, 1986). Furthermore, the concentration factor for tin in the tissue TBT fraction was only about 3×10^4 at 107 ng/l compared with around 10^5 over the 1·5–18·7 ng/l range

(Table 1). Laughlin (1986) suggests that the bioaccumulation of TBT is the resultant of two mechanisms. One involves the partitioning of the compound between the surrounding water and lipids within the organism. For example, it is thought that the increased accumulation of tin observed in females toward the end of the uptake experiments in Fig. 4D and F is related to a rise in body lipid levels associated with increasing sexual development. However, Laughlin suggests that while partitioning appears to predict the relative toxicities of organotin compounds, this process alone seems insufficient to account for the very high concentration factors sometimes observed: it is suggested that a second mechanism involves binding to organic ligands.

Absorption of tin as TBT by *N. lapillus* was observed even in the 'control' tidal tank experiments, since there was a measurable amount in the water (~1·5 ng/l). In addition, there was a concomitant rise in the intensity of imposex in the 'controls' and more obvious effects at higher concentrations. Gibbs & Bryan (1986) have shown that declining populations of *N. lapillus* are characterised by the presence of females that have been rendered sterile through occlusion of the pallial oviduct by vas deferens tissue. In the tidal tank experiments, this condition was reached by one female following exposure to a seawater concentration of 107 ng/l of tin for 1 year. Two similarly affected females were found among animals transplanted for more than 18 months to a harbour locality having a mean seawater tin concentration of 28 ng/l in the TBT fraction. Although the development of imposex to this stage takes many months, it should be borne in mind that the experiments were conducted with adult *N. lapillus*. Experiments with juveniles reared from egg capsules placed in the same tidal tanks are, as yet, incomplete. However, after exposure for about 1 year to a mean seawater tin concentration of 18·7 ng/l as TBT, most females achieving a shell length of 18–22 mm were already sterile. Even in the control tank (~ 1·5 ng/l tin) 40% of similar-sized females were sterile (Gibbs *et al.* 1987). Thus the seawater leachate from TBT-based paint affects developing females far more rapidly and at lower tin concentrations than it does the adults.

Depending on the experimental conditions, the depuration of tin from the TBT tissue fraction occurred with half-times of between about 50 days and more than 100 days. Over experimental periods of up to 9 months there was no evidence that loss of tin leads to any remission of imposex. However, even after 9 months of loss, significant levels of tin remained in the tissues and it is conceivable that a longer exposure to especially clean water might be effective.

In conclusion, the combination of shell-painting experiments, tidal-tank experiments and field transplant experiments shows conclusively that imposex in *N. lapillus* is induced by leachates from TBT-based antifouling paints. Furthermore the sensitivity of *N. lapillus*, especially of growing juveniles, is such that a few ng/l of tin as TBT species appear capable of inducing significant levels of imposex: ultimately this leads to sterility in females and declining populations.

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