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EFFECTS OF TBT ON THE REPRODUCTIVE SUCCESS OF THE DOGWHELK, *Nucella lapillus*

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SUMMARY

Adult dogwhelks (*Nucella lapillus*) were exposed to 2-128 ng/l Tributyltin (TBT) in sea water for up to 12 months, and the number of egg capsules produced was studied. The hatching, short and long term survival of the juveniles from these capsules, held in both clean sea water and sea water containing TBT at 2-128 ng/l, was followed for a further 12 months.

The production of egg capsules was reduced even though the adults had VDSI values of below 4 (ie were not sterile), with a NOEC of 8 ng/l and LOEC of 32 ng/l. The onset of laying of egg capsules was delayed, and the duration of laying was shortened, after exposure of the parent dogwhelks to 32-128 ng/l TBT. The presence of TBT in the hatching water did not affect the number of embryos in the capsules, or the number of juvenile emerging from the capsules, although the hatching period was shortened for egg capsules held in TBT dosed water. The short-term immediate post-hatching mortality of the juvenile dogwhelks (2-4 days) showed no relationship to TBT concentrations, and the 96 hour LC50 for these juveniles was 73,000 ng/l TBT.

Exposure to TBT did not affect the growth of the juvenile dogwhelks as indicated by length/weight relationships.

In juveniles reared in TBT dosed water, the incidence of occurrence of imposex, RPSI and VDSI values show a positive correlation with TBT concentration in the water. Virtually all females at TBT concentrations above 2 ng/l had blocked reproductive tracts although they were unlikely to be sexually mature. Juveniles transferred as eggs from the dosed water system, hatched and reared in clean sea water, show high VDSI values, but relatively low RPSI values, and contained no detectable concentration of TBT. Factors, other than TBT, affected females, eggs or juveniles and led to development of imposex in juveniles in controls/carrier controls.

The concentration of TBT in the tissues of dogwhelks dosed with TBT bore a good correlation with the concentration in the water. The juveniles accumulated less TBT over one year than adults exposed to the same concentrations for the same length of time.

INTRODUCTION

There is a considerable body of literature describing the physiological effects of tributyltin compounds (TBT) on molluscs (Blaber, 1970; Bryan *et al.*, 1986; Gibbs, 1988; Ten Hallers-Tjabbes, 1994; Bower, 1995). Typically, male sexual characteristics are developed by females, a process termed imposex (Smith, 1981). In cases of severe impact, the female reproductive tract can become blocked (Gibbs *et al.*, 1987), (vas deferens stage 5 or 6) and reproduction is prevented by the inability of females to lay egg capsules. Other reported effects of exposure to high concentrations of TBT include rupturing of the capsule gland, spermatogenesis in altered ovarian tissue, and, in males, deformation of the penis (Gibbs *et al.*, 1988)

All these severe conditions very clearly lead to inhibition of reproduction, and therefore potentially to effects on the population structure. It has been reported that unless the female reproductive tract is blocked, ie vas deferens stages 5 or 6 (Gibbs *et al.*, 1987), the breeding capacity of females is not reduced (Gibbs *et al.*, 1988). A study carried out by Bailey *et al.* (1991), exposed adult dogwhelks to TBT concentrations of 2, 8, 32 and 128 ng/l in tanks for 12 months. The higher exposure levels resulted in the development of imposex only to vas deferens stage 4 by the middle of the breeding season and egg capsules were laid in each tank. The current study investigated the effects of TBT on reproduction in, and development of juveniles from, these adult female dogwhelks (*Nucella lapillus*) exposed to TBT, but showing degrees of imposex that would not prevent the laying of egg capsules (ie vas deferens less than stages 5 or 6).

These studies were carried out for the ORTEP organisation at the Scottish Office Agriculture, Environment and Fisheries Department Marine Research Unit, Loch Ewe (Scotland).

MATERIALS

The test compound, Hexa-n-butyldistannoxan batch no Beh 584 was provided by Schering AG in July 1989, and was approximately 97% pure.

The test organisms were the eggs of the dogwhelk, *Nucella lapillus*, which had been laid under the experimental conditions of the previous study (Bailey *et al.*, 1991), and the corresponding hatched juveniles. The juveniles were fed on rope grown mussel spat, *Mytilus edulis*, <10 mm in length, and containing <0.02 mg/kg TBT. New feed mussels were added to tanks every 2-3 weeks as required, to ensure adequate food was always available. Ropes of clean feed mussels were maintained in clean sea water (<1.5 ng/l TBT) until required. Vas deferens stage index (VDSI) values (Table 1) for the parent dogwhelks in the treated tanks were 2.4-4.0 in May 1990, and 3.2-4.3 in August 1990 (Bailey *et al.*, 1991). The imposex data for the parent dogwhelks in May, in the middle of the breeding period, therefore suggest that capsule production should not be significantly inhibited in any of the tanks.

The experiment commenced when eggs started to be laid in the tanks in April and approximately 120 days later the first egg capsules began hatching. The experiment terminated when all the remaining year-old dogwhelks were assessed for imposex in the beginning of September the following year.

METHODS

a) General Experimental Design

The egg capsules had been laid in six large fibreglass aquaria, mostly on rocks or plastic trays used for holding the feed mussels. Prior to the commencement of hatching, the egg capsules were divided between two tank systems one in clean sea water and the other exposed to TBT. Some of the egg capsules in each aquarium (29, 27, 37, 28, 9 and 44% from the two control tanks and tanks dosed with 2, 8, 32 and 132 ng/l TBT respectively) were transferred to small glass aquaria containing clean sea water. The clean sea water aquaria were semi-static systems with daily water exchange and were continuously aerated. The remainder were also transferred to small glass aquaria, in a flow through system, and continued to be exposed to TBT under the same dosage regime as the respective adults. When all egg capsules in the semi-static clean sea water aquaria and dosed flow-through system had hatched and of a size that could be easily seen and maintained, the aquaria were transferred to a larger through flowing system of clean (Tanks 7-12, <1.5 ng/l TBT) or dosed sea water (Tanks 1-6 as appropriate).

b) Tank System Maintenance

Primary TBTO stock solution in ethanol (1,004.3 ppm) was prepared on 5 March 1990 and used for making up the secondary stock solutions. Secondary stock solutions were stabilised by the addition of 2 ml of concentrated hydrochloric acid per litre and were pumped (0.1 ml/min) into the inflowing water (1,500 ml/min) to each fibreglass tank. The secondary stocks were generally replaced on a weekly basis to prevent degradation.

pH, temperature, salinity and dissolved oxygen (DO) were monitored weekly, in each treatment. The dilution water and dosing water flow rates were checked daily. Simulated tidal conditions were maintained initially by halting inflowing water in the non-static tanks, and siphoning off the water from all tanks for several hours each day. After transferal to continuous flow systems, the sea water tanks were siphoned each day with the inflowing water halted. The dosed tanks were drained manually, and after two hours were refilled at a rate of 1,500 ml/min.

Two litre water samples from the control and treatment tanks were taken weekly in polycarbonate bottles, preserved by the addition of 5 ml/l concentrated hydrochloric acid and stored in the dark. Portions of the weekly samples from each tank were composited for analysis on a monthly basis, by solvent extraction and graphite furnace AAS using a modification (Bailey *et al.* 1991) of the method of McKie (1987). The limit of detection was 1.5 ng/l (two standard deviations of repeated analysis of standards at 5 ng/l).

To maintain the correct concentration of TBT in each tank, it was necessary to periodically alter the TBT concentrations in the secondary stocks at the weekly renewal time.

c) Specific Experimental Design

The following studies were carried out using the above experimental animals to investigate the reproductive success of the parents, and the growth and survival of the juveniles.

A Production of Egg Capsules

The number of capsules was estimated, twice per week, by placing a grid over the capsules and counting a sub-sample. The total number of egg capsules present in each tank was counted at the end of the hatching season. The number of adult females present in each tank was determined from records from the previous study (Bailey *et al.*, 1991). This provided an estimate of the mean number of egg capsules produced per female and the rate of production of the capsules through the breeding season.

B Production of Juveniles

i) Viability of egg capsules

To determine the number of capsules and hence the rate of emptying during the hatching period, the number of empty egg capsules found in treatment, transfer and control tanks were counted 34 times during the hatching period (17 weeks). In addition any remaining unhatched capsules were also counted at the end of the hatching period.

ii) Hatching of juveniles

The number of embryos nearing maturity in capsules in all tanks was estimated on two occasions by the dissection of capsules, assessed as being on the point of releasing young, from each tank, (except tank 6). The hatching success was estimated by daily counting of the numbers of young released from small numbers of capsules detached from the rocks on which they were laid and transferred to small hatching tanks where the numbers could be accurately counted. These small hatching tanks were semi-static, with daily exchange of clean sea water (TBT <1.5 ng/l).

C Short-term Survival Rate of Juveniles

i) Post-hatching mortality

To determine the immediate (2-4 day) post-hatching mortality in each treatment, groups of 10 newly hatched juveniles were transferred to glass beakers and maintained under their respective treatment regimes for five days, with daily water exchange. The animals were assessed daily and classified as alive, dead or moribund. The tests were undertaken three times during the hatching period to determine the variation in post-hatching mortality. A limited number of other tests were carried out in which animals were only classified as alive or moribund. It was not possible to transfer newly hatched dogwhelks from tank 6, sea water control on one occasion or from tank 5, sea water control on any occasion except the incomplete tests on 7 August, because insufficient embryos emerged in the 24 hours previous to the start of the experiments.

ii) 96 hour acute toxicity test

Ninety-six hour acute toxicity test of TBT to juvenile dogwhelks was carried out in August 1991. The juveniles were bred from adult dogwhelks collected from Firemore Bay (Loch Ewe) in the spring of 1991. They were maintained in a large fibreglass aquarium, with two simulated tides a day. Capsules laid in the tank were carefully watched as hatching time approached, and newly-emerged juveniles were used for 96 hour LC50 toxicity tests when sufficient hatched in a 24 hour period. The initial concentrations of TBT used were; control, 20, 100, 1,000 and 5,000 ng/l TBT with daily water exchange. However these did not provide sufficient mortalities so the concentrations were increased in a second test to control, 25,000, 40,000, 50,000, 65,000, 75,000, 90,000 and 100,000 ng/l TBT. The test was performed at 10°C in a constant temperature room.

D Long-term (12 months) Development of Juveniles

i) Growth rate

The length and weight of 20 dogwhelks selected at random from each tank were measured five months after hatching in January 1991. In the following June and August (10 and 12 months post hatching), 50 individuals were weighed and measured from all tanks, except tank 5, sea water control, where only 48 were available. The lengths were measured using vernier calipers or a binocular microscope with a calibrated eyepiece scale. Also, the lengths of all the remaining dogwhelks in January were measured from photographs using a digitising tablet attached to an Archimedes computer. The dogwhelks were all returned to their respective tanks after measuring.

ii) Mortality

Dead dogwhelks were removed periodically from each tank. Very small dogwhelks were very fragile and easily broken, so shells were removed infrequently at this stage. The empty shells were dried in an oven at 50°C for 24 hours. Two batches of 50 shells were then counted and weighed and the remainder of the shells weighed to give an estimate of the number of dead shells removed from each tank.

iii) Imposex and tissue TBT concentrations after one year

The examination for imposex followed the methods and classification described by Gibbs *et al.* (1987), and included relative penis size (RPS) and vas deferens sequence (VDS) indices.

Tissue samples were analysed for TBT as described by McKie (1987). The limit of detection was 0.02 mg/kg (two standard deviations of repeated analysis of samples containing 200 ng of tin).

RESULTS

a) Experimental System

The values of the routine environmental parameters were pH 7.55-8.23; DO >51%; temperature <14.4°C; salinity 32.9-34.9‰. There was little variation between tanks and the values are not considered to have adversely affected the dogwhelks. The concentrations

of the TBT in water over the year are summarised in Table 2, and the means closely reflect the target values.

b) **Experimental Results**

A **Production of Egg Capsules**

The first egg capsules were laid in April, prior to the third sampling of adults described in Bailey *et al.* (1991), and continued until the end of July, although most were deposited between late April and mid-June.

Fewer egg capsules were deposited per female (Table 3) in the tanks dosed with 32 ng/l TBT and 128 ng/l TBT (6.4-3.4) than in the control tanks (12.9-15.9). The values for the two low concentrations (2 and 8 ng/l TBT) were similar to or higher than the sea water and carrier controls. The value for tank 3 (2 ng/l TBT) of 19.6 eggs/female was the highest observed value.

The rates of production of the capsules through the breeding season from estimated numbers of egg capsules are shown in Figure 1. Although the first capsule was laid in tank 5 on 2 April 1990, no more capsules were noted until 25 April, when they were present in all tanks except tank 6. April 25 was taken as day 0 (Fig. 1); capsules were present in all tanks on 30 April. The final point on the graph shows the number of egg capsules per female as counted at the end of the hatching period. These indicate that there was generally an under estimation of the number of egg capsules present. The short group of higher estimates seen on all figures (between days 70 and 80) was an artifact and have been disregarded.

There was little production after about day 50 in any tank. The rate of production (the number of egg capsules per female per day) was lowest in tank 6 (128 ng/l TBT) and the highest rate in tank 3 (2 ng/l).

The times taken after the first eggs were laid in any tank for the number of eggs laid to reach the 10th and 50th percentiles of the total number laid and the duration of laying (time between 10th and 90th percentiles) (Table 4) shows that laying started approximately 10 days later in tanks dosed with 32 and 128 ng/l TBT (Tanks 5 and 6 respectively) than in the other tanks. A delay before 50% of the final number was reached in these tanks may also be indicated. The duration of laying was 27 days in those dosed with 32 and 128 ng/l TBT; considerably shorter than the 47 and 52 days found in the two control tanks.

B **Production of Juveniles**

i) <u>Viability of egg capsules</u>

There was no clear relationship between TBT concentration and the total number of capsules that hatched in each tank, or the percentage that hatched (Table 5). Of those egg capsules dosed with TBT the greatest percentage hatched in the highest TBT concentration. Only capsules from tank 3 (2 ng/l TBT) and tank 6 (128 ng/l TBT) hatched better in clean sea water than those left in the respective treated waters. There were particularly low hatching successes in tank 2 (alcohol carrier) hatched in sea water and tank 3 (2 ng/l) capsules hatched in 2 ng/l TBT treated water.

The majority of hatching in all tanks occurred between 20 and 60 days after the first capsules began hatching (Figs 2 and 3). In all tanks, 50% of the egg capsules had hatched earlier in those held in sea water compared to their respective treated tanks (Table 6). The greatest difference was in tank 5 (32 ng/l TBT) in which 50% of the capsules had hatched by day 25 in clean sea water, but in the water dosed with TBT 50% had not hatched until 56 days after start of hatching. The least delay was in tank 6, in which 50% hatched in clean sea water after 39 days, and after 44 days in water dosed with 128 ng/l TBT.

ii) Hatching of juveniles

The numbers of embryos nearing maturity in capsules removed from each treatment when assessed as being on the point of releasing young are shown in Table 7 . An ANOVA on the data from 14 August 1990 indicated a statistical difference between the tanks at the 5% level ($P<0.05$). This difference was caused by the large number of embryos in capsules in tank 4 (8 ng/l TBT). There was no statistical difference between the tanks on the second occasion. Combining the data for the two occasions gave a significant difference at the 5% level, again caused by tank 4. Omitting tank 4, there may be a suggestion of a decrease in the number of animals present in the egg capsules as the TBT level increases, although it is not statistically significant.

Not all of the capsules which were detached from the rocks on which they were laid and set up in small static sea water hatching tanks, released embryos. The average number of juveniles hatching from the capsules in each static tank (Table 8) is lower than the figures obtained from dissecting the unopened capsules (Table 7). Similar numbers of juveniles emerged from the control tank capsules, and from the two highest dose tank capsules, indicating little influence of TBT exposure on the numbers of embryos maturing and emerging from capsules.

C Short-term Survival Rate of Juveniles

An ANOVA performed on data describing the 2-4 day post hatching mortality in each treatment (Table 9) showed no significant differences between the six tanks. The hatching conditions of clean sea water and exposure to TBT concentrations up to 128 ng/l did not affect the short-term survival of the juveniles.

The 96 hour LC50 for TBT to juvenile dogwhelks (less than 24 hours old) was estimated to be 73×10^3 ng/l with 95% confidence limits of 55×10^3 and 120×10^3 ng/l.

D Long-term Development of Juveniles

i) Growth rates of juveniles over one year

The lengths of all the dogwhelks in each tank were measured on three occasions during the year; January, June and August 1991 (5, 10 and 12 months). The data are summarised for each occasion in Table 10, and box and whisker plots of the data from each tank on each occasion (Fig. 4) suggest that there are differences between tanks 1-6 (dosed tanks) and tanks 7-12 (sea water tanks). A two way analysis of variance was carried out on the data obtained from the measurements after one year (August), with parentage as one factor and treatment after hatching (ie dosed water or sea water) as the other. Both factors, and the interaction, were significant. The large sample sizes in some tanks caused relatively small differences in average length to be significant. A two-way analysis of variance on mean

shell lengths shows a highly significant effect for treatment after hatching but not parentage. This implies that some effect other than exposure of the parent dogwhelks to TBT is causing the differences observed in length. Mean length and the total number of dogwhelks present in each tank are negatively correlated (Fig. 5), which suggests that population densities in the aquaria influenced the growth rate.

The weight and length of dogwhelks selected at random from each tank were measured on three occasions. The growth curves obtained from these data were similar for all tanks, and do not indicate any effects of exposure to TBT on the length/weight relationship. However, the mean shell lengths in animals from tanks 7-12 (clean water) are all greater than the animals from tanks 1-6 (dosed water). The data indicate that the dogwhelks grew better in clean water than in water containing 32-128 ng/l TBT.

ii) Determination of mortalities over one year

The weight of dried empty dogwhelk shells removed from each tank during the cleaning session in February/March 1991 is given in Table 11, with the calculated total number of dead dogwhelks.

The percentage survival in each tank to Feb/March and August 1991 (Table 12) show greater than 90% mortality in all but two of the tanks in the first six months after hatching, and the overall mortality greater than 90% in all but one tank (tank 6 dosed, 84.6% mortality). Greatest mortality occurred when the dogwhelks were small, in the first six months after hatching. In the second six months survival was between 40.8% and 64.9% in the dosed tanks, but was 79.8% to 100% in the sea water tanks. This difference is unlikely to be caused by TBT exposure, as both control tanks show similar differences.

iii) Determination of imposex (RPSI and VDSI and incidence of occurrence) after one year

Sufficient growth had occurred after one year for the RPSI and VDSI of dogwhelks in each tank to be determined (Table 13).

a) **Incidence of Occurrence**

The number of juveniles showing a VDSI of stage 2 or above in each tank (Table 13) indicates that in the tanks which were continuously dosed with TBT only three individuals had a VDSI stage lower than 2, one each in tanks 3, 4 and 6 (2, 8 and 128 ng/l TBT). The two control tanks showed lower incidences of occurrence than the dosed tanks, but the beach sample showed fewer instances of occurrence than the controls.

All the juveniles raised in clean sea water also showed high incidences of occurrence of imposex. The lowest was tank 8, (53.1%). The high level of incidence of occurrence in the sea water control aquarium which contained individuals transferred from the large sea water control tank, may imply that there has been some contamination in the large tank. However, this is unlikely, because if the inflowing water had been contaminated, all the control tanks would show a similar high level of occurrence. Similarly, the lower incidence in Tank 2 of the dosed group than in Tank 8 of the sea water group, but as both received the same carrier solvent it indicates that this is not causing imposex development.

b) **Relative Penis Size Index (RPSI)**

The RPSI in the continuously dosed tanks show an increase with increasing concentration of TBT, with the beach sample being the lowest (0.01%), the two controls having low values (0.07% and 0.04%) and the tank dosed with the highest concentration of TBT, 128 ng/l, having the highest RPSI value (117%).

The dogwhelks which were hatched and raised in clean sea water (<1.5 ng/l TBT) showed much lower RPSI values. The RPSI increased with increasing concentration of TBT in the tanks in which the eggs were laid, except for tank 12 (4%). The RPSI value in tank 12 is lower than in any of the clean sea water tanks, except for the alcohol control. The sea water control tank 7 shows a high RPSI, higher than the dogwhelks hatched from adults exposed to 2 ng/l TBT and 128 ng/l TBT, and also higher than the control tanks for the dosed tanks.

c) **Vas Deferens Sequence Index (VDSI)**

The VDSI values show similar patterns to the RPSI values. The continuously dosed tanks show increasing values with increasing concentrations of TBT. The beach sample has the lowest value (0.04) and the tanks dosed with the higher concentrations of TBT (32 and 128 ng/l TBT) contained only one individual whose reproductive tract was blocked. Most females in tank 4 (8 ng/l TBT) had blocked reproductive tracts.

Those hatched into clean sea water showed unexpectedly high values, although the reproductive tracts of most females were not blocked. The control tank 7 has a VDSI value less than tanks 9, 10 and 11 but greater than that from tank 12, whilst dogwhelks from the alcohol control have the lowest VDSI value.

d) **Tissue TBT Concentrations**

The concentration of TBT in the tissues of dogwhelks (mg/kg wet weight) sampled from each tank after one year (Table 13) show a positive correlation between TBT concentrations in the water and in the tissue. The juveniles which were maintained in clean sea water over the year showed no detectable levels of TBT in the tissues.

After one year exposure the juveniles accumulated less TBT than the adults, although the incidence of occurrence of imposex, the VDSI and RPSI are all higher in the juveniles than in adults (cf Table 1).

DISCUSSION

Gibbs *et al.* (1988) investigated the effects of exposure to TBT for two years from hatching to maturity on female dogwhelks. They found that inhibition of capsule production occurred in females that had reached VDS stages five and six, but that at lower VDS stages capsule production proceeded normally. Inhibition was found at TBT concentrations in water of 8-12 ng/l TBT. At higher concentrations (25 to 250 ng/l TBT) progressive alteration of the ovaries occurred, from suppression of oogenesis, initiation of spermatogenesis, ripe sperm present in the vesicula seminalis, and the lack of a sperm ingesting gland.

The current experiment is concerned with effects of TBT on reproduction at degrees of imposex less than those associated with the masculinisation processes described by Gibbs

et al. (1988). Histological examination of the adult female dogwhelks in the current experiment (in prep), did not show any tendency to spermatogenesis.

a) Production of Egg Capsules

At concentrations of 128 and 32 ng/l TBT there was a reduction in the number of egg capsules produced per female, but at 2 and 8 ng/l TBT the number produced per female was similar to that in the controls, indicating a NOEC of 8 ng/l, and a LOEC of 32 ng/l over the first 50 days after laying commenced.

The duration of laying, and the time taken to reach 10% of eggs laid, in each tank shows that laying commenced earlier in the lower TBT concentrations and the controls than in the higher concentration, and the duration of laying was longer. The total number of capsules laid per female were reduced through delay of the start of laying, shortening of the laying period, and reduction in the rate of capsule production in the middle of the capsule production period at VDSI levels of 3.9-4.0. These females showed reduced condition (body weight) compared to controls at the end of the breeding season (Bailey *et al.*, 1991)

b) Production of Juveniles

The percentage of egg capsules hatching shows no relation to the presence/absence or concentration of TBT, indicating that TBT in the surrounding water does not affect the ability of the capsules to release their juvenile dogwhelks.

The timing of hatching of the egg capsules followed a sigmoid curve in all tanks. In almost all cases, the first 10% of the egg capsules opened earlier in clean sea water than in TBT-dosed water. The length of time for 90% to hatch is similar in all tanks, except treatment 6 where the hatching time is shorter, both for those hatched in sea water and in dosed water. Hatching was therefore not delayed by the presence of TBT in the water. The hatching period, however, was shorter for capsules laid in high concentrations of TBT, and this effect was not ameliorated by placing the developing capsules in clean sea water. The NOEC and LOEC for effects of TBT on the length of the hatching period were 32 ng/l and 128 ng/l respectively. Exposure during capsule production determines the scale of the effect, which was found in capsules from in breeding females of VDSI 4.0.

The number of embryos per capsule nearing maturity was very variable in all tanks, and was not related to TBT exposure. Further, the presence of TBT before or during hatching did not affect the number of dogwhelks hatching from each individual egg capsule. The egg capsules that did not hatch contained substances presumed to be eggs and nurse eggs. The estimated numbers that hatched, derived from counts of live and dead animals, are less than those from dissecting the capsules, suggesting that, in dissected capsules, eggs that would be used as nurse eggs for the developing juveniles may have been counted as potential hatchlings. The numbers counted to have hatched were similar to those found by Robertson (1991) in Sweden. The low number in tank 3 appears to be due to factors unrelated to presence of TBT.

c) Short-term Survival of Juveniles

There was no difference in the survival of juvenile dogwhelks in the 2-4 days immediately after hatching between those individuals hatched in clean sea water and those under the TBT dosing regime.

The 96 hour LC50 value (73,000 ng/l TBT) confirmed that the acutely toxic concentration to juveniles was much higher than that which leads to sublethal and probably lethal effects in the form of imposex (eg Gibbs *et al.*, 1988).

d) Long-term Development of Juveniles

The differences in growth of juvenile dogwhelks between tanks were not simply related to exposure to TBT, but were confounded by population density effects. Allowing for density effects, dogwhelks held in 28-132 ng/l TBT were smaller than those held in clean water.

The length/weight relationships showed little variability, either between tanks or over the time period studied, indicating that TBT did not affect this aspect of growth. In more detail, dogwhelks held in dosed tanks showed slower growth early in the year, and increased rate of growth as the year progressed, compared to growth patterns in the control tanks.

The mortalities in all tanks were high (Table 12), and the greatest mortality occurred in the first six months after hatching. Almost all tanks showed 90% mortality or greater in this period. Tank 6 (dosed) had rather less mortality, possibly due to fewer dogwhelks being present. After the first six months, the mortality rate was greatly reduced, and in the course of one year as adults in tanks the mortality was also low (Bailey *et al.*, 1991). The overall mortalities show that less than 10% survived for one year. The survival rate (Table 14) is probably better than in the wild, because of the constant availability of food and the lack of predators. The relatively good survival in Tank 6 (128 ng/l TBT) is probably a further reflection of the density dependent effect observed on growth, but the data suggest that exposure to TBT was not detrimental to survival.

Incidence of occurrence of imposex, RPSI and VDSI was much lower in the beach samples than in the control or dosed groups (tanks 1-6). The control groups (tanks 1-2) showed imposex comparable to the control parent animals exposed as adults (Bailey *et al.*, 1991). In juveniles from tank 3 (2 ng/l) to 6 (128 ng/l) the RPSI and the VDSI values together with the concentrations of TBT in the tissue increased with increasing concentrations of TBT in the surrounding water. The RPSI values in all dosed tanks were significantly greater than those in the controls. The VDSI values for tanks 4-6 indicate that most of the females had blocked reproductive tracts. Many of them had not developed a full reproductive system, even though some of them were of a similar size to adults. The relationships between RPSI, VDSI and TBT concentrations are similar to those observed in the field (eg Gibbs *et al.*, 1988).

The results for the juveniles raised in clean water (tanks 7-12) show a different pattern. Incidences of occurrence of imposex was 95% and higher in all groups except the animals obtained from parents held in the solvent control. The VDSI in the juveniles was between 3.21 and 4.35 without correlation to the TBT concentration in which their parents were held. Only the solvent control had low VDS indices. The juveniles in tanks 7-12 contained no detectable concentrations of TBT. If these animals had accumulated some TBT during their embryo stage at all, it was either very small or was metabolised during the course of the year of the experiment.

The lack of detectable TBT in juveniles from tanks 7-12 is consistent with data from tanks 1-6, and many field studies. However, the VDSI values in excess of 4 seen in juveniles from tanks 9-11, would normally be associated with measurable concentrations of TBT in the tissues. Similarly, VDSI values in excess of 4 would normally be associated with RPSI

values greater than 20%, perhaps up to 70%. The imposex effect seen in tanks 7-12 therefore has characteristics, notably high VDSI values in comparison to RPSI values and TBT concentrations, which differ from the normal expression of imposex in dogwhelks.

The results suggest that the effects of exposure of adults to TBT may be detectable as an imposex-like response, in their progeny, possibly through TBT accumulated in the eggs. however, this does not explain the results from control tanks 7 and 8. The differences between the effects in tanks 7-12 and the usual expression of imposex suggests that other aspects of the experimental conditions also have affected the development of imposex, or a pseudo hermaphroditic condition. The only period of obviously different experimental regimes was before hatching completed, when the juveniles in tanks 1 to 6 were exposed under flow-through conditions, while the others were held under a semi-static regime. There is no obvious reason, in what way this may have effected directly imposex development.

However, there may have been another yet unidentified cause affecting the general condition of the juvenile animals, taking into account the length differences in the groups from tanks 1-6 and 7-12. It appears that the group of animals, which had higher weights, were more susceptable to imposex development. This may also explain why imposex in the beach population was generally lower than in the laboratory controls, although feeding and temperature conditions appear to be more favourable for the laboratory animals.

CONCLUSIONS

A In comparison to unexposed controls, adult dogwhelks exposed to TBT concentrations of 32 ng/l or more, with VDSI values of 3.9-4.0:

i) Produced less egg capsules per female;
ii) Showed delayed commencement of capsule laying;
iii) Had a shortened laying period;
iv) Laid capsules more slowly.

B There was no relationship between the TBT concentration (up to 128 ng/l) to which adult dogwhelks were exposed during breeding and:

i) Release of juveniles from egg capsules;
ii) The number of embryos maturing in each egg capsule;
iii) The number of juveniles emerging from capsules.

Exposure of adults to 128 ng/l TBT shortened the hatching period of egg capsules, irrespective of the TBT concentration in the incubation medium (up to 128 ng/l).

C The 96 hour LC50 for TBT to newly-hatched dogwhelks was 73,000 ng/l. Immediate 2-4 day post hatching mortality of juveniles was not affected by TBT concentrations up to 128 ng/l.

D TBT exposure of juveniles to 32-128 ng/l reduced the growth of juveniles over 12 months. No effects were detected on juvenile mortality arising from exposure of adults or juveniles to up to 128 ng/l TBT.

Imposex values were positively correlated with TBT exposure concentration of juveniles. There are indications that exposure of adult dogwhelks to TBT can adversely affect the imposex status of their progeny, even when raised in uncontaminated (<1.5 ng/l TBT) sea water. In contrast, the juveniles raised in uncontaminated (<1.5 ng/l) sea water showed relatively severe imposex without being related to the TBT concentrations to which the parents were exposed. The results suggest that other aspects of the experimental conditions have also affected imposex development, and the relationship between penis and vas deferens growth in females held in clean water particularly since the juveniles obtained from the parents from the control group also showed severe imposex development.

ACKNOWLEDGEMENTS

This work was funded by the Organotin Environmental Programme Association, to which the authors extend their thanks. The authors also thank G Rodger for editing and preparing this manuscript.

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TABLE 1

Degree of imposex in the breeding adult (parent) dogwhelks (Bailey *et al.*, 1991)

		Incidence of occurrence %	May		% sterile	Tissue TBT*	Incidence of occurrence %	August		Tissue TBT*	% sterile
			RPSI %	VDSI				RPSI %	VDSI		
Tank 1	Control	48	0.13	1.56	0	<0.02	37	0.10	1.06	<0.02	0
Tank 2	Alcohol control	37	0.03	1.32	0	<0.02	24	0.04	0.70	<0.02	0
Tank 3	2 ng/l TBT	76	0.73	2.36	0	0.07	92	5.33	3.15	0.07	0
Tank 4	8 ng/l TBT	96	7.23	3.50	0	0.21	100	20.84	3.97	0.22	3
Tank 5	32 ng/l TBT	93	26.64	3.87	0	0.57	99	42.08	4.33	0.61	33
Tank 6	128 ng/l TBT	100	49.62	4.00	2	0.94	100	63.40	4.25	0.97	24

*Mean male and female

TABLE 2

Concentration of TBT (ng/l) in experimental tanks

Tank No		Number of observations	Target concentration	Mean	SE
1	Control	15	<1.5	<1.5	0
2	Alcohol control	14	<1.5	<1.5	0
3*	2 ng/l TBT	20	2	2.57	0.16
4	8 ng/l TBT	24	8	7.41	0.63
5	32 ng/l TBT	20	32	27.82	2.38
6	128 ng/l TBT	23	128	107.74	7.71

Points not included in calculation = <1.5
* includes two values <1.5

TABLE 3

Production of egg capsules during breeding season (54 days)

Tank		A	B	C	D	Egg capsules per female	Mean capsules per female per day
1	Control	753	2,801	255	205	12.94	0.24
2	Alcohol control	430	3,003	234	183	15.90	0.29
3	2 ng/l TBT	473	2,204	162	104	19.56	0.36
4	8 ng/l TBT	449	1,643	148	99	15.09	0.28
5	32 ng/l TBT	22	646	140	99	6.46	0.12
6	128 ng/l TBT	45	359	151	102	3.38	0.06

A = Number of egg capsules before third sampling
B = Number of egg capsules after third sampling
C = Number of females before third sampling
D = Number of females after third sampling

$$\text{Egg capsules per female} = \frac{A}{C} + \frac{(B - A)}{D}$$

TABLE 4

Production of egg capsules (estimated from Fig. 1)

Tank		Day 10% laid	Day 50% laid	Duration of laying 10-90% (days)
1	Control	5	17	47
2	Alcohol control	6	32	52
3	2 ng/l TBT	7	19	33
4	8 ng/l TBT	3	16	45
5	32 ng/l TBT	15	35	27
6	128 ng/l TBT	15	30	27

TABLE 5

Total number of egg capsules counted and percentage hatched in each tank at the end of the hatching

Tank number TBT dosage (ngl^{-1})		No of capsules hatched	No of capsules present	Percent hatched
Hatched in clean sea water				
1	Control	566	819	69.1
2	Alcohol control	387	812	47.7
3	2 ng/l TBT	594	816	72.8
4	8 ng/l TBT	303	464	65.3
5	32 ng/l TBT	39	61	63.9
6	128 ng/l TBT	153	157	97.5
Hatched under dosage regime				
7	Control	1,665	1,982	84.0
8	Alcohol control	1,517	2,191	69.2
9	2 ng/l TBT	673	1,388	48.5
10	8 ng/l TBT	1,034	1,179	87.7
11	32 ng/l TBT	438	585	74.9
12	128 ng/l TBT	193	202	95.5

TABLE 6

Time (days) for 0-10%, 10-50%, and 50-90% of eggs capsules to open under sea water or dosed conditions each tank

Tank		Dosed			Tank		Clean sea water		
		10%	50%	90%			10%	50%	90%
1	Control	30	20	45	7	Control	12	23	51
2	Alcohol control	35	16	40	8	Alcohol control	25	13	33
3	2 ng/l TBT	32	22	55	9	2 ng/l TBT	12	25	43
4	8 ng/l TBT	38	9	44	10	8 ng/l TBT	13	25	44
5	32 ng/l TBT	38	18	52	11	32 ng/l TBT	15	10	60
6	128 ng/l TBT	28	16	27	12	128 ng/l TBT	30	9	25

TABLE 7

Number of dogwhelks/egg capsules dissected

14 August						
Tank	1	2	3	4	5	6
TBT conc ng/l	Sea water control	Alcohol control	2	8	32	128
	18	30	18	25	22	12
	13	23	23	56	23	24
	25	16	17	43	11	15
	25	11	23	28	29	21
	27	13	12	26	28	21
	33	26	37	24	16	16
Mean	23.5	19.8	21.7	33.7	21.5	18.2

3 October					
Tank	1	2	3	4	5
TBT conc ng/l	Sea water control	Alcohol control	2	8	32
	20	13	14	25	15
	21	17	23	24	21
	33	22	30	17	11
	28	20	13	18	25
	32	20	32	36	12
Mean	26.8	18.4	22.4	24.0	16.8

Overall mean	25.0	19.2	22.0	29.3	19.4

TABLE 8

Hatching success obtained from counts of capsules and juveniles in the small hatching aquaria

Tank		No hatched	No of empty capsules	Juveniles/ capsule	Juveniles/ capsule (estimate)
1	Control	45	3	15.0	8.4
2	Alcohol control	159	10	15.9	8.5
3	2 ng/l TBT	60	9	6.7	12.9
4	8 ng/l TBT	23	1	23.0	13.8
5	32 ng/l TBT	164	10	16.4	8.8
6	128 ng/l TBT	138	10	13.8	9.0

$$\text{Estimate} = \frac{\text{Total No of dogwhelks}}{\text{No of capsules hatched}}$$

TABLE 9

Two to four day post-hatching mortality on three occasions (20, 10 and 10 animals used respectively)

Tank	Hatching conditions	% Dead			Mean
		28 Aug	11 Sept	25 Sept	
1	Sea water	0	10	0	3
2	Carrier	0	20	30	17
3	2 ng/l TBT	0	0	20	7
	8 ng/l TBT	5	0	40	15
5	32 ng/l TBT	15	30	20	22
6	128 ng/l TBT	15	10	40	22
7	Clean water	5	30	0	12
8	Clean water	10	10	20	13
9	Clean water	5	50	10	22
10	Clean water	5	20	10	12
11	Clean water	*	*	*	*
12	Clean water	10	10	*	10

*No hatchlings available to test

TABLE 10

Summary of dogwhelk shell length measurements during their first year

Tank No	Condition (TBT ng/l)	No of observations	Mean (mm)	Standard deviation
January - 5 months				
1	Control	862	6.89	4.623
2	Alcohol control	604	7.84	5.280
3	2	680	8.13	4.985
4	8	659	6.93	4.492
5	32	282	5.56	3.701
6	128	308	6.19	3.102
7	Clean sea water	189	10.96	4.316
8	Clean sea water	84	8.23	3.381
9	Clean sea water	203	11.41	5.109
10	Clean sea water	221	11.56	4.815
11	Clean sea water	48	15.19	5.510
12	Clean sea water	121	13.39	4.182
June - 10 months				
1	Control	560	14.19	6.368
2	Alcohol control	406	14.85	6.947
3	2	480	14.95	6.097
4	8	478	13.72	6.728
5	32	160	14.56	6.193
6	128	212	14.64	6.154
7	Clean sea water	155	19.56	4.913
8	Clean sea water	69	17.29	5.311
9	Clean sea water	179	19.49	5.120
10	Clean sea water	198	19.16	5.254
11	Clean sea water	47	20.65	5.788
12	Clean sea water	117	20.20	4.723
August - 12 months				
1	Control	417	19.71	6.056
2	Alcohol control	288	20.50	5.813
3	2	347	20.54	5.575
4	8	356	19.33	5.834
5	32	115	23.25	6.243
6	128	167	23.13	5.430
7	Clean sea water	152	25.40	3.700
8	Clean sea water	67	25.90	4.317
9	Clean sea water	173	24.44	3.668
10	Clean sea water	188	25.09	3.424
11	Clean sea water	48	26.69	5.027
12	Clean sea water	114	26.02	3.655

TABLE 11

Mortalities determined from weight of dead dogwhelks

Tank	Weight of dead dried dogwhelks (g)	Calculated No of dead dogwhelks present (Jan 1991)	Weight/ dogwhelk (mg)
Maintained in dosed regime			
1	2.6422	7,861	0.336
2	3.2446	10,419	0.349
3	2.6549	6,755	0.393
4	4.0675	11,330	0.359
5	0.6822	2,481	0.275
6	0.3513	774	0.454
Maintained in clean sea water			
7	4.4647	9,298	0.480
8	1.7454	4,171	0.419
9	2.1726	8,302	0.262
10	3.2384	6,259	0.517
11	0.6127	1,401	0.437
12	0.6484	1,927	0.337

TABLE 12

Percent survival of juvenile dogwhelks

Tank	Percent survival		% survival over second six months
	February/March	August	
Dosed			
1	9.88	4.78	48.4
2	5.48	2.61	47.7
3	9.15	5.93	64.9
4	5.50	2.94	53.4
5	10.21	4.16	40.8
6	28.47	15.43	54.2
Sea water			
7	1.99	1.60	80.4
8	1.97	1.57	79.8
9	2.39	2.03	85.2
10	3.41	2.90	85.1
11	3.31	3.31	100.0
12	5.91	5.57	94.2

TABLE 13

RPSI, VDSI, incidence of occurrence and tissue TBT concentration in each tank after one year

Tank	No of females	No of males	Incidence of occurrence %	RPSI	VDSI	Tissue concentration mg/kg net
Maintained in dosed regime						
Beach	104	96	7	0.01	0.04	
1	95	102	42	0.07	1.28	<0.02, <0.02
2	96	104	38	0.04	1.14	<0.02
3	89	111	99	64.0	3.98	<0.02, <0.02
4	84	116	99	88.6	4.96	0.08, 0.07
5	55	60	100	90.9	5.00	0.21, 0.24
6	75	89	99	117.7	4.99	0.66, 0.67
Maintained in clean sea water						
7	70	82	96	8.8	3.81	<0.02
8	32	35	53	0.3	1.45	<0.02, <0.02
9	83	90	95	7.6	4.28	<0.02, <0.02
10	80	108	98	10.0	4.35	<0.02
11	24	24	96	10.4	4.30	<0.02
12	52	62	98	4.0	3.21	<0.02

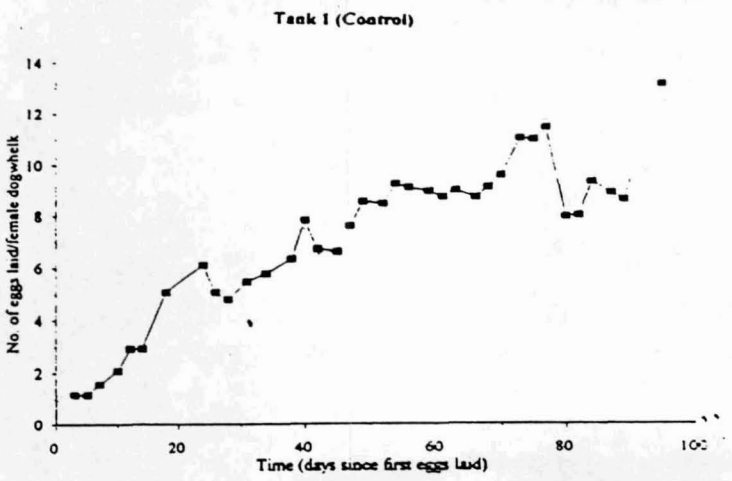
TABLE 14

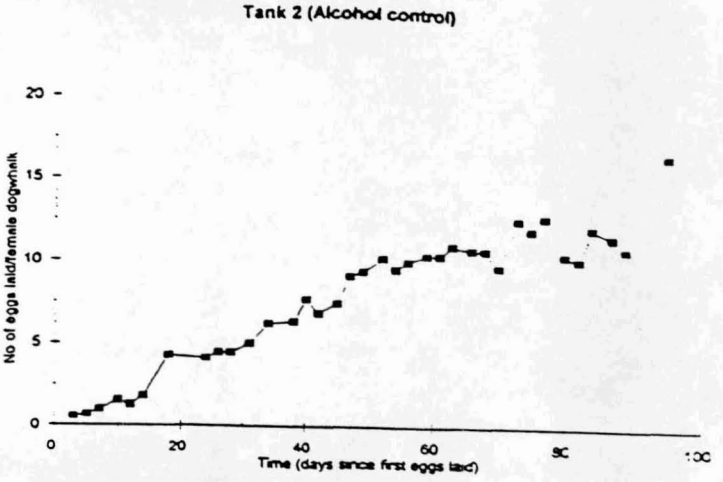
Survival of juveniles over one year

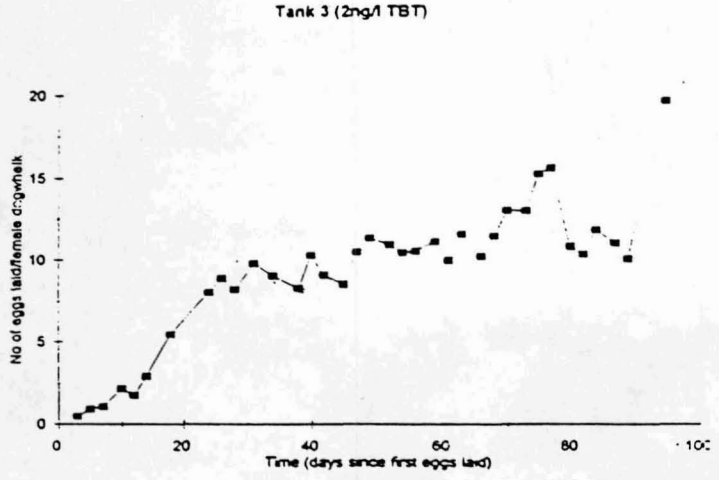
Tank	Juveniles/ female	Survival/ capsule laid	Survival/capsule hatched
1	2.63	0.20	0.26
2	1.88	0.12	0.19
3	5.45	0.28	0.40
4	4.96	0.33	0.40
5	1.63	0.25	0.34
6	2.65	0.78	0.81

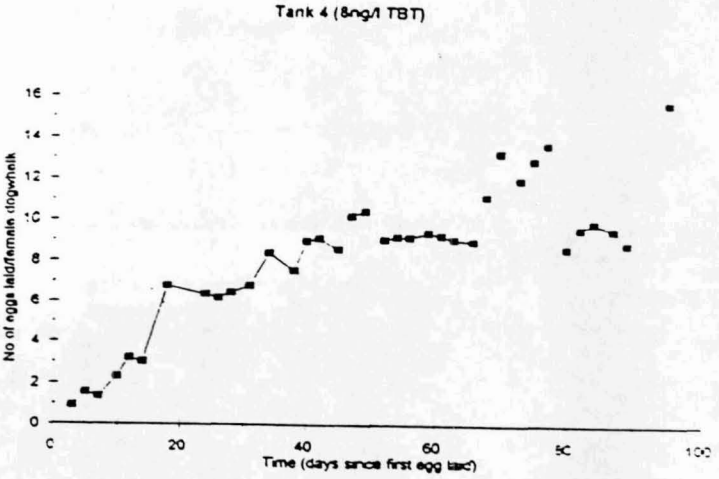
LIST OF FIGURES

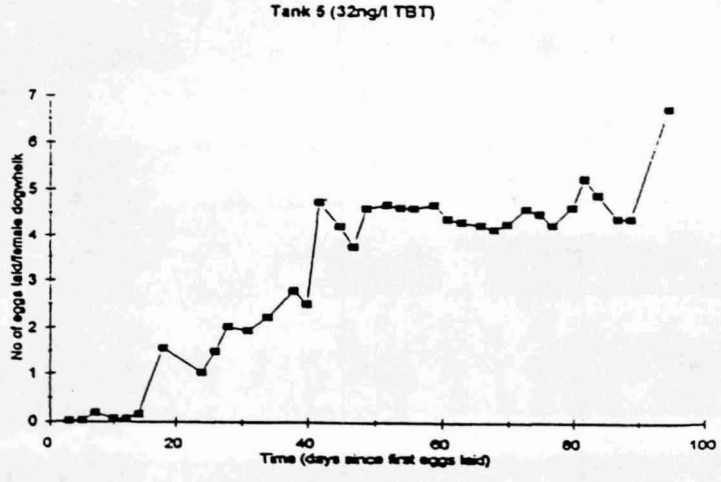
Figure 1 Eggs laid/female dogwhelk











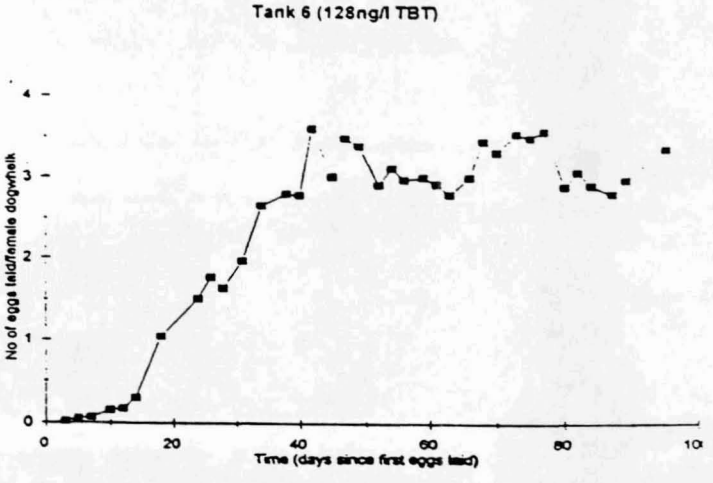
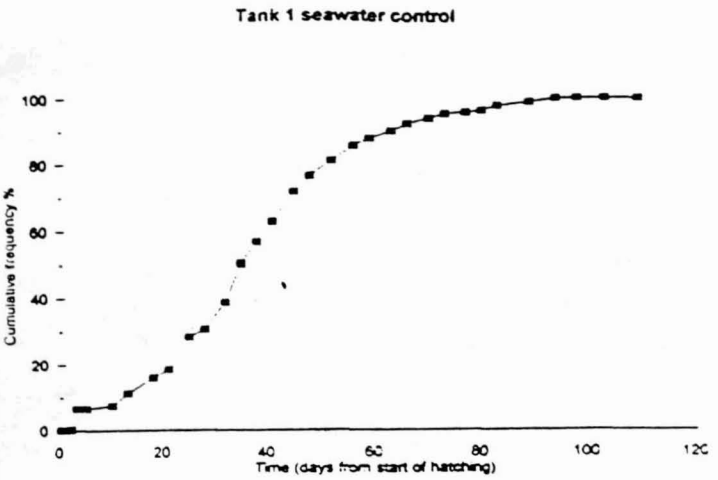
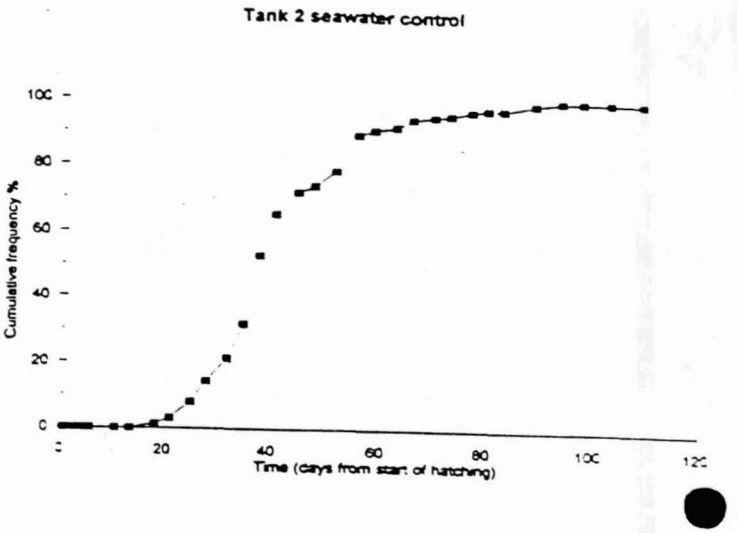
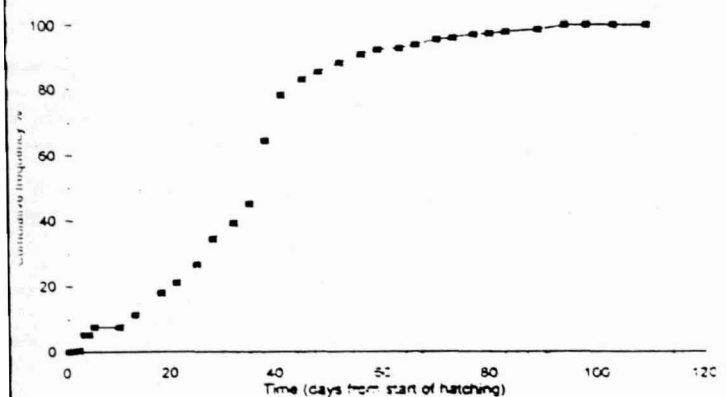
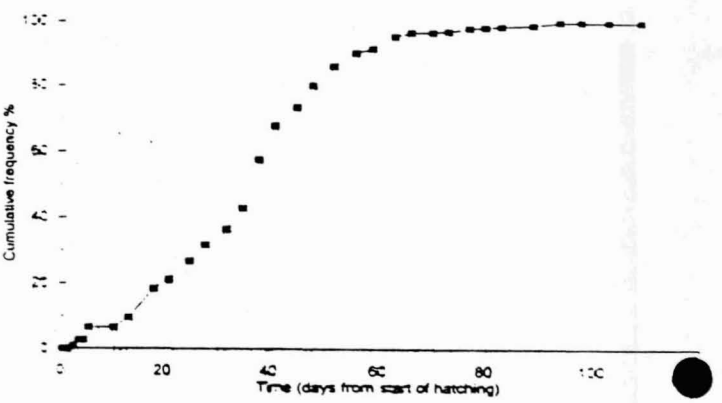


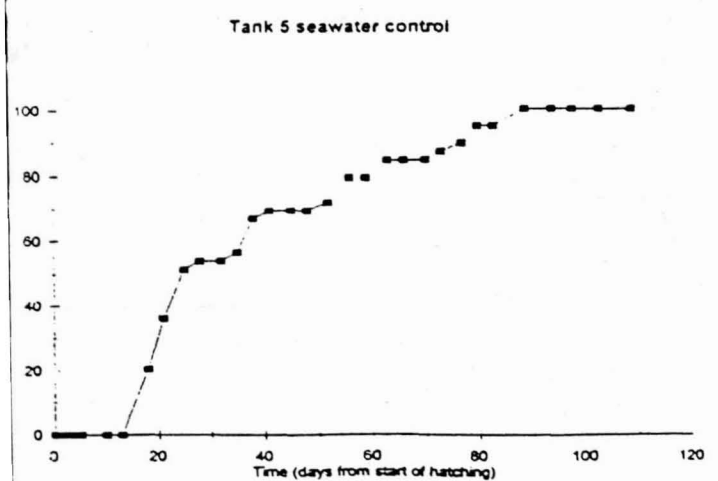
Figure 2 Dogwhelks hatched in sea water











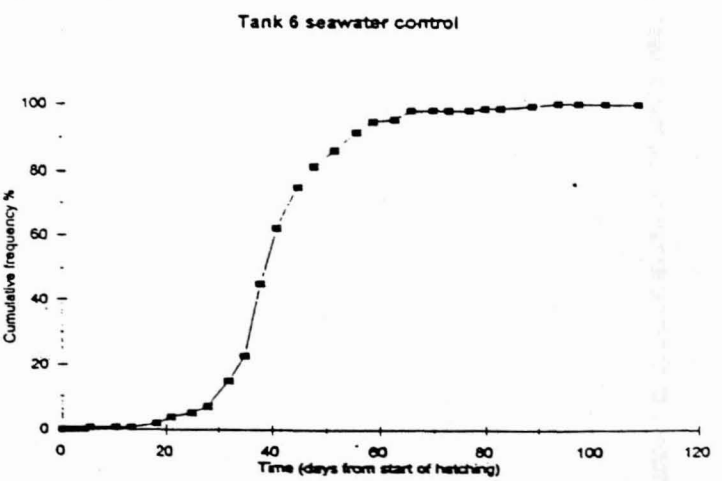
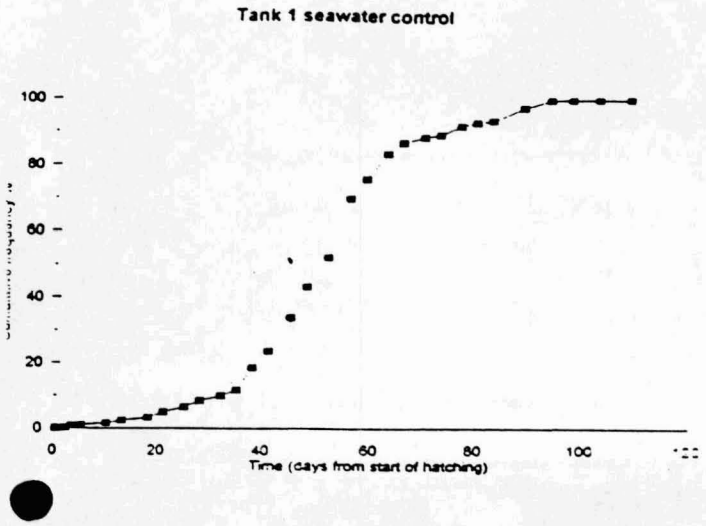
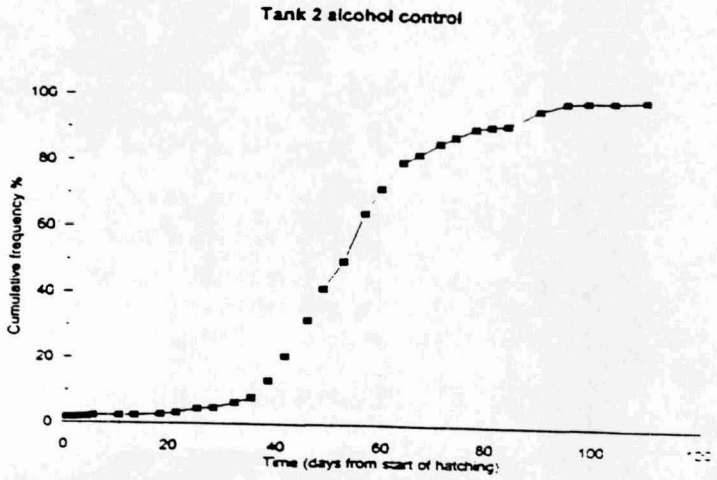
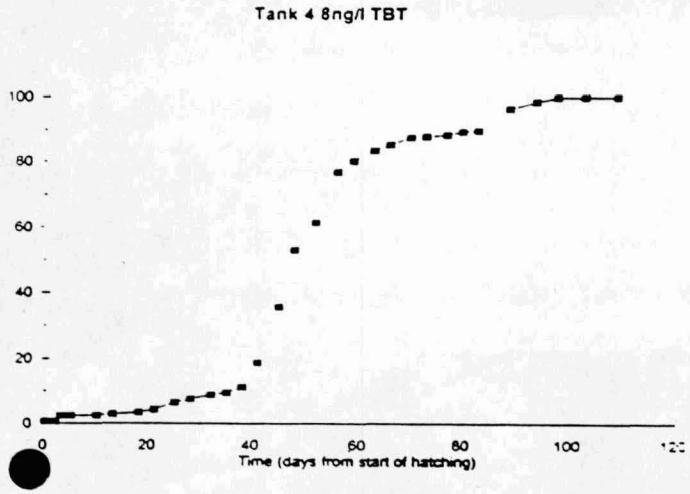
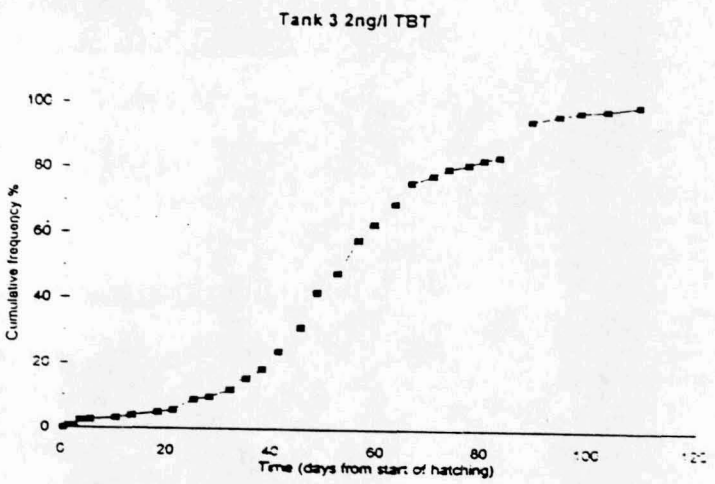


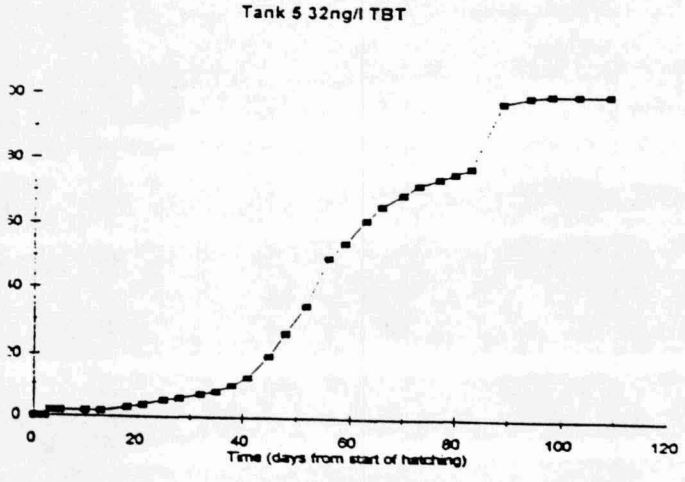
Figure 3 Dogwhelks hatched in dosed water











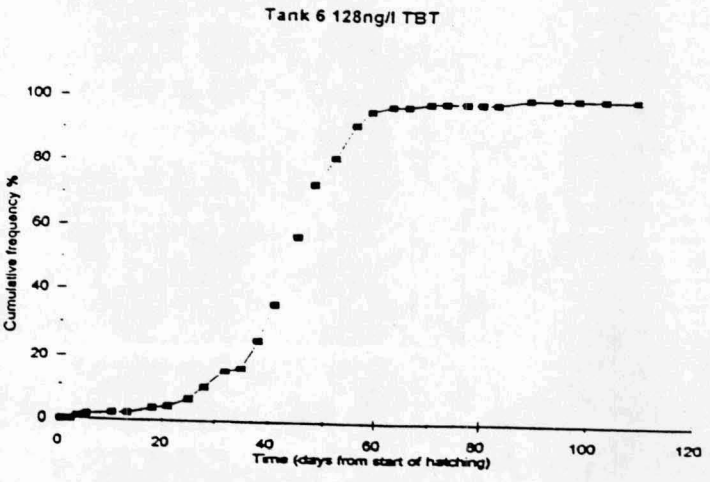


Figure 4

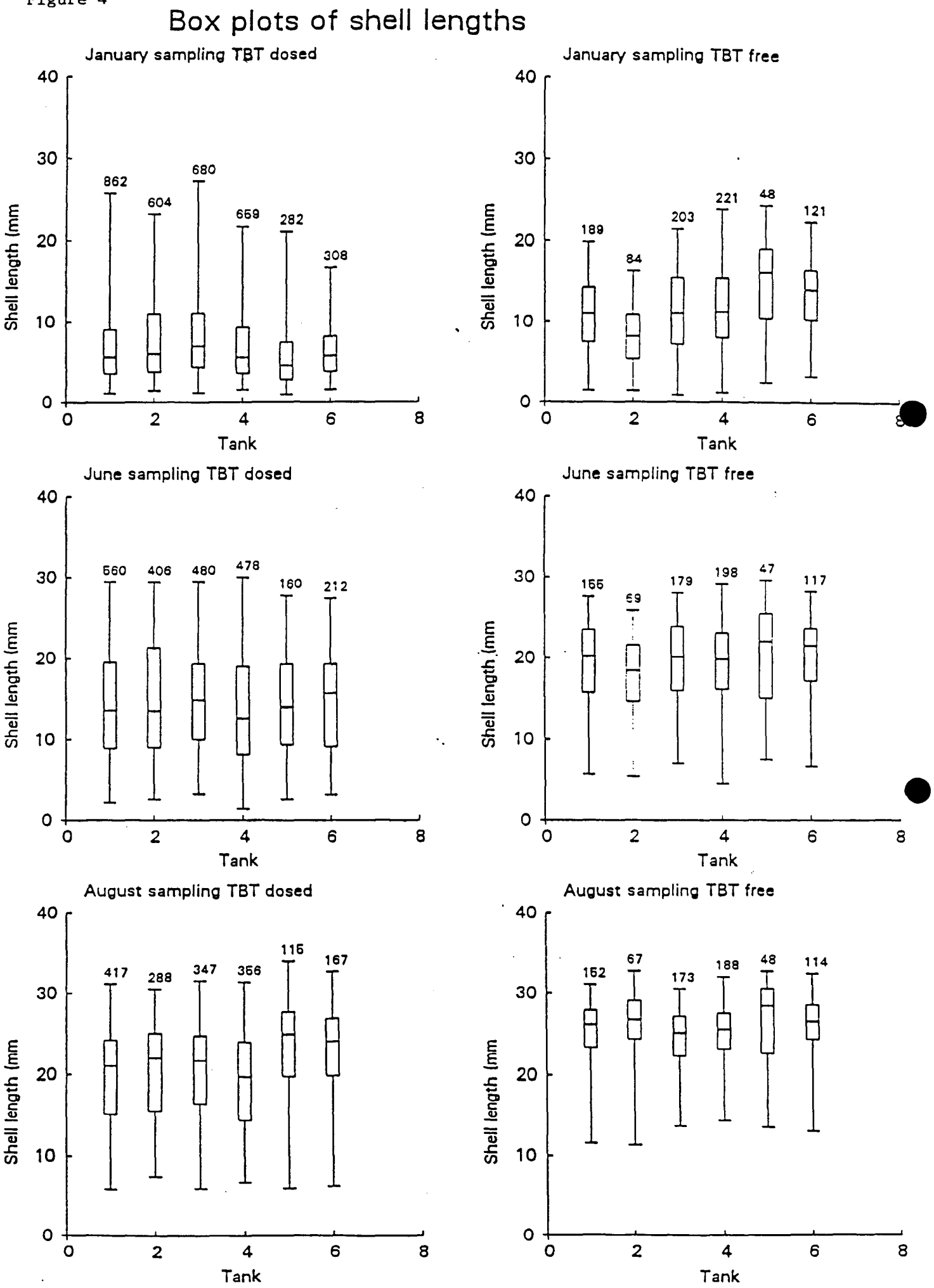
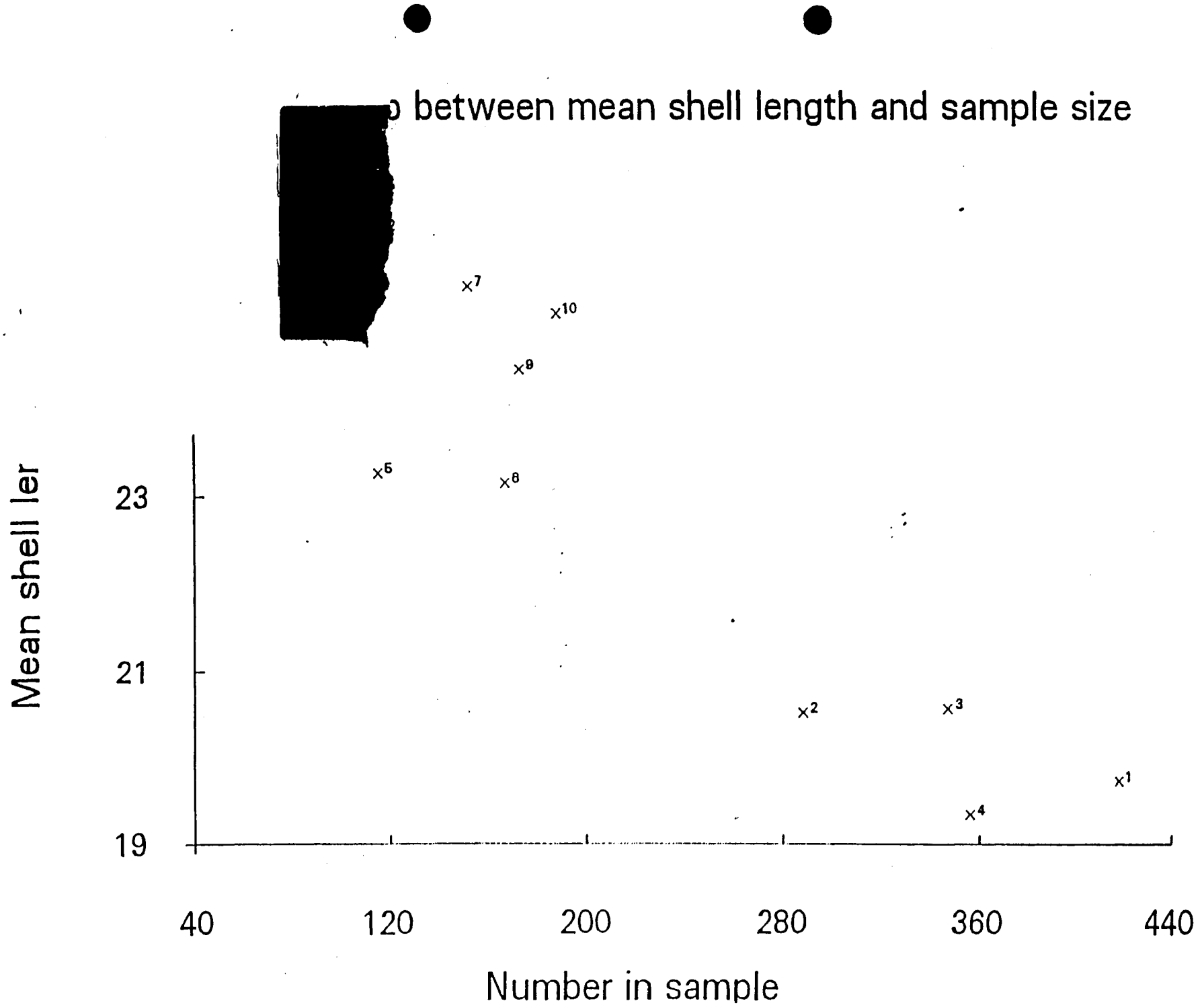

Box plots of shell lengths
January sampling TBT dosed
862
604
680
659
282
308
January sampling TBT free
189
84
203
221
48
121
June sampling TBT dosed
660
406
480
478
160
212
June sampling TBT free
155
69
179
198
47
117
August sampling TBT dosed
417
288
347
366
115
167
August sampling TBT free
152
67
173
188
48
114
Shell length (mm
Tank
0
10
20
30
40
0
2
4
6
8

Figure 5


between mean shell length and sample size
Mean shell ler
23
21
19
40
120
200
280
360
440
Number in sample
x1
x2
x3
x4
x5
x6
x7
x8
x9
x10