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Metallic pollution in estuaries, with special reference to the effects of tributyltin (TBT) and copper on the early life stages of *Scrobicularia plana* (Mollusca: Bivalvia)

by

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Metallic pollution in estuaries, with special reference to the effects of tributyltin (TBT) and copper on the early life stages of *Scrobicularia plana* (Mollusca: Bivalvia).

Jose M. Ruiz

During the 1980s a decline in populations of the bivalve *Scrobicularia plana* was noted in several U.K. estuaries: tributyltin (TBT) was suspected of being the cause although its toxicity to adults could not be demonstrated except at elevated concentrations; disappearance of clam populations has been also observed in other European countries.

Laboratory tests have revealed that: **i)** D-larvae hatching from embryos after 48 hours in TBT concentrations of 188 ngSn/l amounted to < 50% of control values, and doses of 364 ngSn/l or 20 µgCu/l prevented normal development in ≈ 90% of embryos. **ii)** planktonic veliger larvae exposed for 10 days to nominal TBT doses ≥ 50 ngSn/l grew at rates which, at maximum, were one third of that exhibited by controls. **iii)** settling pediveligers subjected for 30 days to levels of TBT ≥ 70 ngSn/l suffered significant mortalities, and postlarvae kept at 23 ngSn/l displayed some shell growth which was both substantially reduced and grossly abnormal. **iv)** exposure for 30 days to ≥ 300 ngSn/l or ≥ 20 µgCu/l impaired the burying activity in sand of small spat, and juveniles reared in TBT solutions at ≥ 28 ngSn/l grew significantly less than those in the control treatment. **v)** while small spat held in heavy metal polluted sediment suffered massive mortalities in 12 days, juveniles exposed for 36 days to butyltin contaminated sediment (0.4 µgSn/g) did not display any limited survival, but both their growth and burying activity were significantly reduced relative to those of juveniles kept in control sediments.

It is concluded that in U.K. coastal areas where TBT in water during the summer-autumn months ranged from ≈ 20 ngSn/l to ≈ 200 ngSn/l (i.e. ≈ 50-500 ngTBT/l) and *Scrobicularia plana* populations disappeared or declined markedly, a cause-effect relationship is most likely to exist between the former and the latter through the deleterious effects of the chemical on the early life stages of the clam; in addition, the continued presence of sediment-bound TBT may render mudflats unsuitable for the development of larval and juvenile bivalves.

List of contents

List of Tables and Illustrations

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Presentations and Conferences attended:

- October 1990. Metallic contamination in sediments from Spanish estuaries. University of Plymouth. Seminar.
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- April 1992. Ruiz, J.M. & Saiz Salinas, J.I. Bioavailability of heavy metals in two estuaries of the Basque Country. III Colloque d'Oceanographie du Golfe de Gascogne, Arcachon (France). Oral communication.
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- September 1992. Ruiz, J.M. & Saiz Salinas, J.I. Bioindicators of metallic pollution in sediments: application to estuaries in North Atlantic Spain. Estuarine and Coastal Sciences Association 22 Symposium, Plymouth. Oral communication.
- September 1992. Ruiz, J.M., Gibbs, P.E., Bryan, G.W. & Wigham, G.D. Effects of TBT and Cu exposure on the 48 hours embryonic development of clams, *Scrobicularia plana*. ECSA 22 Conference, Plymouth. Poster communication.
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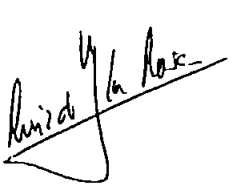


Signed: **Jose M. Ruiz.**

Date: 1.4.93

To Marisol, she knows all about it

Chapter 1: General introduction and aims.

1.1. Assessing the biological effects of contaminants.

Metallic waste from industrial and urban activities has been traditionally dumped into rivers in the belief that it would be carried to the open sea and then conveniently dispersed; however, precipitation processes due to estuarine mixing result in metallic accumulation in sediments (Turekian, 1977; Bryan, 1980) where concentrations of metallic compounds reach levels usually several orders of magnitude higher than those in overlaying waters (see for instance Bryan and Langston, 1992). Furthermore, it is recently being realized that sediments may act as reservoirs of some contaminants which can exert toxic effects even in the absence of further input, in such a way that environmental degradation is occurring in areas where water quality criteria are not being exceeded (Chapman, 1989).

Since the concern of marine pollution monitoring and control is largely with the deleterious effects that contaminants may have on the biota within a given ecosystem, it is commonly agreed that strategies including biological methods provide the most appropriate means of assessing their impact; there is no viable alternative at present to determine metal bioavailability that excludes the use of biota (Waldichuk, 1985). Thus, models of bioavailability based on chemical measurements require validation with appropriate organisms, at least in the first instance, and it is self-evident that a contaminant having no biological effect is of no biological significance (Stebbing *et al.*, 1980). Assessing the relevant biological effects of contaminants would necessarily involve a compound approach including field work and laboratory experiments.

Due to the difficulty and complexity of field monitoring relying upon analyses of waters and sediments, the use of biomonitors is now the most widely employed method to monitor trace elements in coastal waters (see review by Phillips, 1990). Biomonitors are usually employed to quantify field pollutant abundance or bioavailability by virtue of their tissue concentration of contaminants, but substance-specific biological responses (such as those induced by tributyltin, see section 1.2 below) have also been successfully utilised. While bioindicators of dissolved contaminants have long been employed even for

large-scale programmes (the "Mussel Watch", see Goldberg, 1980), sediment biomonitors seem comparatively less applied; however, increasing recognition of the hazard constituted by pollutants associated with sediments may promote their wider use.

Laboratory toxicity tests and bioassays are necessary since it is only within an experimental context that the possibility exists of positively identifying those contaminants which have toxic effects, because it is only in controlled experiments that individual factors can be isolated and their importance determined (Stebbing *et al.*, 1980). Further, where several chemicals interact, the integration of effects by biological material is the only reliable evidence for predicting or detecting adverse impacts (Cairns and Pratt, 1989). Since the key to the biological success or failure of any species in affected environments is provided by the effect of a pollutant at the most sensitive stage of the life cycle, usually the earlier ones, bioassays should, if at all possible, test the lethal and sublethal effects of pollutants on these early stages (i.e. embryonic, larval, juvenile). When the organism used is the species of concern in a given situation, bioassays become particularly relevant in order to establish causal relationships between toxic pollutants and their biological effects.

1.2. Tributyltin.

Tributyltin (TBT: $[CH_3(CH_2)_3]_3Sn^+$) started being widely used as the biocide agent of antifouling paints in the early 1960s (Champ and Pugh, 1987). By the late 1970s it became evident that TBT leached from these formulations affected organisms other than those originally targeted. The amount of unequivocal evidence relating TBT with detrimental effects occurring in the estuarine environment gathered in the 1980s (MAP, 1989) led to its partial ban in countries all over the world (see for instance Cardwell and Meador, 1989).

Measurements of TBT levels in U.K. estuaries in the 1980s revealed a number of principles applicable elsewhere: i) concentrations were generally lowest in winter, increasing rapidly as newly-painted boats were launched during the spring; they remained high over the summer, then declining gradually during the autumn. Thus, concentrations of the ion TBT^+ detected in subsurface waters covered the range < 1 ng/l to > 1000 ng/l

(see for instance Waldock *et al.*, 1987); levels of TBT were usually higher than those of its less toxic degradation product, dibutyltin (DBT). **ii)** concentrations in the surface microlayer exceeded those just below the surface by factors of 1.9 to 27 (Cleary and Stebbing, 1987). This was an important observation since biota inhabiting the surface layer (i.e. neuston) include not only microorganisms but also eggs and planktotrophic larval stages of many fish and, possibly, invertebrates (Hardy *et al.*, 1987). **iii)** following the partial restrictions on TBT usage (1987 in the U.K.), concentrations in waters declined; however, levels in some sediments remained high, posing unknown risks for environmental quality (Langston and Burt, 1991; Waite *et al.*, 1991; Dowson *et al.*, 1992).

TBT has been said to be "probably the most toxic substance ever introduced deliberately in the marine environment" (Mee and Fowler, 1991), and has been shown to be particularly noxious to molluscs, even at relatively low concentrations (see review by Bryan and Gibbs, 1991). Apart from its acute effects on a range of marine organisms, notably their early stages, the possibly best known and documented deleterious actions specifically induced by TBT are: **i)** imposition of male characters in female neogastropods ("imposex") leading to eventual demise of populations of certain species (see review by Gibbs *et al.*, 1991), and **ii)** shell thickening in oysters, especially *Crassostrea gigas* (Thunberg) (see for instance Alzieu *et al.*, 1986). Although in the field organotins are much more bioavailable than inorganic tin (Langston *et al.*, 1990) and laboratory experiments demonstrated acute toxicity of relatively-high levels to adults (Beaumont *et al.*, 1989; Langston and Burt, 1991), conclusive proof of TBT ecotoxicity to sediment-dwelling bivalves such as *Scrobicularia plana* (da Costa) is still required.

1.3. *Scrobicularia plana.*

Habitat. The tellinid bivalve *Scrobicularia plana* is an infaunal species commonly inhabiting the soft bottoms of Northeast Atlantic estuaries, from the Norwegian sea into the Mediterranean and south to Senegal (Tebble, 1966); it is typically intertidal, but subtidal populations have occasionally been found (Rasmussen, 1973; Wolff, 1973). *S. plana* lives buried in the sediment, feeding by means of extensible

siphons on the material around the burrow entrance during low tide (see for instance Hughes, 1969; Zwarts, 1986); this activity leaves an star-shaped mark on the sediment which reveals the location of the specimen. However, since the distinction between the suspension- and deposit-feeding mode of life is only one of degree (Moore, 1977), it may reduce its deposit-feeding activity because of natural causes such as low mud temperatures during winter (Hughes, 1969; Zwarts and Wanink, 1989) and possibly to avoid severely-contaminated deposits (Bryan and Gibbs, 1983).

Reproduction and gametes. In the Northern region of its distribution range, *S. plana* reproduces once a year (Essink *et al.*, 1991), but Southern populations usually display two reproduction seasons (Paes-da-Franca, 1956; Bachelet, 1982). In waters of North Wales the gamete follicles of *S. plana* are empty between October and March (Hughes, 1971), and natural spawning in U.K. is likely to be concentrated around July-August.

S. plana is usually gonochoristic, although some hermaphrodites have been reported from a Portuguese population (Paes-da-Franca, 1956). The gonads of ripening specimens ramify from the top of the digestive gland downwards, their colour ranging from dirty-white to yellow-orange; no definite correlation between colour and sex has been established. However, the gonadal follicles have a different shape in each sex, and with experience it is possible to distinguish the tubular spermatogenic follicles of the male from the more rounded oocytic follicles of the female. An approximately equal sex ratio is assumed for *S. plana*, but deviations may occur when part of the population has spawned (Hughes, 1971).

Each *S. plana* ovum is surrounded by a thick and clear membrane or chorion displaying a cicatricule; this is probably the trace of the ovum's peduncle (Frenkiel and Mouëza, 1979) rather than a true micropyle to facilitate the entry of spermatozoa (Hughes, 1971). While the average diameter of the egg plus chorion is agreed to be 140-150 µm, authors do not coincide on the measures of the egg itself: Hughes measurements of stripped eggs average 91 µm in diameter, whilst those of Frenkiel and Mouëza of spawned ova are 75-80 µm Ø; it is likely that the maturation, only obtained through spawning, results in a cytoplasmic contraction which is accentuated after

fertilization. The sickle-shaped sperm heads are reported to be of a length between 20-30 µm (Hughes, 1971; Frenkiel and Mouëza, 1979).

Biological cycle. Despite intensive efforts, notably by Hughes (1971) and Mengus (1978), reproduction of *S. plana* in the laboratory remained elusive till Frenkiel and Mouëza (1979) described its early development; since no other work has been found in the literature challenging their observations, data below summarizing *S. plana* embryonic and larval development at 16-18 ºC are taken from this source.

After external fertilization, zygotes of *S. plana* follow an embryonic development common to many other bivalves (see for instance Verdonk *et al.*, 1983), leading to planktotrophic veliger larvae. This process, however, is characteristic in that embryos undergo the whole of such development within the mucous chorion described above, which affords effective protection against variable estuarine conditions (Frenkiel and Mouëza, 1979). A ciliated blastula which rotates within the chorion is formed at ≈ 12 h; after 24 h, the typical pear-shaped trochophore continues to rotate inside the chorion (dilated to ≈ 200 µm) and begins to form the shell. Some 48 h after fertilization the larva has achieved the prodissoconch I stage; since the shell's contour resembles the capital letter D, this bivalve stage is commonly termed "D-larva"; with a mean length of 106 µm, D-larvae continue rotating within chorion until hatching occurs between 2½ and 4 days. Free D-larvae swim and feed on phytoplankton by means of a ciliated velum and develop the umbones at 10-12 days. The foot is formed some 18-20 days after hatching, and when it becomes fully active the larvae reach the pediveliger stage (≈ 30 days, shell 250-270 µm long). Pediveligers alternate periods of swimming by means of the progressively less functional velum with periods of crawling "on foot", probing the bottom substratum; if a suitable sediment is found, pediveligers bury and sedentarization begins. Spat develop the exhalant (superior) siphon when about 600-700 µm long, and when they reach 900 µm the two siphons are formed and can be withdrawn into the siphonal space; this marks the conclusion of metamorphosis and the incorporation into the juvenile stage. No explicit information is given by Frenkiel and Mouëza (1979) on the time taken by pediveligers to complete metamorphosis, but a period of at least another 2-3 weeks can be inferred from their text.

Burying depth of bivalves mainly depends on size of siphons (Zwarts and Wanink, 1989). When living within the top few centimetres of the sediment, juveniles are likely to be exposed every so often as a consequence of disturbance, notably by turbulence in stormy weather (Hughes, 1970). When dug out, *S. plana* juveniles would promptly rebury to avoid surface predators, but unsuitable conditions may promote byssus thread production and subsequent drifting (own laboratory observations); thread-drifting plays a most relevant role in the migration and secondary settlement of the related species *Macoma balthica* (Linnaeus), and spat of up to 1 cm shell length has been caught in plankton nets (Beukema and de Vlas, 1989). Animals mature during their second summer after settlement, corresponding to a shell length of about 20 mm (North Wales population, Hughes, 1971). Adult animals frequently reach > 40 mm in length, and may live up to 18 years (Essink *et al.*, 1991).

Population dynamics as governed by natural factors. Intertidal mudflats offer a multitude of microhabitats, usually resulting in patchy distributions of *S. plana* which are difficult to explain (Hughes 1970). However, given the ubiquity and local abundance of *S. plana,* monitoring of populations has been widespread over North Atlantic Europe and produced a considerable body of information. In a review by Essink *et al.* (1991) it is concluded that severity of winter is the natural abiotic factor most important in governing population density and success of recruitment, leading even to transient disappearance of some populations. Nevertheless, it is pointed out that adult specimens living deeper than about 10 cm are less affected by low winter temperatures and ice, and that these enduring long-living specimens are responsible for maintenance of populations.

Other natural factors causing decrease of abundance in clam populations are high river run-off (Marchant *et al.*, 1983 in Essink *et al.*, 1991) and predation by birds. Hughes (1970) and Zwarts (1986) estimated that the main predator of *S. plana* adults is the oystercatcher *Haematopus ostralegus* (L.), which may account for a mortality of up to 10%; in addition, the clam is known to be an important component of the diet of a number of other avian species which may have suffered the effects of *S. plana* declining numbers in certain areas (e.g. the knot *Calidris canutus* L., see Zwarts *et al.*, 1992). In this respect, the observation that *S. plana* buries at almost double the depth during

winter than during summer is not considered to be an adaptation to reduce the risk of being eaten by birds (Zwarts and Wanink, 1989); it may be speculated that greater winter depth is achieved to cope with unfavourable winter temperatures.

Population dynamics: caused by pollution stress?. In some occasions it is possible to attribute decrease in biota abundance to episodic man-made alterations of local conditions such as hydrodynamics (Ducrotoy and Desprez, 1986). However, pollution-induced disturbances of biological communities are more often than not very difficult to identify, particularly in estuarine and coastal environments where multitude of chemicals may display similar gradients of contamination. Some of the few conclusively established cause-effect relationships between pollutant and specific biological response are those linking TBT and declining molluscan populations (see section 1.2 above).

In a laudable initiative, the European programme COST 647 ("Coopération européene dans la domaine de la Recherche Scientifique et Technique") was established in 1979 to look into the problems of accurately evaluating biological changes in coastal marine benthos (Keegan, 1986). Its Intertidal Sediment Working Group designated *S. plana* as one of the "key species" of this habitat; investigation of spatial and temporal scales of natural variability of these key species would assist in understanding and predicting the consequences of events induced by man. Monitoring of relatively clean habitats, free from man-induced interferences was aimed at differentiating between natural and anthropogenically imposed factors. However, it is acknowledged that "regrettably, many of the original study sites were, and continue to be, exposed to more gradual or subtle pollution and disturbance, and that the programme's interpretative base needs to be consolidated and expanded through the inclusion of hitherto under-utilised disciplines such as biogeochemistry" (Keegan, 1991).

As far as the population dynamics of *S. plana* is concerned, the review by Essink *et al.* (1991) compiles COST 647's data sets on numerical densities of the clam in several European locations relating to periods of up to 18 years. While decreasing densities in some areas are ascribed to factors such as hard winters and sediment instability, "Indications were found for a gradual decline or even disappearance of the populations of *S. plana* in the northern part of the German and Danish Wadden Sea, the cause of

which is unknown" (Essink *et al.*, 1991). In summary, three groups can be distinguished in the studied sites: **i)** those suffering winter decrease, **ii)** those showing marked non-winter decrease, and **iii)** those where previously documented populations have not had any spatfall or have even disappeared over the 1970s and 1980s (see also Zwarts and Wanink, 1989; Zwarts *et al.*, 1992). TBT in some of these localities has been detected at considerable concentrations over the 1980s (up to 600 ng/l), and disappearance of a TBT biomonitor (the gastropod *Nucella lapillus* L.) has been concurrently noticed (Eastern Scheldt, Ritsema *et al.*, 1991); no data on TBT levels at the other sites have been found in the literature.

In the U.K., *S. plana* has extensively been used as a biomonitor of metallic pollution (Bryan et al., 1985), and repeated quantitative and semi-quantitative observations made at a number of sites for more than a decade show that populations: **i)** have virtually disappeared in many estuarine locations bordering the Solent, **ii)** present a discontinuous, perturbed structure (Poole Harbour) or **iii)** comprise only a few old individuals (Langston *et al.*, 1987, 1990). Environments at these sites suffered substantial levels of TBT contamination over the 1980s, both in water (< 1 to > 1000 ng/l, Waldock *et al.*, 1987) and sediments (< 0.03 to > 1.29 µg/g dry weight), suggesting an inverse correlation between levels of TBT and abundance of bivalves (Langston *et al.*, 1990). European locations where *S. plana* populations are reported to be declining are depicted in figure 1.1.

***S. plana* in Bilbao estuary**. The estuary of Bilbao in the East Cantabrian Coast of Spain has historically received considerable amounts of metallic waste but very little attention has been directed towards assessing its impact. While the little information available describes the water and particulated matter far from the estuary itself as considerably contaminated (Guerrero Perez *et al.*, 1988) and the sediments within the estuary as severely polluted by heavy metals (Seebold *et al.*, 1982; Swindlehurst and Johnston, 1991), no data have been found dealing with levels and effects on local macrobenthic populations. Unpublished data report that, even though the intertidal mudflats in Bilbao are largely devoid of fauna common to other estuaries in the area, there is one known population of *Scrobicularia plana* which, although limited in extent,

it is apparently well established. The almost total absence of pleasure crafts and of high boating activity of any other kind in the inner estuary support the assumption that TBT levels in Bilbao are far from those causing concern in other European estuaries (see section 1.2). Survival of *S. plana* in Bilbao despite heavy metal pollution indicates that this is not likely to cause depopulation elsewhere, and that interesting comparisons to the matter above may arise from studying the metallic contamination in Bilbao estuary and its effects on the local *S. plana* population.

1.4. Aims.

Considering all the above circumstantial evidence (mostly field data) suggesting a cause-effect relationship between TBT and the observed lack of recruitment into populations of *Scrobicularia plana* in European estuaries, there are clearly sufficient grounds for pursuing related investigations. These should ideally test the effects of environmentally relevant levels (i.e. up to ≈ 1000 ng/l) of TBT on the early life stages of the clam by means of an approach including the pertinent laboratory toxicity tests and bioassays. If possible, effects of Cu should also be tested since restrictions on TBT have promoted a return to the usage of this metal as the biocide component of antifouling paints. The goals of the present work are:

- To assess the extent of metallic pollution in Bilbao estuary and its effects on the indigenous population of *S. plana.*
- To investigate the effects of field TBT exposure on the reproductive function of *Scrobicularia plana.*
- To test the effects of TBT (and Cu) exposure on the early life stages of *S. plana,* namely:
    - **i)** embryonic development (planktonic).
    - **ii)** larval stages: from hatching to settlement (planktonic) and then on to metamorphosis (benthic).
    - **iii)** juvenile (benthic).
- To determine the suitability of contaminated sediments (either with TBT or heavy metals) for the successful establishment of *S. plana* juveniles.

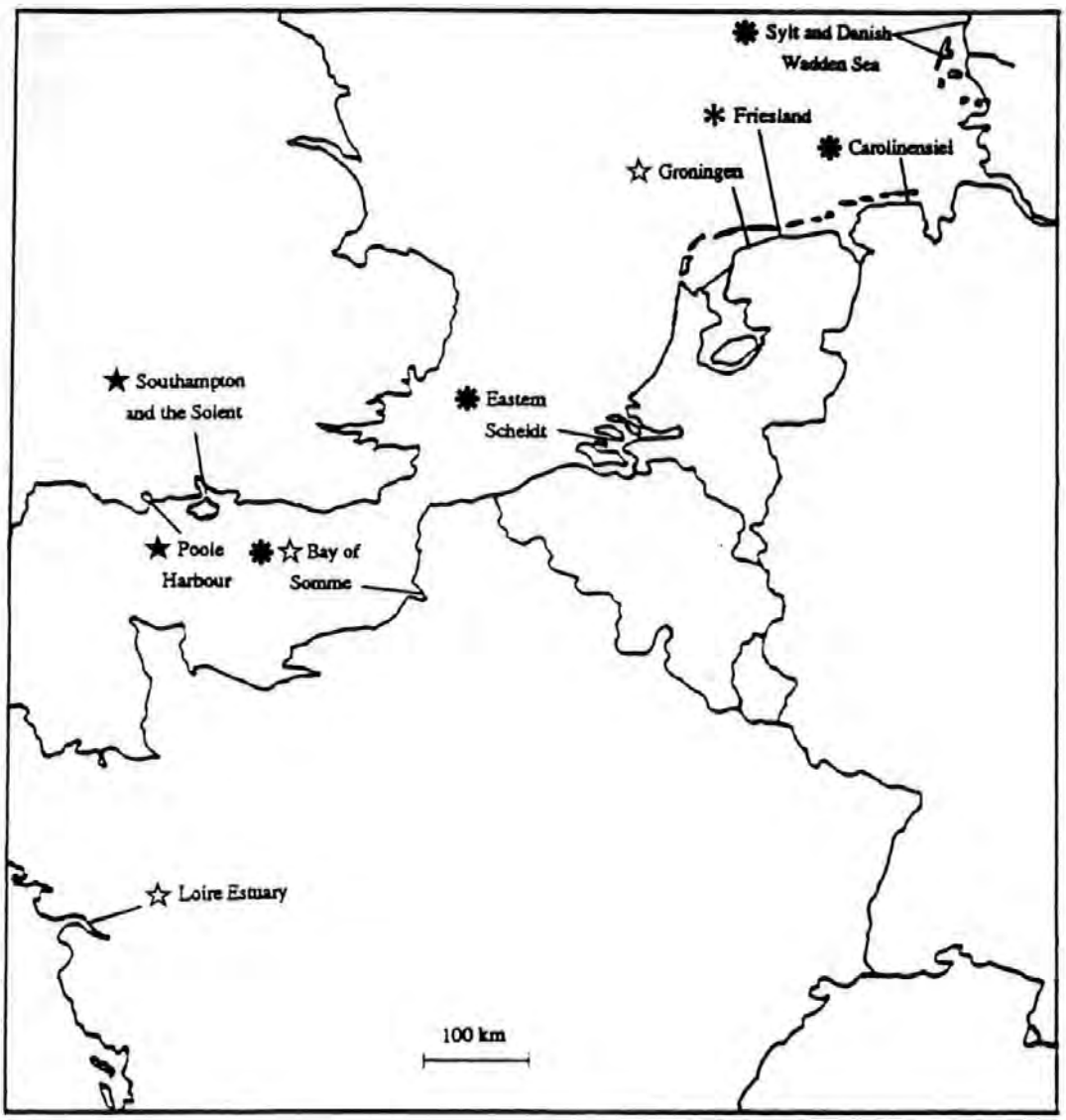


Figure 1.1: European sites where populations of *Scrobicularia plana* have been reported to be declining over the 1980s. Key: ✸ Site: disappeared (Essink *et al.*, 1991); ☆ Site: unexplained non-winter mortality (Essink *et al.*, 1991); ✻ Site: not significant spatfall during 1976-1986 (Zwarts and Wanink, 1989; Zwarts *et al.*, 1992); ★ Site: transient juvenile settlement or disappeared (Langston *et al.*, 1990).

Chapter 2. Assessment of metallic pollution in the estuary of Bilbao and its effects on *Scrobicularia plana*.

2.1. Introduction.

The estuary of the Nervion river in Bilbao has traditionally received contaminating domestic sewage and industrial waste water (Azkona *et al.*, 1984) as a result of which offshore sediments and waters are far more polluted with heavy metals than the average for the rest of the Spanish Cantabrian Coast (Guerrero Perez *et al.*, 1988). In addition, levels of metals in sediments within the estuary have been reported to be considerable (Seebold *et al.*, 1982; Swindlehurst and Johnston, 1991) but, to date, there is no information concerning the possible effects of this metallic load on infaunal populations. Although macrofauna is scarce, clams *Scrobicularia plana* and polychaetes *Nereis diversicolor* O. F. Müller inhabit some of the intertidal mudflats along the estuary. These well documented bioindicator species (Bryan *et al.*, 1985) have been used to assess the extent of heavy metal contamination in Bilbao; the study includes reference to other estuaries in the area such as Santoña and Guernica, both important feeding areas for migratory birds. Research has been focused mainly on the populations of *S. plana* in Bilbao, and its characteristics in relation to metallic pollution in the estuary.

2.2. Materials and methods.

2.2.1. Area of study and sampling.

In a first instance (December 1989), 5 estuaries in the Eastern Cantabrian Coast of Spain (from West to East: Agüera, Bilbao, Guernica, Deva and Orio) were explored by means of collecting superficial sediment during low tides. Two sites separated by ≥ 2 km were sampled in each estuary, except for Bilbao where 3 localities were selected (Lamiaco, Asua-1 and Deusto); sites within Bilbao estuary are shown in figure 2.1.a, and position and coordinates for the other sites are given in figure 2.1.b and Table 2.1.a., respectively. Result of sediments analyses (Table 2.1.a) showed diverse heavy metal contamination in all estuaries, including the Agüera which had been considered a potential control site; it was then decided to concentrate efforts in Bilbao and Guernica

(with 2 upstream added sites (G-3 - 43° 21' 22" N, 02° 40' 20" W - and G-4 - 43° 19' 20" N, 02° 40' 29" W -), selecting a new control estuary further westwards, Santoña (only one site, S - 43° 26' 17" N, 03° 29' 03" W-). Preliminary explorations were made in Bilbao in May 1990 as well, collecting sediment samples (in Lamiaco, Asua-1, Asua-2 and Asua-3, see figure 2.1) for heavy metal and organotin analyses.

Bioindicators from these three estuaries were collected throughout 1991, in January, May and December, and additional material was collected at 2 new sites in Bilbao where *S. plana* populations were found in February 1992; also, surficial sediments in an area about 2 m^2 in Lamiaco (a man-made terrace at ≈ mid-intertidal level) were sieved *in situ* on 3.1.92, and the material retained by a 1 mm pore mesh kept in 70% ethanol to estimate bivalve recruitment. Samples (listed in Appendix 1) consisted mostly of sediments and *S. plana*, but other species were also collected to assess heavy metal impact. In addition, ≈ 200 clams were collected in January 1991 at site G-2 and held in the laboratory for a few days while their length was measured to the nearest mm with some callipers. They were marked on both valves with water-proof ink and subsequently redeployed in lots to the field, either within plastic boxes (30 x 21 x 15 cm l.w.h) with perforated lids or free by burying them in holes previously dug in the sediment; animals were transplanted to Santoña (only free clams), Asua-1 and Lamiaco in Bilbao (only boxed clams) and also replaced at G-2 (both free and boxed clams). Finally, 25 G-2 clams were similarly measured, marked and transplanted free to Lamiaco in May 1991, and the reverse (63 individuals from Lamiaco to G-2) was done in July 1991 (see also Appendix 1.B for transplant scheme).

2.2.2. Treatment and metal analyses of samples.

Samples were taken and processed following closely those methods standardised by Bryan *et al.* (1985) and summarised below; organotin content of samples was determined as detailed in Bryan *et al.* (1986) and outlined in section 3.2.6.

2.2.2.1. Sediments.

Surficial sediments (top 1 cm layer) were scraped off during low water at approximately mid-intertidal level and wet sieved through a 100 µm pore polythene mesh

in 50% seawater; after settling overnight, most of the water was decanted and the sediment mixed to form a slurry which was poured into plastic vials. All samples were kept frozen for their transport to the Plymouth Marine Laboratory (PML). Sediments were thawed in the fridge for 1 day, and 4 ml aliquots of the resulting slurry taken into a 100 ml conical flask and dried in an oven (≈ 85 °C) overnight; then, about 20 ml HNO_3 (high purity, usually Aristar, BDH) were added per gram of dried sample. The flask was covered with a glass bubble and heated on a hot plate (≈ 100 °C) for 1-2 days until a pale yellow solution was obtained; results of this digestion will be referred to as total metallic content because it seems unlikely that metals remaining insoluble after this treatment will ever become bioavailable. After removing the bubble, the acid was slowly evaporated and the residue dissolved in concentrated high purity HCl and later diluted with distilled water to give 10% or approximately 1N HCl; finally, after ≈ 1 h, the extracts were decanted into test tubes and insoluble particles allow to settle. Separate sediment aliquots were generally also processed with hydrochloric acid to characterise the fraction of metals potentially bioavailable (Luoma and Bryan, 1981) as follows: 2 ml slurry are pipetted into glass liquid-scintillation vials and extracted for 2 h with 20 ml of 1 N HCl (high purity) while continuously stirred with a magnetic flea. The vial supernatant is then decanted into a glass syringe connected to a plastic membrane filter (0.45 µm) holder; pressure is applied with the plunger and, after discarding the first few ml, the extract is separated from the sediment and collected in another vial. Both total and bioavailable extracts were analysed for 10 heavy metals (Ag, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) with flame (Varian SpectrAA-20) or graphite furnace (Varian SpectrAA-300 Zeeman) Atomic Absorption Spectrometry (AA); all results will be given in a dry weight (salt-free) basis.

Sediment organic content was also usually determined from the loss in weight of dry (85 °C) aliquots heated at 400 °C for 6 h; since the sediments had been treated with 50% seawater and its water content was known, the results are corrected for the loss in weight of seawater salts at 400 °C.

2.2.2.2. Fauna.

Once in the laboratory, all faunal samples were depurated of ingested sediment for 1 week: bivalves and polychaetes in aerated 50% seawater (the worms were held in

acid-washed sand for the first 6 days) and crustaceans in clean seaweed *Fucus sp.*. Samples were either frozen (bivalves and worms) or dried (crustaceans) for their transport; they were later thawed, extracted with nitric acid (bivalves only soft-parts) and their heavy metal content determined as described in section above for sediments. The number of individuals pooled per replicated sample of *S. plana* and their mean valve length is given in Appendix 2.

In addition, some specimens of *S. plana* from Lamiaco were brought alive to PML in May 1991. Different tissues (digestive gland, kidney, mantle, gill, adductor muscle and relatively developed gonad) from 4 animals were independently pooled and analysed for some metals with flame (Cu, Fe and Zn) and furnace (Cd and Pb) AA. The tissues of other 5 individuals were cryofixed by K. Ryan (see Ryan, 1991) and examined with X-ray electron microscopy by J. Nott. All handling water was disposed of on soil and left to evaporate.

2.3. Results and discussion.

Results of analyses of samples collected in May 1990 (Table 2.1.b) confirmed the occurrence of heavy metal pollution in Bilbao intertidal sediments indicated by a preliminary survey (see Table 2.1.a); Cd, Cu, Pb and Zn values agree with those reported by Swindlehurst and Johnston (1991) for samples collected in November 1990, and show that severity of sediment metallic contamination in the Asua river (see figure 2.1) increases upstream. As for organotin in sediments (see also Table 2.1.b), levels in Lamiaco are low while those along the Asua are considerable and approach the threshold suspected to be detrimental to infaunal bivalves (i.e. 0.3 µgSn/g as TBT, Langston *et al.*, 1990). Given the almost total absence of pleasure crafts in Bilbao estuary and the unnavigability of the Asua, it is thought that TBT originates mainly from industrial activities and, possibly, urban sewage (Fent, 1989).

Results of the sampling in January 1991 are listed in Table 2.2; figure 2.2 plots Cd, Cu, Pb and Zn values for Santoña, Guernica-2 and Lamiaco sites. It is evident that Lamiaco sediments are far more contaminated than those from the other estuaries for the majority of metals analysed and, since values exceed those of the Regional Geochemical

Background (see Seebold *et al.*, 1982), it can surely be concluded that pollution stems from anthropogenic local sources. A considerable fraction of that contamination seems to be bioavailable and, thus, *S. plana* in Lamiaco (the only population of the clam known in Bilbao up to February 1992) accumulates much more Cd, Cu, Pb and Zn than in either Guernica-2 or Santoña (see figure 2.2.b); notably, the level of Cd in clam tissues (mean of 3 replicates = 100 ppm) is more than double the maximum known to have been ever recorded in U.K. estuaries (see for instance Bryan *et al.*, 1985). Pollution is also reflected in the heavy metal content of *N. diversicolor* (figure 2.2.c); however, the polychaete is known to regulate its body burden of Zn (see for instance Bryan and Gibbs, 1983) and, even in Asua-1 where most metals in tissues reach maxima, levels in worms are below 300 ppm Zn despite high concentration in sediments (see Table 2.2).

Undetectable or low levels of organotin were found in all sediment samples analysed (Table 2.2) and, although TBT concentrations in clams from Lamiaco (0.5 μgSn/g) deserve some consideration, they are far from the several ppm Sn accumulated by *S. plana* in areas where clam populations are thought to be adversely affected by organotin pollution (Langston *et al.*, 1990).

Levels of most metals in the sediments of Guernica estuary clearly increase upstream to reach maxima in G-4 (the closest to the village), and for some elements they reach (Cr, Ni) and even surpass (Ag, Mn) concentrations found in Lamiaco (see Table 2.2). Values of Ag in *S. plana* tissues follow that gradient (see figure 2.3.a), and the levels of this metal are biomagnified wherever the clam is present; a similar instance has been previously found in U.K. estuaries (see for instance Bryan and Langston, 1992) and also for *Macoma balthica* in North America, where it was suggested to be due to the experimental fact that Ag bioavailability is raised by the association of the metal with extracellular polymers formed by bacteria (Harvey and Luoma, 1985). On the contrary, while Cu levels in sediments (extracted with either nitric or hydrochloric acid) also increase upstream (see figure 2.3.b), the flux of actually available metal is reversed and values in bioindicators (clams and worms) are highest in the more downstream site, G-2; both *S. plana* and *N. diversicolor* are well documented bioaccumulators of Cu (see for instance Bryan *et al.*, 1985), but it is unknown what local factors enhance uptake of Cu

by biota at G-2. Results plotted in figure 2.3 constitute yet another example of how metal bioavailability does not necessarily relate directly to the chemical load in the physical environment. It has to be noted, however, that use of frozen and possibly anoxic sediments for extraction schemes as in the present case casts doubts on their validity since such schemes are generally only valid for oxic, unpreserved material. Thus, the fact that levels of "bioavailable" Cu in sediments from all three sites within Bilbao estuary (see Table 2.1) are orders of magnitude lower than those referred to as total is probably a reflection of the sediments being high in sulphide (i.e. anoxic).

Because of high metal levels along the Asua, special efforts were made in May 1991 to collect all possible macrofauna in Asua-1; the results of the whole sampling campaign undertaken this month are given in Table 2.3. No reference on heavy metals in *Orchestia sp.* has been found in the literature, nor could this amphipod be collected elsewhere for comparison. However, the potential of supra-littoral isopods (including *Ligia oceanica* L.) as bioindicators of metallic pollution was studied by Hopkin *et al.* (1985); they found that *L. oceanica* from the Severn estuary contained significantly more Cd, Cu and Zn than animals from a control site. Interestingly, levels of the same metals in isopods from Asua-1 are 2-3 times (and levels of Pb up to 1 order of magnitude) higher than in the most affected sites in the Severn (mean for Asua-1 = 25, 460, 33 and 415 ppm, respectively for Cd, Cu, Pb and Zn). Although no data are available on metals in *L. oceanica* from other Cantabrian estuaries, it is likely that these high concentrations are not characteristic of cleaner areas; since no macroalgae are distributed in the upper reaches of Bilbao estuary, it is suggested that isopods in Asua-1 uptake most of their metallic burden from leaf litter and other terrestrial sources.

The polychaete collected in Asua-1 in May 1990 can be referred to as *Lycastopsis sp.* and, since this genus is not commonly distributed in North Atlantic waters, its occurrence in Bilbao is probably the result of an accidental import (P. Gibbs, personal communication). As for its metallic content, *Lycastopsis sp.* shows higher mean levels of Cd, Cu and Pb accumulation than any other polychaete sample (including *N. diversicolor* collected in Asua-1 in January 1990), and concentrations are much elevated than those reported for a related species in India (Athalye and Gokhale, 1991); on the

contrary, its levels of Zn are the lowest of all worm samples, indicating that, like other nereids, *Lycastopsis sp.* is probably able to regulate the body burden of this metal.

The distribution of some metals among different tissues in *S. plana* from Lamiaco is also shown in Table 2.3. Levels of Cd, Pb and Zn in the digestive gland are ≈ 1 order of magnitude higher than in any other tissue and, therefore, it can be concluded that this is the preferred organ for metal storage in the clam. However, Fe is found mostly in the mantle, while Cu seems to be more evenly distributed over all soft parts examined but concentrating in the gill and kidney; the latter does not agree with the higher Cu levels in kidney and digestive gland reported by Bryan and Gibbs (1983). Despite high Cd levels in tissues (458 µg/g dry wt in the digestive gland), X-ray microanalysis in the electron microscope failed to locate any Cd-containing lysosomes or other granules; X-ray microanalytical spectra from granules distributed in samples (figure 2.4) revealed some major accumulations of Fe and Zn and trace amounts of Co, Cr, Mn, Ni and Pb, but no trace of Cd in any tissue. Since, in addition, no metallothionein (MT) -like proteins for Cd-binding have been found in *S. plana* (Langston and Zhou, 1987), it would seem that this clam does not resort to any combination of the two detoxification mechanisms common in marine molluscs (see for instance Carmichael *et al.*, 1980; Nott and Langston, 1987). Alternatively, it is possible that Cd in a labile form was present *in vivo* but translocated during processing for electron microscopy or, as suggested by Langston and Zhou (1987) for *Macoma balthica*, some unidentified proteins in the high molecular weight pool execute the MT protective role. Clearly, further research on the mechanisms allowing *S. plana* to tolerate severe heavy metal pollution in Lamiaco is required.

Results of analyses of samples collected in December 1991 and February 1992 are listed in Table 2.4. Figure 2.5 plots Cu, Pb and Zn values for sediments and *S. plana* collected throughout 1991 (January, May and December) in Santoña and Guernica-2 sites. While concentrations in sediments from both estuaries show little variation over time, a decrease in heavy metal levels in tissues of Santoña clams becomes obvious from January to May, returning to higher levels the next winter (figure 2.5.a); this is most likely due to the fact that, at this latitude, *S. plana* is already in some advanced stage of gamete development and, therefore, body metal concentrations are diluted with the

growing gonad during the reproduction season (Bryan *et al.*, 1985). This clear "drop and recovery" feature of metals displayed by clams from a relatively-unaffected area is, however, obscured in Guernica-2 (figure 2.5.b); it is thought that as a result of reduced fresh water inputs during summer and subsequent higher ambient metal levels, the natural diluting trend of the clam is counterbalanced by seasonal environmental fluctuations coupled with a constant rate of man-produced contamination.

Figure 2.6 summarises all heavy metal data for sediments and *S. plana* in Lamiaco. Sediment level of Cd increased from 14 ppm in December 1989 up to a maximum of 112 ppm a year later and, after that date, they sharply dropped down to 4 ppm in the last sample collected, December 1991; levels of Cu, Pb and Zn during these 2 years followed a parallel evolution. Although there are no data available for fauna in 1989 and 1990, metal levels in clam tissues (figure 2.6.b) match the descending trend of the sediment load over 1991; yet, the decrease is less acute, from , for instance, 100 ppm Cd in January to 50 ppm in May and 30 ppm in December. It was thought that the drought known to have affected the Spanish Cantabrian Coast and the South West of France during 1989 and 1990 (which resulted in ≈ one million people in Bilbao suffering severe water restrictions for nearly a year) may have some relation with those remarkable dynamics shown by heavy metal contamination. Flow data of the Nervion river (the main water course draining into Bilbao estuary) were obtained from a recording station of the county authority (Diputacion de Vizcaya) situated upstream the upper tidal limit. Figure 2.7 plots monthly average values from January 1988 (a year regarded as typical for the area) to February 1991; unfortunately, no record is available after this last month due to computer malfunctioning in the monitoring station. However, it can be noted that water restrictions were lifted in Bilbao in March 1991 as a result of abundant rainfall in the area which continued during the spring of 1991.

A cause-effect relationship between data in figure 2.7 and those in figure 2.6 is cautiously suggested: after two years of a river flow substantially reduced (and uninterrupted contaminating inputs to the system) the levels of heavy metals in sediments and clams reached an impressive maximum which otherwise is not usual for the area. After resumption of the normal rainfall, increased water flow in the estuary washed the

excess metal accumulated in surficial sediments rather quickly, and allowed clam detoxification which, however, proceeded at a slower pace. It is important to note, though, that the granulometry of sediments may have varied with time and also between sites and could explain some of the variance observed. Figure 2.8 plots metal levels at three sites along the Bilbao estuary (see figure 2.1 for location) on about the end of 1991, and it shows that sediment load increases markedly in a downstream direction. This may be an indication of the normal river flow resumed in early spring flushing down the sediment-associated metal out of the estuary; however, lack of samples prior to February 1992 prevents conclusions on whether data for Sestao and Arriluce plotted in figure 2.8.a are typical for these sites as a consequence of their very low situation in the intertidal level. Since all *S. plana* populations known to exist within Bilbao estuary (those of Sestao and Arriluce only discovered in February 1992) showed very similar soft-parts metal levels at the turn of the year 1991 (figure 2.8.b), it is possible that these are actually the values characteristic for the area; clams in the two more downstream sites had not accumulated more metals because higher levels in sediments (figure 2.8.a) would only be a short-term situation product of the transient sediment flush-out.

Some bivalve spat were sieved out of the surficial sediment at Lamiaco in January 1992; they were identified as juveniles of *S. plana* (12 specimens from 2.5 mm to 7.3 mm in length, mean 3.6 mm) and of *Cerastoderma sp.* (1 individual 2.6 mm long). Since no adult cockle has been found in Lamiaco, it is thought that spat of *Cerastoderma* are imported to this site from elsewhere but die out without completing their biological cycle; the origin cockle population is likely to be that of *Cerastoderma edule* (L.) in Arriluce showing a considerable degree of Ni contamination (see Table 2.4), for which metal the species is a good indicator (Bryan *et al.*, 1985). As for the spat of *S. plana*, because no population of the clam is established in the outer part of Bilbao Sound (Ruiz, 1986), they constitute evidence that the populations within the inner reaches of Bilbao estuary are able to produce recruits and survive despite severe heavy metal pollution.

Finally, results of the successful *S. plana* transplants from Guernica-2 to Santoña and Lamiaco are plotted in figure 2.9. Some other clams were transplanted in boxes -at a density well below the 400 individuals/m^2 reported as safe by Hughes (1970)- because it

was feared that unrestricted animals taken to polluted sites may move and, thus, be lost; unfortunately, none of the animals transplanted in boxes survived 5 months in Asua-1 or Lamiaco (14 individuals per duplicated box to each site), and only 4 out of 36 implanted in 2 boxes in G-2 were recovered alive in May 1991. However, sufficient clams transplanted free to Santoña in January 1991 were recovered alive the following May; their heavy metal content -notably Ag- was then higher than that of Santoña natives, but lower than levels of G-2 natives (figure 2.9.a, G-2 natives = mean of G-2 collected for the first time and G-2 clams implanted free in January 1991, see Table 2.3). On the contrary, not one of 63 specimens taken from Lamiaco to G-2 in July was found in December while, somehow surprisingly, 16 out of 25 clams transplanted on 13.5.91 from G-2 were recovered alive on 7.12.91 (2 were killed during the recovery process) after having been in Lamiaco for nearly 7 months. These G-2 survivors in Lamiaco altered the composition of their metallic burden, taking up considerable Cd, Pb and Zn but eliminating most of their Ag load (figure 2.9.b, G-2 natives = mean of G-2 collected for the first time and G-2 clams implanted free in January 1991, see Table 2.4); however, transplants probably did not reach an equilibrium with respect to all the metals in the new environment since more than a year is need in some instances such as Cd and Pb (Bryan *et al.*, 1985). Absence of siphon marks on the sediment around the burrow entrances of both native and transplanted clams indicates that *S. plana* in Lamiaco do not deposit-feed to avoid ingesting metallic sediment; this was also suggested by Bryan and Gibbs (1983) for clams transplanted to a polluted site within Restronguet Creek. In addition, it is possible that transplants underwent other physiological changes to survive in Lamiaco (see Worrall and Widdows, 1983).

2.4. Conclusions.

The intertidal sediments and infauna within Bilbao estuary are considerably affected by heavy metal pollution, notably those along the Asua river. Of the three estuaries studied (i.e. Santoña, Guernica and Bilbao), metal levels are far higher in Bilbao samples with the exception of Ag, Cr, Mn and Ni in Guernica; this also applies to butyltins which, however, do not reach concentrations of concern in *S. plana*. Pollution

was severest at the climax of a 2-year drought (i.e. January 1991) which resulted in unprecedented metal levels in the clam (up to 100 ppm Cd); it is unknown what detoxifying mechanisms are employed by *S. plana* to tolerate pollutants concentrations, but elimination of heavy metals accumulated in tissues once the normal river flow was restored proceeded more slowly than clean-up of sediments. At the turn of the year 1991, some 9 months after the drought was over, the three different *S. plana* populations found within Bilbao showed very similar levels of heavy metals (for instance ≈ 28 ppm Cd); these are thought to be the normal concentrations for the estuary. Despite heavy metal pollution, the environmental conditions in Bilbao are not acutely toxic to non-native clams, and the local populations of *S. plana* are able to breed.

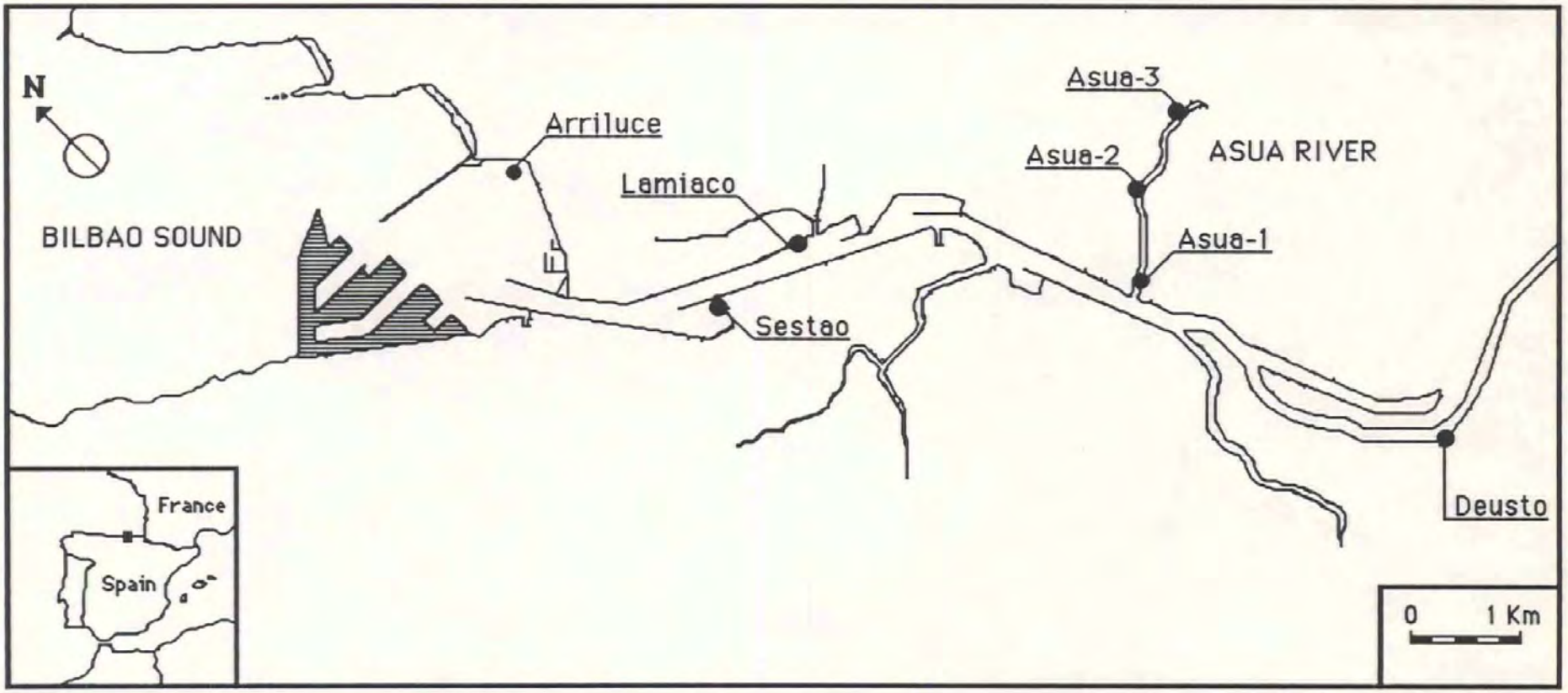


Figure 2.1.a: Sampling sites (●) in Bilbao Estuary.

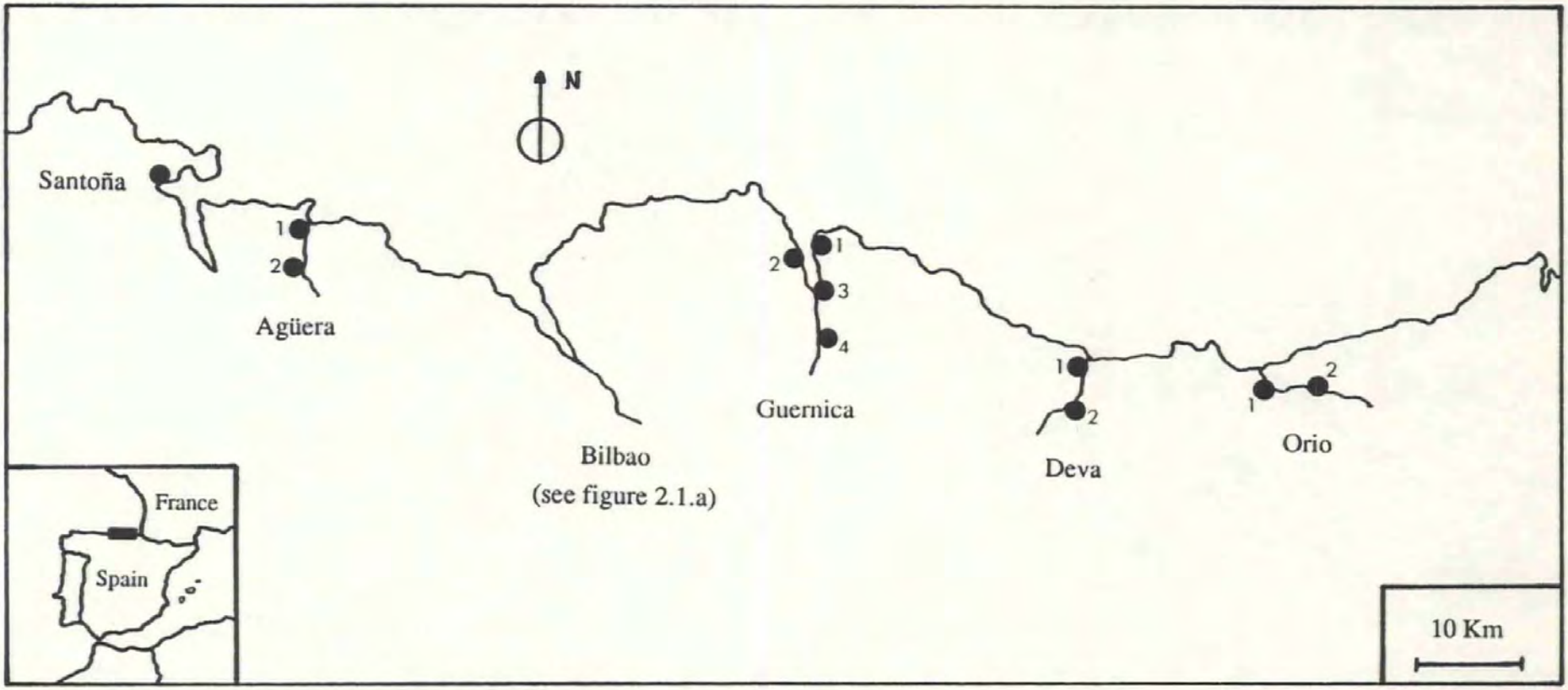


Figure 2.1.b: Sampling sites (●) in Cantabrian Estuaries.

Table 2.1: Total (nitric extracted) and bioavailable (1N hydrochloric extracted) concentrations (µg/g dry wt, Fe %) of heavy metals in surficial sediments collected in Cantabrian estuaries in (a) December 1989 (%Org.: percentage of organic matter), and (b) May 1990 (including organotins - µgSn/g dry wt -).

(a) December of 1989

Bioavailable

	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn	Latitude	Longitude
Agüera-1	0,05	1,99	5	15	13	1,15	142	8	94	301	43° 23' 36"	03° 19' 07"
Agüera-2	0,02	2,20	3	2	10	0,69	69	26	49	130	43° 22' 21"	03° 18' 58"
Lamiaco	0,01	4,15	7	35	0,50	1,27	149	27	87	278	see figure 2.1	
Asua-1	0,02	50	55	77	0,30	1,20	90	45	283	1555		
Deusto	0,03	2,55	9	161	0,90	2,28	325	33	108	1150		
Guernica-1	0,09	1,40	5	20	22	0,73	165	7	57	139	43° 23' 47"	02° 40' 59"
Guernica-2	0,41	0,35	5	31	31	0,91	343	27	43	117	43° 22' 14"	02° 40' 53"
Deva-1	0,12	2,29	6	129	63	1,28	215	50	99	807	43° 17' 53"	02° 21' 21"
Deva-2	0,17	3,84	<1	250	77	2,88	1716	121	100	1319	43° 16' 37"	02° 22' 24 "
Orio-1	0,06	0,60	4	17	60	0,91	114	75	65	217	43° 16' 37"	02° 07' 51"
Orio-2	0,10	1,09	7	47	73	1,73	223	44	88	386	43° 16' 55"	02° 05' 36"

Total

	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn	%Org.
Agüera-1	0,09	1,70	21	13	18	2,39	171	50	70	288	3.64
Agüera-2	0,09	6,16	8	11	24	2,72	189	19	80	268	4.35
Lamiaco	5,92	14	16	139	600	2,67	235	52	557	586	5.7
Asua-1	36	231	58	463	1584	6,46	502	232	2018	7837	11,32
Deusto	3,51	4,18	16	195	161	3,97	392	73	116	1326	8.79
Guernica-1	0,48	0,39	7	52	45	2,74	267	23	58	183	4.41
Guernica-2	0,35	0,44	9	79	52	2,91	456	29	47	165	5.08
Deva-1	0,89	2,14	9	158	146	3,68	307	81	95	784	4.79
Deva-2	5,58	5,77	8	336	350	6,07	2188	180	107	1871	8.68
Orio-1	0,64	0,96	10	64	166	3,71	290	40	89	462	6.41
Orio-2	0,85	1,09	12	83	116	3,52	309	42	91	477	7.36

(b) May of 1990

Total

	Cd	Cu	Pb	Zn	TBT	DBT	Total	%TBT
Lamiaco	38	1032	700	3653	0,02	0,03	0.05	41
Asua 1	87	879	1341	3473	0,10	0,23	0.33	29
Asua 2	138	1564	1427	4827	0,07	0,19	0.26	28
Asua 3	215	5934	3752	13642	0,04	0,41	0.45	8

Table 2.2: Concentrations of heavy metals (µg/g dry wt, Fe %) and organotins (TBT and DBT: µgSn/g dry wt) in sediment and faunal (replicates a, b, c) samples collected in Cantabrian estuaries in January 1991. %Org.: percentage of organic matter. n.d.: not detectable. Clams: *Scrobicularia plana*. Worms: *Nereis diversicolor*.

Surface sediments

	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn	%Org.
Bioavailable											
Santoña	0.35	2.59	3	6	8	1.49	53	5	41	367	6.57
Guernica-2	1.76	1.15	24	40	34	2.54	137	21	30	251	5.71
Guernica-3	5.03	2.41	3	59	47	2.38	155	22	26	170	6.1
Guernica-4	8.78	4.77	19	57	57	2.7	427	30	22	220	6.92
Lamiaco	4.96	108	24	99	1862	9.19	574	30	1525	5917	8.37
Asua-1	3.89	33	13	95	579	2.08	213	43	877	2063	8.45

											TBT	DBT
Total												
Santoña	0.38	1.54	5	23	17	2.85	142	19	57	336	n.d.	n.d.
Guernica-2	1.52	1.56	8	90	52	2.84	249	30	40	150		
Guernica-3	5.81	2.2	8	133	80	3.31	287	43	36	155	n.d.	n.d.
Guernica-4	14.53	5.55	13	143	105	3.81	509	55	30	188		
Lamiaco	5.07	112	50	157	1785	7.37	409	55	1112	5261	n.d.	0.11
Asua-1	7.59	70	21	376	1322	3.04	358	146	1680	3520		

Clams

	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn	TBT	DBT
Santoña a	0.3	1.1	6	2	33	0.12	31	5	28	659	0.05	n.d.
Santoña b	0.2	1.0	5	2	16	0.15	23	5	27	725		
Santoña c	0.1	1.6	9	3	16	0.13	26	7	35	829		
Guernica-2 a	7.8	2.9	9	8	80	0.15	36	9	19	1080		
Guernica-2 b	5.5	2.1	6	6	44	0.11	29	7	12	864		
Guernica-2 c	6.1	2.7	8	7	65	0.13	29	9	19	913		
Guernica-3 a	22	5.8	9	18	31	0.17	20	10	15	1138	0.09	0.13
Guernica-3 b	17	4.4	8	15	26	0.17	16	10	15	991		
Guernica-3 c	24	4.8	7	16	32	0.20	20	10	14	937		
Lamiaco a	1.6	97	28	4	162	0.21	9	3	192	4278	0.5	0.2
Lamiaco b	1.4	97	29	4	158	0.23	11	3	197	4069		
Lamiaco c	1.5	108	32	4	164	0.27	9	4	205	4072		

Worms

	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Santoña a	0.6	0.4	3.2	< 0.5	11	0.04	11.2	1.3	2.2	227
Santoña b	0.5	0.2	3.3	< 0.5	11	0.04	10.4	2.3	2.0	239
Guernica-2 a	2.8	0.6	4.5	< 0.5	16	0.07	12.7	3.5	2.3	202
Guernica-2 b	2.8	0.6	4.2	< 0.5	15	0.07	10.5	3.5	1.3	198
Guernica-3 a	2.6	1.0	2.2	< 0.5	11	0.04	11.3	4.5	0.6	169
Guernica-3 b	2.9	0.8	1.9	< 0.5	12	0.04	13.5	4.5	0.7	160
Lamiaco	1.5	1.5	12.5	< 0.5	62	0.06	8.0	2.3	4.0	247
Asua-1	8.8	5.4	10.3	< 0.5	153	0.06	12.3	5.8	12.3	267

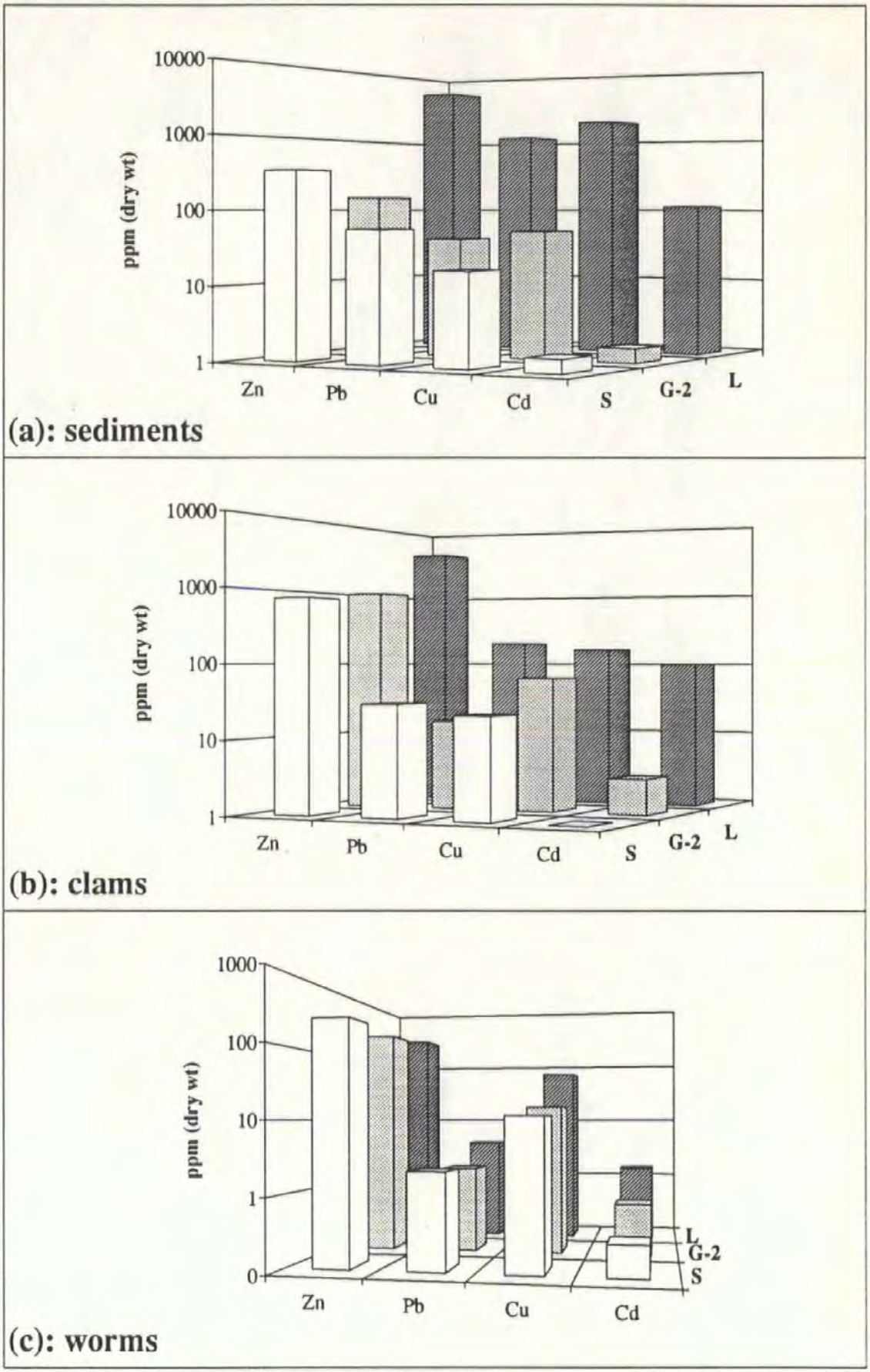


Figure 2.2: Mean levels of heavy metals in Santoña (S), Guernica-2 (G-2) and Lamiaco (L), samples collected in January 1991. Clams: *Scrobicularia plana*. Worms: *Nereis diversicolor*.

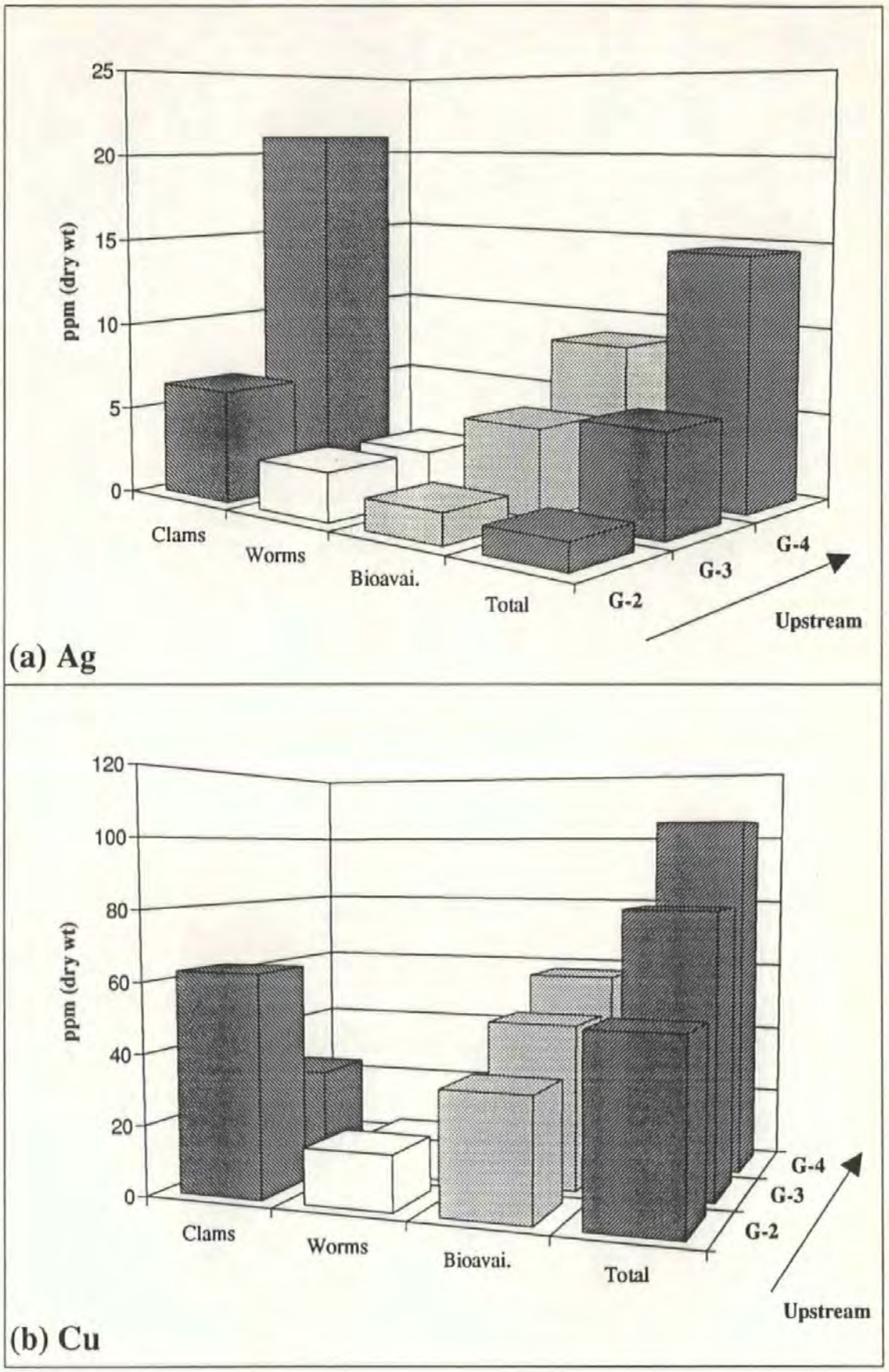


Figure 2.3: Mean levels of heavy metals in sediments (total and bioavailable) and fauna collected in sites (G-2, G-3, G-4) within the Guernica estuary in January 1991. Clams: *Scrobicularia plana*. Worms: *Nereis diversicolor*.

Table 2.3: Concentrations of heavy metals (µg/g dry wt, Fe %) in sediment and faunal (replicates a, b, c, d, e) samples collected in Cantabrian estuaries in May 1991. %Org.: percentage of organic matter. Clams: *Scrobicularia plana*.

Surface sediments

	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn	
Bioavailable											**%Org.**
Santoña	0,01	0,33	26	0,01	6	0,34	37	14	41	117	5,12
Guernica-2	0,63	0,24	8	15	25	0,49	163	9	25	60	3,96
Lamiaco	0,25	3,39	5	33	20	0,75	208	10	99	391	4,62
Asua-1	0,01	23	14	225	5	1,29	197	61	263	2491	9,46
Total											
Santoña	0,61	0,58	4	36	13	1,76	110	16	43	269	
Guernica-2	1,95	0,45	7	73	33	2,47	249	25	27	130	
Lamiaco	2,38	5,1	9	93	163	2,72	305	27	167	656	
Asua-1	14	87	25	497	1518	3,9	361	219	1568	4050	

Clams

	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Santoña	0,1	0,1	1	1	8	0,06	20	2	7	356
transplant to Sant. a	1,2	0,4	3	3	18	0,05	28	4	9	528
transplant to Sant. b	2,2	0,5	4	3	22	0,05	25	4	9	609
Guernica-2 a	5,3	1,0	7	7	46	0,11	40	8	15	819
Guernica-2 b	9,2	1,0	10	8	64	0,11	38	12	14	893
transp. free to G-2 a	5,3	1,0	6	6	42	0,10	38	8	12	649
transp. free to G-2 b	7,8	1,3	9	9	60	0,14	47	11	18	866
transp. boxed to G-2	7,8	0,9	8	7	71	0,12	32	8	18	759
Lamiaco a	1,2	51	21	5	87	0,25	18	6	145	2484
Lamiaco b	1,2	48	25	4	74	0,24	17	7	126	2335

Tissues of Lamiaco clams

	Cd	Cu	Fe	Pb	Zn
Digestive gland	458	46	0,19	1160	8014
Kidney	47	70	0,07	196	413
Mantle	7	50	0,27	35	370
Gill	3	97	0,10	1	496
Adductor muscle	15	45	0,06	137	147
Gonad	3	58	0,08	19	348
Whole	50	81	0,24	136	2410

Other fauna in Asua-1

		Cd	Cu	Fe	Pb	Zn
Ligia oceanica a		31	386		13	318
Ligia oceanica b		27	605		38	663
Ligia oceanica c	n=1	21	490		35	305
Ligia oceanica d		19	324		29	287
Ligia oceanica e		29	496		47	504
Orchestia sp. a	n=3	6	85		5	230
Orchestia sp. b		11	73		3	242
Lycastopsis sp. a	n=10	8	165	0,06	37	144
Lycastopsis sp. b		8	179	0,06	48	152

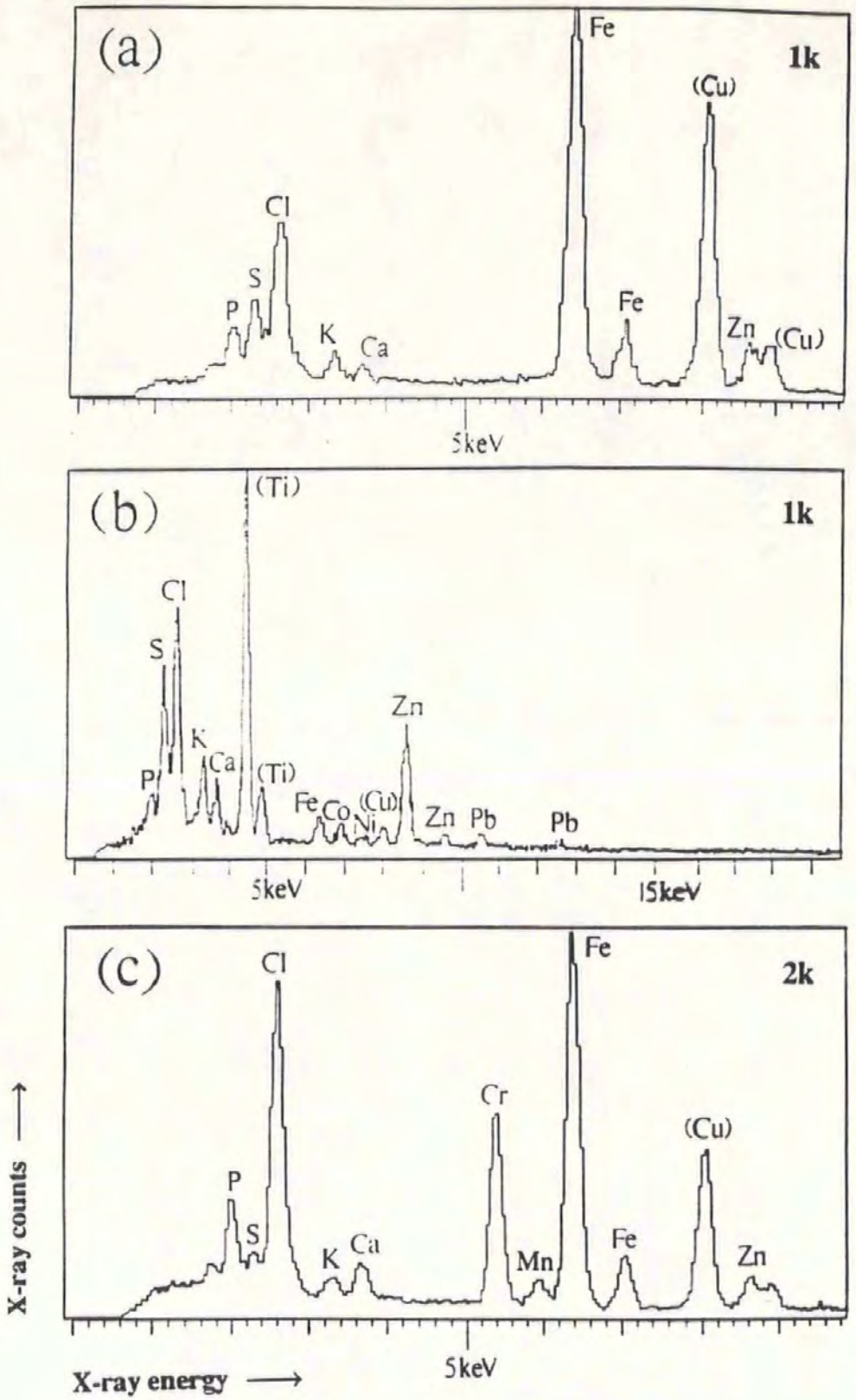


Figure 2.4: *Scrobicularia plana*, specimens from Lamiaco. X-ray microanalytical spectra from concretions in (a) digestive gland, (b) kidney and (c) gill. Elements in brackets denote contamination from the grid used.

Table 2.4: Concentrations of heavy metals (µg/g dry wt, Fe %) in sediment and faunal (replicates a, b) samples collected in Cantabrian estuaries in December 1991 (except *, February 1992). %Org.: percentage of organic matter. Clams: *Scrobicularia plana*. Cockles: *Cerastoderma edule*.

Surface sediments

	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn	
Bioavailable											**%Org.**
Santoña	0,01	0,55	7	2	9	0,63	53	4	52	180	6,65
Guernica-2	1,58	0,22	10	45	37	0,8	231	15	33	83	5,05
Guernica-3	2,6	0,28	8	53	35	0,65	195	18	19	58	4,12
Guernica-4	0,8	1,09	11	117	32	0,77	310	60	45	151	9,9
* Arriluce	1,07	21	16	135	96	3,19	335	14	531	2090	9,51
* Sestao	0,47	7,76	11	106	104	1,86	327	12	292	1008	8,39
Lamiaco	0,78	3,74	3	53	71	0,71	189	7	110	479	3,69
Total											
Santoña	0,43	0,76	5	41	15	2,54	114	17	46	315	
Guernica-2	3,58	0,49	7	91	41	2,55	279	26	30	146	
Guernica-3	15	0,48	7	106	44	2,62	294	31	22	116	
Guernica-4	51	3,93	11	230	136	2,01	400	88	44	236	
* Arriluce	5,85	26	18	203	578	5,76	464	32	595	2596	
* Sestao	4,76	8,93	11	168	274	4,86	460	30	337	1345	
Lamiaco	1,72	3,94	7	106	118	2,69	299	22	126	646	

Clams

	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Santoña	0,2	0,1	4	2	24	0,08	26	3	19	748
Guernica-2 a	13	1,2	13	10	92	0,13	43	14	19	985
Guernica-2 b	13	1,3	8	7	80	0,11	31	10	16	864
transplant to G-2	16	0,8	10	8	90	0,13	46	8	18	877
Guernica-3 a	39	1,6	4	5	52	0,15	29	7	9	553
Guernica-3 b	26	1,3	4	6	41	0,09	28	8	8	445
* Arriluce a	0,8	26	9	3	39	0,13	8	5	76	1806
* Arriluce b	0,7	25	11	4	45	0,17	10	6	68	2372
* Sestao a	1,1	22	11	5	48	0,15	10	5	82	1856
* Sestao b	2,0	32	10	6	56	0,18	10	4	114	2121
Lamiaco a	1,4	31	13	5	63	0,17	13	6	93	1829
Lamiaco b	1,4	30	11	5	74	0,14	11	5	80	1578
transplant to Lam. a	3,9	8,5	10	8	76	0,13	14	9	60	1552
transplant to Lam. b	2,8	5,6	8	6	47	0,07	9	6	37	1131

Cockles

* Arriluce a	n=16	0,1	2,2	2	2	10	0,09	17	54	6	172
* Arriluce b		0,2	2,3	3	2	11	0,10	25	63	7	225

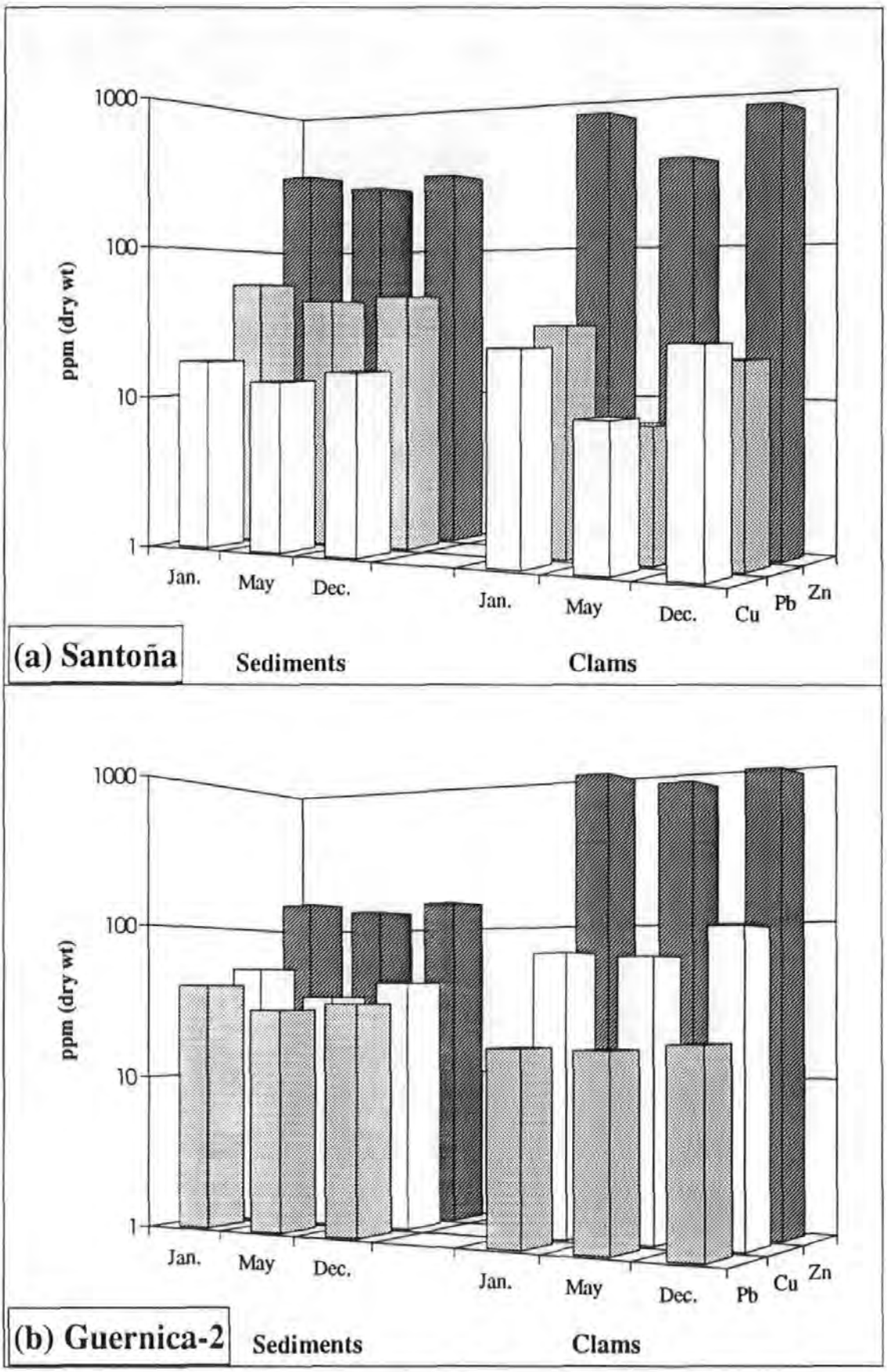


Figure 2.5: Mean levels of heavy metals in sediments and clams *Scrobicularia plana* collected in Santoña and Guernica-2 sites throughout 1991.

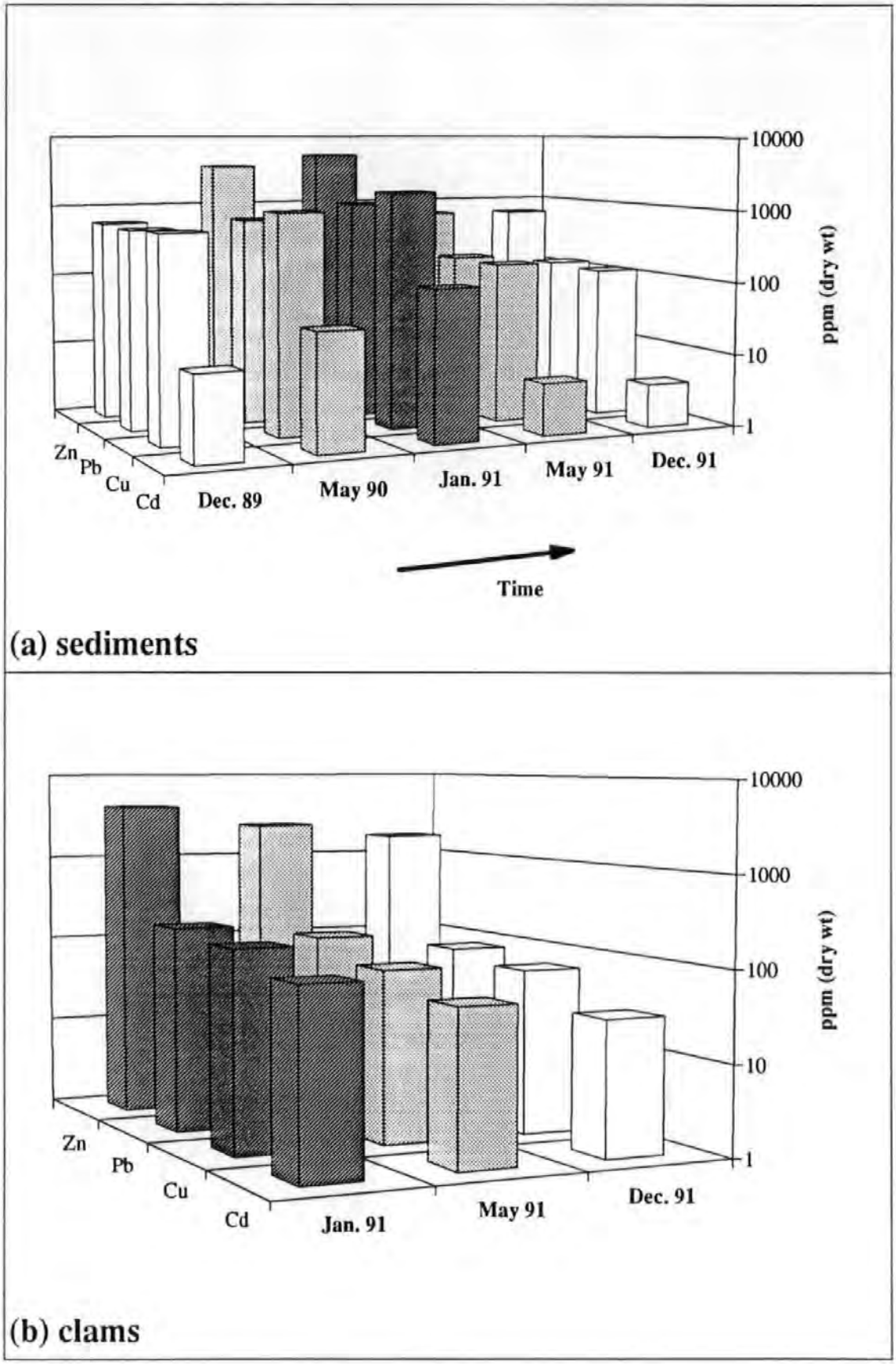


Figure 2.6: Evolution of heavy metal levels in Lamiaco site (Bilbao estuary) as monitored during one (clams) or two years (sediments). Clams: *Scrobicularia plana*.

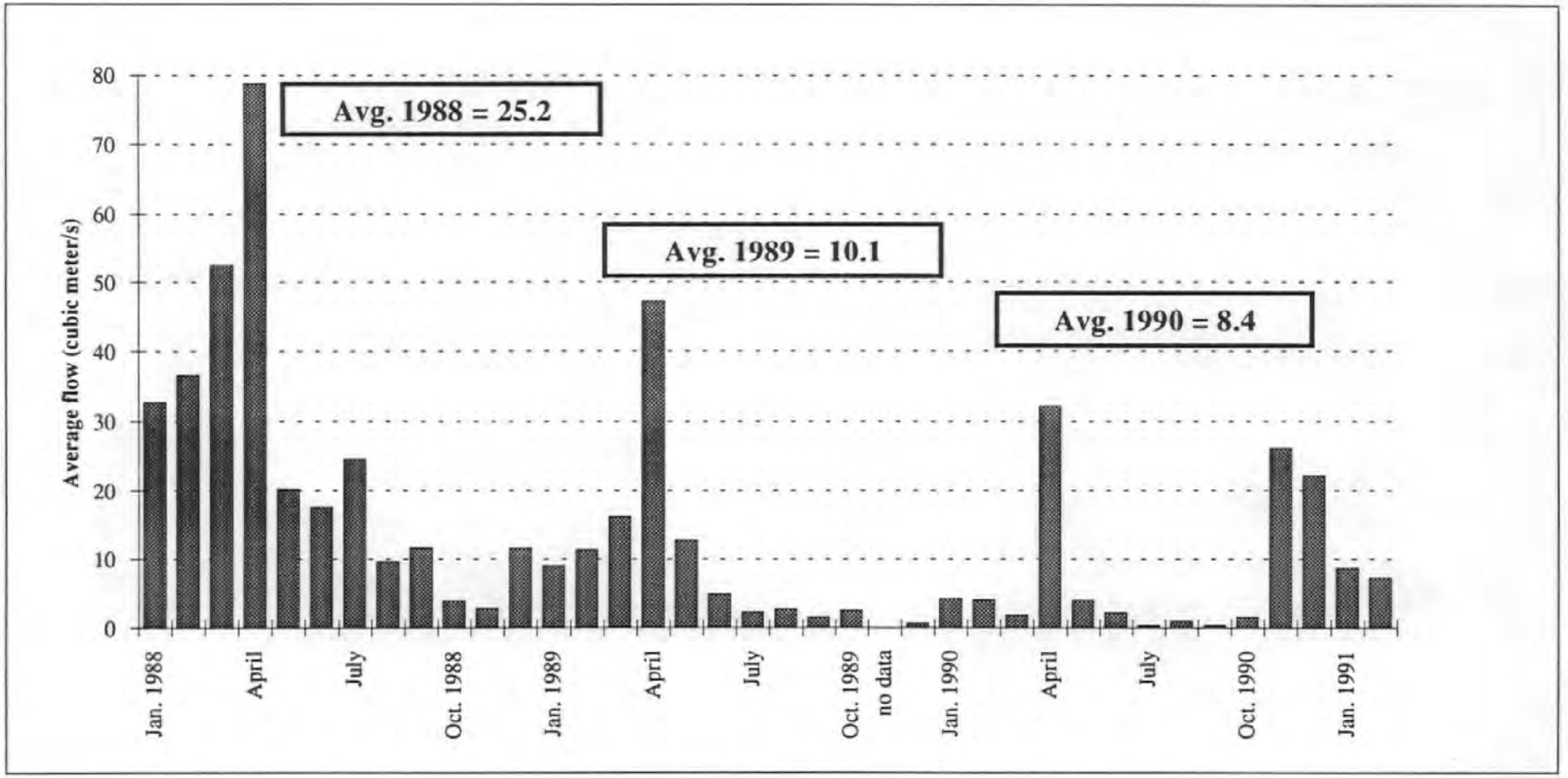


Figure 2.7: Monthly average water flow of the main tributary to Bilbao estuary (Nervion river) from January 1988 to February 1991, data from recording station of Diputacion de Vizcaya.

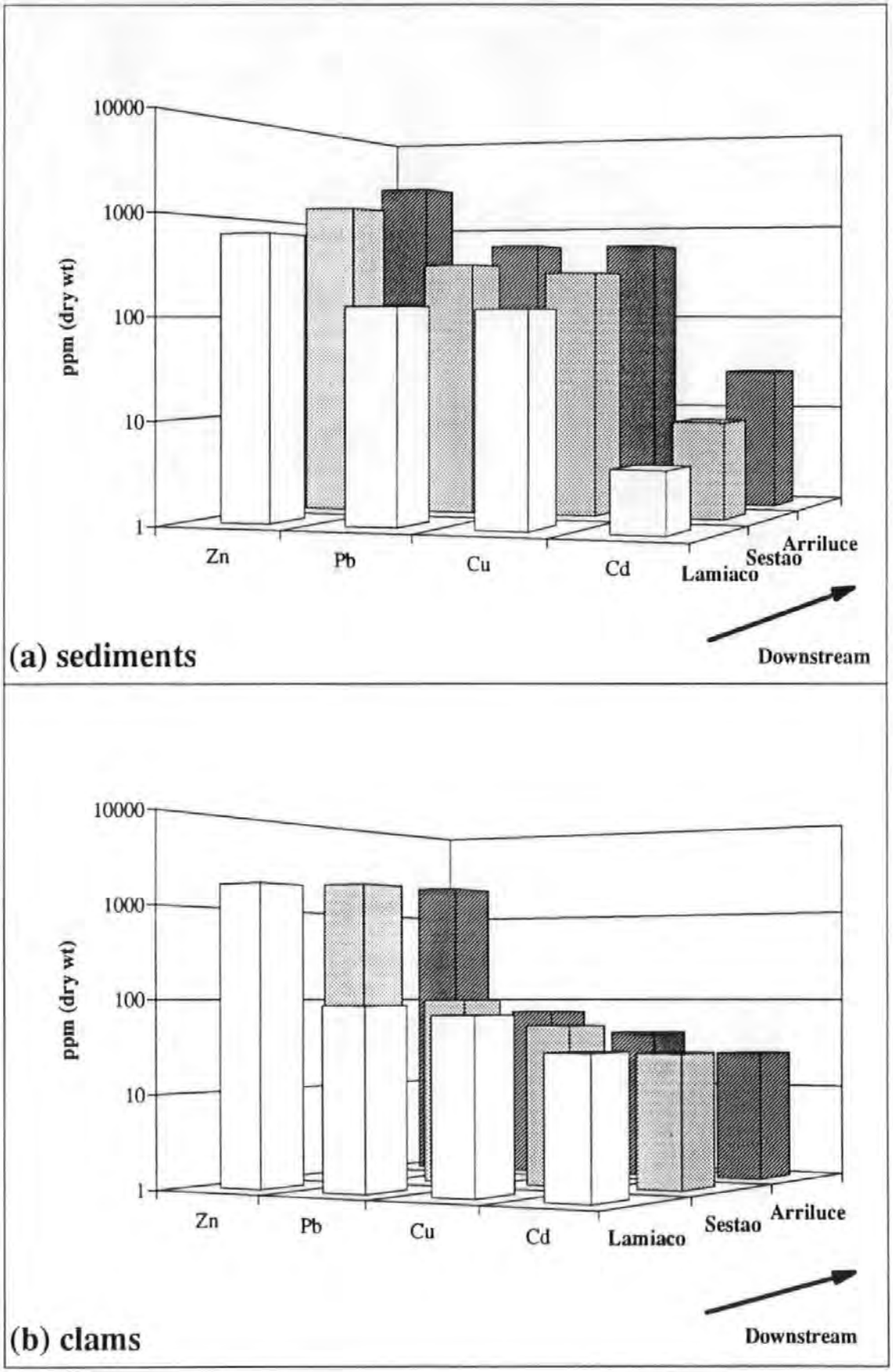


Figure 2.8: Mean levels of heavy metals in sediments and clams collected in sites within Bilbao estuary in December 1991 (Lamiaco) and February 1992 (Sestao and Arriluce). Clams: *Scrobicularia plana*.

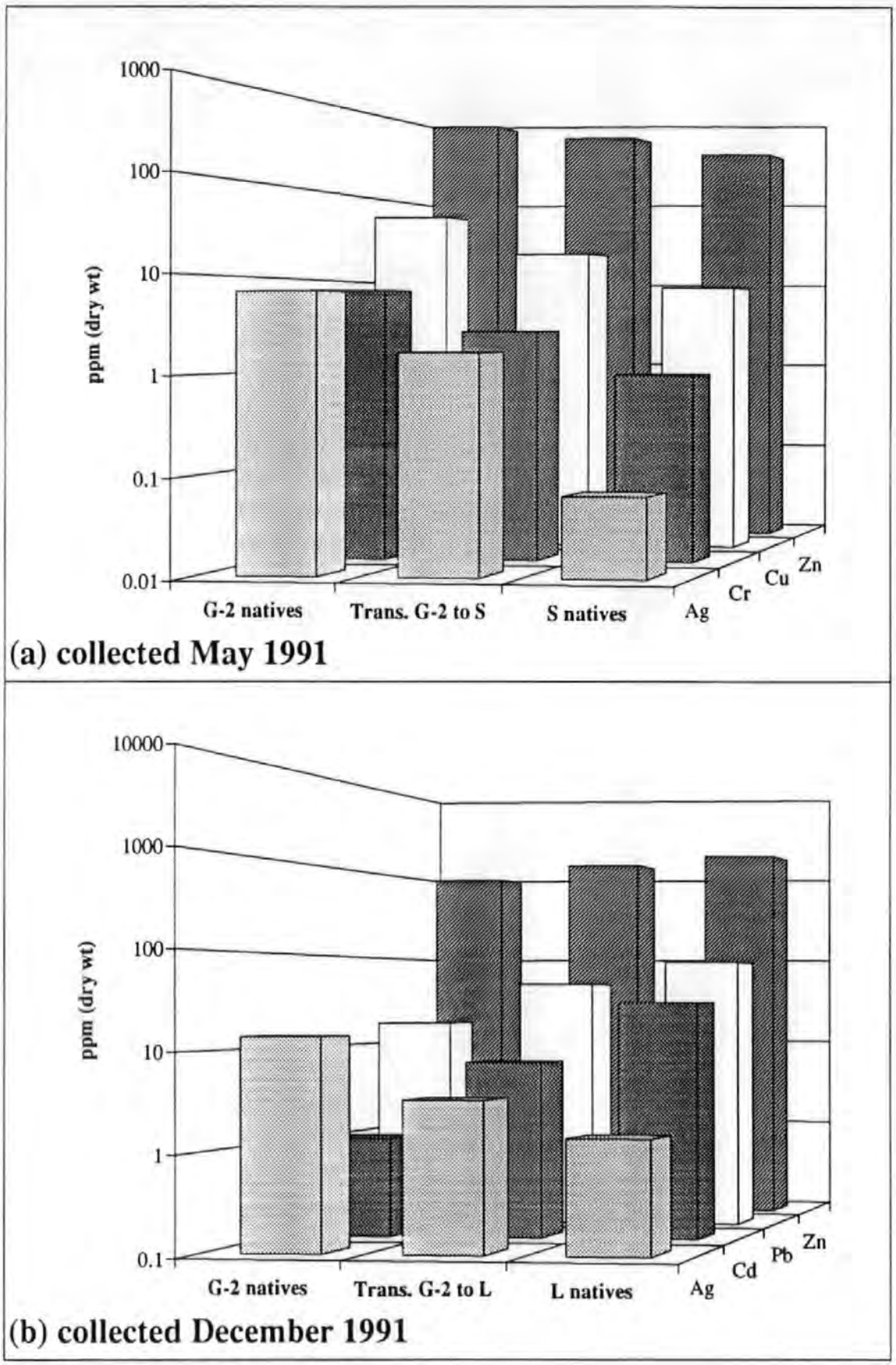


Figure 2.9: *Scrobicularia plana*. Levels of heavy metals in Santoña (S), Guernica-2 (G-2) and Lamiaco (L) natives, and concentrations of G-2 clams transplanted to S for 5 months (a) and to L for 7 months (b).

Chapter 3: Effects of field TBT exposure on the reproductive function of *Scrobicularia plana.*

3.1. Introduction.

Metallic compounds are known to have a range of toxic effects on marine life (see for instance Langston, 1990) of which short-term lethality is the most obvious; however, the relevance of acute toxicity studies to pollution impact assessment is of a lesser importance since concentrations lethal to marine biota are, with the exception of a few heavily polluted sites, unusually found in the field. On the contrary, subtle sublethal effects on more susceptible processes such as growth, reproduction and recruitment are likely to be more widespread and can, in time, equally affect populations.

The best documented reproductive anomaly induced by TBT is imposex, a unique malformation occurring in up to 40-50 species of neogastropods (Gibbs *et al.*, 1991). Other noxious effects include impairment of egg production in copepods (Johansen and Møhlenberg, 1987), reduced viability of fish embryos (Walker *et al.*, 1989), and reduced release of viable brood in mysids (Davidson *et al.*, 1986). The latter was also found in oysters *Ostrea edulis* as a result of retarded sex change during the gametogenic cycle (Thain and Waldock, 1986), the only bivalves known to have suffered some sort of reproductive dysfunction due to TBT.

The present chapter describes efforts directed to investigate possible effects of field TBT exposure on the reproduction of *Scrobicularia plana.* Since no morphological anomaly has been observed in adult clams, the most feasible approach of those available (see Dixon, 1983) was to compare the success of artificially-fertilized embryos from variously TBT-affected populations in yielding normal D-larvae after development in standard laboratory conditions.

3.2. Material and methods.

3.2.1. Collection and holding of broodstock.

Adult specimens of *Scrobicularia plana* about 40 mm long were collected at approximately mid-intertidal level on field sites in the Plym (South Devon, O.S. Grid

reference SX508555) and Torridge (North Devon, O.S. Grid reference SS453308) estuaries from June to September 1990, and on the Torridge site only from July to September 1991 (see Table 3.1). A sample of surficial sediment was taken together with clams during 1990.

Each lot of animals collected during 1990 was held in a single bowl with aerated 24 ± 2 ppt seawater (SW) at outdoor temperature while batches of them were progressively used to attempt artificial fertilizations (see Table 3.1); in such conditions animals began to die within 20 days. Although a similar holding protocol was used for animals collected during 1991, the following modifications were introduced: **i)** A technique to ascertain sex of clams before fertilization was developed: a hypodermic needle was injected through a gap between both valves at the posterior end of the animal to reach and gently puncture its gonad; sometimes a wedge needed to be inserted between valves to create and/or maintain this gap. The tissue withdrawn into the barrel of the needle was then washed down onto a slide and the resulting biopsy examined at the microscope. Material extracted in a single operation usually contained abundant gametes so that sexes could be clearly identified and kept in separate bowls. Animals failing to be sexed in this way were not used. **ii)** In an attempt to improve holding conditions and to delay deterioration of clams, bowls to contain separate sexes were filled up with a layer ≈ 10 cm thick of sediment from the native site. Sexed clams were then transferred to these bowls and allowed to bury into the sediment; most clams did so overnight and stayed buried till used for fertilizations. About 6 g of cornflour dissolved in SW were added once a week to each bowl as supplementary food.

Conditioning. During 1990 it was found that clams collected in the field produced gametes for successful fertilizations only over a few weeks in August. Thus, an attempt was made to lengthen experimental period by conditioning *S. plana* in the laboratory during spring-summer of 1991. About 200 clams and sand were collected in May from both Plym and Torridge sites and maintained in 4 plastic tanks (62 x 40 x 43 cm l.w.h) with aerated SW at outdoor temperature until September. A layer of Plym sand about 15 cm thick was made up in 2 tanks and 100 Plym clams deposited in each; Torridge sand and animals were similarly distributed. Almost every day the tanks were

emptied in the morning and refilled in the evening; clams found dead or moribund on the surface were removed. Some 12 g of cornflour (to give a concentration of ≈ 170 mg/l) dissolved in SW were added as food to each tank twice a week. Fine Plym surficial sediment was also added to both Plym tanks to form a layer ≈ 1.5 cm deep on top of the sand; after a couple of weeks conditions in these tanks became noticeably anoxic (dark colour, strong smell) and the top fine sediment was removed as far as possible. On 27 September 1991 all four tanks were dismantled; surviving clams from duplicated tanks were pooled according to origin, sexed and transferred to separate bowls until they were used to attempt fertilizations.

3.2.2. Procurement of gametes.

Ova. Preliminary experiences during 1989 had showed that ova stripped off the gonad were rarely fertilizable and produced erratic results, if any, when cultured. Efforts were therefore devoted to obtain spawned ova, but no attempt was made to induce spawning by methods usually successful with other bivalve species and known to have failed when applied to *S. plana*, e.g. temperature increments coupled with addition of algal foodstuff, electric shocks, injection of inorganic salts, addition of sperm suspensions (Hughes, 1971); and emersion for several hours or thermal stimulation (Mengus, 1978). Rather, the method of Frenkiel and Mouëza (1979) was followed and repeatedly tried.

A 0.1 M solution of ammonium hydroxide in SW was prepared from standard ammonia solution (≈ 35 % by wt, 0.88 specific gravity, Analar, BDH) and injected with a hypodermic syringe into the visceral mass of about a dozen clams of unknown sex (batches in 1990) or 6 females (batches in 1991); volume injected ranged from 1 to 2 ml depending on the size of each individual, and animals were left dry after injection. Response of clams to injection was usually conspicuous: they gaped valves, extended siphons and sometimes ejected water containing gametes. Animals displaying such a spectacular reaction to NH_4OH were placed in individual spawning bowls with SW shortly after injection (i.e. 5-10 min), but those reacting less intensely were left in air for a period of up to 30 min. Once in water, the behaviour of clams was uneven: while some did not display any further activity for up to 3 h (maximum period of observation), most

of them released gametes (see Table 3.1). Spawning clams extended both siphons to some extent and produced a jet of either packets of eggs or milky masses of sperm through the superior (exhalant) siphon. Pressure applied to this jet was variable: some clams were observed to spin on their valve as a result of the strong recoil and some other animals just let gametes drop off the tip of the siphon. Spawning could last from a few minutes up to 3 h, being usually maintained at a relatively constant pace. Amount and quality of gametes was also variable between and within batches.

Eggs within freshly spawned packets looked compressed and somewhat polyhedral, but they tended to get detached and to round off after a few minutes in water. A sample of ova in each individual bowl was examined at the microscope and their quality assessed *de visu*. If most ova from at least one female were considered regular in shape (spherical) and size (≈ 80 µm Ø), fertilization and culture of embryos was attempted as detailed below. Table 3.1 lists the number of occasions in which this was carried out. Every animal used was eventually sacrificed, sexed and an arbitrary score corresponding to the stage of their gonadal development given to each.

Sperm. Sperm freshly spawned by males injected with NH_4OH was usually motile but it was not used for fertilizations since these were to take place 2-3 hours after injections and by that time activity of spermatozoids was greatly reduced. Instead, up to 1.7.91 freshly stripped sperm was used. Two male clams were selected and their gonads stripped off into a common bowl to obtain a thick suspension of sperm. However, fertilizations with animals collected from 24.7.91 onwards employed sperm spawned by the action of serotonin following a method modified from Gibbons & Castagna (1985); this molluscan neurotransmitter was not tested on females since it is reported to be less effective on females of several other species of bivalves (Castagna *et al.*, 1985). About 1 ml of a 2 mM solution of serotonin (5 hydroxytryptamine, creatinine sulphate complex, BDH) in SW was injected in male clams (usually 4 individuals) as described above for NH_4OH; animals were left dry for some 5 min and then placed in individual bowls with SW. Reaction to serotonin injection was much less violent than the usual to NH_4OH, and only minimal activity of the foot was occasionally observed. Spawning behaviour followed a common pattern: siphons were not protruded at all, and sperm was not

expelled in jets; rather, sperm was quietly released and accumulated adjacent to the posterior end of the valves, forming a dense and clearly defined cloud which became larger as more sperm was produced.

All freshly obtained sperm consisted of clumps of spermatozoids which broke up after a few minutes due to the activity of their flagella. Sperm to be used in fertilizations was a mixture from 2 males; its suitability was checked under the microscope and rarely had to be rejected because of its poor motility. Sperm spawned by males injected with serotonin is considered to have been the liveliest.

3.2.3. Fertilization and seeding procedure.

When possible, fertilization attempts involved ova from 2 females and sperm from 2 males (see Table 3.2). Once quality of ova had been assessed, contents of separate bowls in which selected females had spawned were sieved through a 300 µm pore mesh (to retain debris and large pieces of tissue) and pooled in a 500 ml bowl with SW. Small volumes of the freshly obtained sperm mixture were added and the gamete suspension gently agitated. After 2-3 minutes the solution was sampled and the ratio spermatozoids/egg estimated with a microscope, x 100 magnification; this was kept at about 5 spermatozoids visible around each egg to prevent polyspermia (Gruffydd and Beaumont, 1972), and the addition of more than one sperm suspension was sometimes required to achieve that proportion. After allowing some 20-30 min for fertilization to be accomplished, the gamete mixture was sieved through a 64 µm pore mesh to retain eggs and avoid detrimental effects of decomposing sperm (Loosanoff and Davis, 1963).

Retained ova were rinsed with freshly filtered (0.45 µm) 24 ± 2 ppt sea water (FSW) and resuspended in a 100 ml cylinder. The suspension was mixed by the gentle action of a perforated plastic plunger (see for instance Woelke, 1972; ASTM, 1989) and aliquots of 0.5 or 1 ml (depending on the concentration of the suspension) placed in a Sedgewick-Rafter chamber with an automatic micropipette; the number of eggs in each aliquot was counted and the density in the cylinder's suspension ascertained as the mean of three countings. Volumes estimated to contain the desired number of ova were taken with the micropipette while mixing, and seeded in containers with FSW to complete

undisturbed incubation . In the present chapter, only vessels not spiked with any chemical are considered from those involved in embryo toxicity tests (water controls, see chapter 4).

3.2.4. Culture of embryos.

Temperature. Preliminary experiences before August 1990 had showed that development of *S. plana* embryos reared at ≈ 15 °C was prolonged and asynchronous (while some larvae hatched by day 3 some other embryos were still rotating within chorion 5 days after fertilization). However, embryos in simultaneous cultures left at room temperature displayed a quicker and more even development. It was then decided that cultures originating from fertilizations performed from August 1990 on were to be kept for 2 days at 20.5 ± 1 °C in a constant temperature room (year 1990) or in a water bath fitted with thermostat-heater and circulating pump (year 1991). At that temperature, no living (i.e. rotating) embryo was observed to be enclosed within chorion 48 h after fertilization.

Density. Following recommendations of keeping density of cultures below 30 embryo/ml (see for instance Woelke, 1972), a constant density of 25 embryo/ml was chosen and maintained in rearing vessels. Occasionally, however, some concurrent cultures were run at 125 egg/ml (see Table 3.2).

Containers. Rearing vessels were Pyrex finger bowls (9 cm Ø) with 200 ml FSW in which 5000 eggs were seeded; however, for culturing large amount of ova at a density of 25 egg/ml (to obtain sufficient larvae to culture, see chapter 5) or rearing ova at higher densities, 400 ml plastic beakers were employed. Glassware used was washed in laboratory detergent (Deacon), acid (≈ 1 N HCl) and twice rinsed in distilled water before each culturing exercise.

3.2.5. Recovery and examination of larvae.

Some 48 h after fertilization contents of individual rearing vessels were sieved through a 64 µm pore mesh and with a small funnel concentrated in a 100 ml cylinder (≈ 20 cm high) with FSW. Due to the obviously reduced proportion of larvae obtained from

the amount of eggs reared, it was considered necessary to recover all of them and not just a sample as is usual when assessing the success of cultures of commercial bivalves (ASTM, 1989). Volume in the cylinder for one replicate of each culture subjected to different conditions (density and/or container, see Table 3.2) was made up to 100 ml; after gentle mixing with the perforated plunger, the top 20 ml were immediately transferred to a glass scintillation-counting vial and this labelled 'live'. Addition to this vial of 2 ml buffered (20 g *di*-sodium tetraborate -Analar, BDH- per litre) 40% formaldehyde (Analar, BDH) killed living larvae instantly and resulted in a final concentration of ≈ 3.5%; dead fully-shelled larvae were observed to sink readily in a still water column (≈ 5 cm/min). Another 8 ml 40% buffered formalin were added to the 80 ml left in the cylinder and, after waiting 5 min for the newly killed larvae to sink, the 20 ml at the bottom of the cylinder were transferred to a second vial labelled 'dead'. Volume in the cylinder for the other replicated vessel of each culture conditions was made up to 90 ml FSW; after adding 9 ml 40% buffered formalin and waiting 6 min, the bottom 20 ml were similarly transferred to a third vial labelled 'total'. All vials were kept for examination.

Examination of larvae consisted of switching, stepwise, the contents of vials to a Sedgewick-Rafter chamber and with aid of a microscope counting the numbers of larvae found; annotation was made of both fully-shelled D-larvae (ventral edge of valves reaching each other and covering all soft parts) and of larvae which had developed an abnormal or incomplete shell (i.e. some portion of soft parts was exposed). Lengths of a sample of D-larvae in each successful fertilization (see Table 3.2 for figures) were measured to the nearest 6 µm by a microscope, x 100 magnification, fitted with an eye-piece graticule calibrated against a micrometer slide.

3.2.6. Metallic content of sediments and *S. plana*.

Since twice a year is usually considered a sampling frequency sufficient to estimate the heavy metal levels in sediments and biota from a given area (Bryan *et al.*, 1985), only one sample of surficial sediment (the fraction < 100 µm) and *S. plana* of those collected at each site during summer 1990 was analysed. Techniques used for

treatment of samples followed those standardised by Bryan *et al.* (1985), and a summary of them can be found in section 2.2. However, since levels of total organotin and the proportion of TBT in relation with that of its derivative DBT are more variable in estuarine environments (see for instance Langston *et al.*, 1987), a more intense analytical program processed aliquots of almost every sample of sediment and clams collected during summer 1990.

The determination of tin as tributyl or dibutyl species in clams and sediments closely followed the method employed by Bryan *et al.* (1986). In summary, three aliquots of homogenated tissue containing the equivalent of about 0.04 g of dry tissue were placed in 30 ml stoppered boiling tubes; for sediment, however, the aliquots of the fraction < 100 µm were to be the equivalent of about 0.4 g of dry sediment. A standard of 0.2 µg of tin as TBT oxide in ethanol was added to one sample and a standard of 0.2 µg of tin as DBT dichloride to another, and left for 2 h. Aliquots (5 ml) of concentrated hydrochloric acid were added to the homogenates, which were shaken at intervals for 30 min. Following the addition of 5 ml of hexane, the tubes were placed on an automatic shaker for 15 min and then centrifuged for a few minutes: 5 ml of distilled water were added and, after swirling briefly, the tubes were recentrifuged. Finally, 1 ml of the clear hexane extract was removed and shaken with 0.5 ml of 1 N sodium hydroxide solution to separate the dibutyltin from the tributyltin fraction. Reagent blanks were put through the same procedure. Tin was measured in a Perkin-Elmer 76 B graphite furnace attached to a Perkin-Elmer 603 atomic absorption instrument, settings being those detailed in Bryan *et al.* (1986). Tin as tributyltin was determined in the NaOH-treated extracts: concentrations of tin as dibutyltin were calculated following subtraction of the tributyltin data from that for the untreated hexane extracts. Detection limits were of the order of 0.02 µgSn/g for tissues and 0.005 µgSn/g for sediments, on a dry weight basis.

3.3. Results.

3.3.1. Holding of broodstock.

All Plym clams held in conditioning tanks were dead by 27.9.91, and only 48 (36+12) animals survived in Torridge tanks. None of the 400 clams collected in May and

kept for 4 months was found to have developed a swollen, ripe gonad, and no fertilization and culture could be attempted using these animals.

3.3.2. Reproduction in the field.

Table 3.1 summarizes the work carried out on the reproduction of *Scrobicularia plana* during the summers of 1990 and 1991. Greater effort was made during 1990, which included work on both populations in question and resulted in handling of more than 500 clams; the year 1991 was devoted to the Torridge population since embryo toxicity tests were wanted to be performed on Torridge material to be compared with those carried out on Plym embryos during 1990 (see chapter 4). According to observations on sacrificed animals, gonadal development in the Plym population is progressive from June and peaks in August. In the Torridge population it is earlier and more asynchronous, and by early August a considerable proportion of the population is already spent; these spent individuals may account for the sex ratio deviating from unity in the population in 1990. Earlier natural spawning in the Torridge could be related to the different texture of the sediment at each site: in the Plym it is muddy throughout, but in the Torridge it is more sandy in places and would therefore permit a quicker warming of the subsurface layers.

3.3.3. Reproduction in the laboratory.

Overall, ≈ 50% of animals injected with either NH_4OH or serotonin released some gametes (see Table 3.1). A greater proportion of female response to spawning stimulus was usually achieved with animals collected during August 1990 in the Plym site and during July-early August 1991 in the Torridge site. In view of this and, more importantly, the proportion of successful fertilizations in relation to those attempted, it is considered that animals collected in the Plym on 7-8.8.90 and in the Torridge on 6.8.90 and on 3.8.91 were the only individuals in a mature condition; however, the number and quality of eggs spawned (see Table 3.2) was variable.

After 48 h at 20.5 ± 1 °C, all rearing vessels contained a majority of inert, dead material enclosed within chorion, and a small percentage of live, free-swimming larvae.

Dead material included unfertilized eggs, embryos in which cleavage had stopped at different stages and a few trochophora larvae which had reached some degree of shell formation. Live larvae consisted mostly of fully-shelled D-larvae and very few partially-shelled larvae. Larvae possessing a complete but conspicuously misshapen shell (i.e. abnormal larvae) were very rarely observed (see Loosanoff and Davis, 1963, and Le Pennec and Le Roux, 1979, for a description of several types of shell malformation); larvae having a shell either incomplete and/or abnormal were grouped as abnormal larvae. The number of the D-larvae obtained in a given culture defines its percentage of successful larval development (%SLD = (no. of D-larvae/no. of ova seeded) x 100); this parameter is of major importance since it characterises the fitness of gametes to produce potential recruits to the population. Partially-shelled larvae, dead embryos and all the other stages found in cultures do not constitute any benefit for the population recruitment, and are therefore ignored.

The %SLD of all successful fertilizations is given in Table 3.2. The results for cultures originating from the same broodstock and run at the same density are pooled together independently of the volume of the rearing vessel since no major difference seems to exist between their %SLD and the size of their D-larvae. Although no such difference is apparent either between cultures run at different densities, their results are separated for clarity. The %SLD of all fertilizations is consistently low; notwithstanding this fact, the ≈ 14% achieved with Plym animals is the maximum obtained from the rearing of *S. plana*; in this respect, the results obtained are in total agreement with the observations of Frenkiel and Mouëza (1979): "while near all the eggs have undertaken segmentation, a high percentage of embryos stopped development at the morula stage and less than 10% of eggs produce larvae: on the contrary, all the larvae hatched appear to be normal and active".

The %SLD is also variable, both between and within populations, and may even vary within lots collected at the same time on the same site. While the latter may be due somehow to the holding period spent in the laboratory (and subsequent variation of original condition), it is probably a reflection of the particular state of maturation of individual clams. The maximum %SLD achieved with Torridge cultures (≈ 10, see Table

3.2) is undefined since not all D-larvae were retrieved due to the main aim of the culture, namely further rearing of the larvae obtained (see chapter 5).

Although recovery procedure allowed ample time for the formalin-killed D-larvae to sink down the still water column in the cylinder, incompletely-shelled free larvae may not be well represented in "dead" and "total" vials because of their lighter weight (i.e. slower sinking speed). Therefore, only larvae in the "live" vials are considered to estimate the percentage of abnormality in the culture (%Abn.: (no. of abnormal larvae/no. of total larvae) x 100). This is consistently low for all the fertilizations, and even if dead and total vials were considered, the %Abn. would still be $\leq 5\%$ for all the cultures independently of origin of clams and culture conditions; however, D-larvae hatched in cultures run at the higher density (125 ova/ml) looked less active and translucent than those originating from concurrent cultures at 25 ova/ml. Embryonic aberrations of the type described by Dixon and Pollard (1985) for *Littorina "saxatilis"* were seldom observed.

Finally, all D-larvae measured (minimum 5% of those produced in each culture) fell into the category of 90 µm of length: this argues for a remarkable uniformity of the size of the recently-hatched larvae, which would only differ in the few µm allowed by the examination procedure. However, this consistent length of 90 µm disagrees markedly with that reported by Frenkiel and Mouëza (1979) of 106 µm; this would probably be due to the different temperatures at which embryos were cultured: while in the present case a constant temperature of 20.5 ± 1 °C in the rearing vessels promoted a relatively synchronous hatching by 48 h, the temperature in Frenkiel and Mouëza cultures was set at 16 °C and resulted in slower development with hatching occurring between 2½ and 4 days. Two days difference of age between larvae, particularly at these very early stages, is a significant period and can explain that 16 µm longer size of older hatch.

3.3.4. Metallic content of sediments and *S. plana*.

The heavy metals content of *S. plana* tissues and surficial sediments in August 1990 at both sites is shown in Table 3.3. Levels of Ag, Co, Cu, Mn and Ni are higher in the Torridge than in the Plym, and conversely for Cd and Zn. It can be concluded that,

globally, both sites are not severely affected by metallic pollution, and that those metals are not likely to exert any acute toxicity on the local *S. plana* populations.

Levels of organotin in clams and sediments during the 1990 reproduction season of *S. plana* are compiled in Table 3.4. Concentrations in tissues are one order of magnitude higher than those in sediments for each site, and although increasing with time, both are negligible for Torridge samples. Values in Plym clams are considerable, and also slightly stepping up from June to September, but still far from those typical of areas regarded as seriously TBT-polluted where levels found in tissues of *S. plana* are in the area of several parts per million (see for instance Langston *et al.*, 1990).

The most interesting feature observed is the variation of the proportion of TBT and DBT in clam tissues (Table 3.4 and figure 3.1). On 5.6.90 in the Plym population, when the reproduction season is just started and even some animals do not show any gonadal development (see Table 3.1), the percentage of TBT and DBT with respect to the total organotin (defined as TBT + DBT) is about 50%-50%. As time passes by and gonads increase in maturity, the proportion of TBT increases and that of DBT decreases to reach 97% and 3% on 21.8.90, respectively, when every animal in the field sample was still to spawn (see Table 3.1). By 17.9.90 (all animals spent), the percentages of both species of organotin approach those at the beginning of the season (see figure. 3.1.b). These dynamics of TBT and DBT are also clear but less marked in the Torridge population (figure. 3.1.a), probably because some animals had spawned sooner than in the Plym; however, the post-season sample in the Torridge (collected in February 1991) provides a good example of winter relative levels of TBT and DBT at a site with very limited organotin contamination, arguing for a major proportion of DBT in animals at a zero reproductive stage.

3.4. Discussion.

3.4.1. Holding of broodstock.

Holding in native sediment and addition of cornflour as described in section 3.2.1 may be useful in maintaining *Scrobicularia plana* in the laboratory for periods of about 1 month. Sexing procedure saved time and clams for fertilizations attempts; it nevertheless

seemed to relate to findings of a small proportion (up to 5%) of moribund clams in holding bowls shortly after they had been sexed. As this proportion decreased in successive runs, it is concluded that, if care is taken to insert the needle into the gonad and not other surrounding organs, this sexing technique is effective and safe.

The attempt to condition a broodstock of *S. plana* in the laboratory proved a complete failure. Since density of clams in tanks (100 per tank ≈ 400 clams/m^2) had been reported as safe (i.e. no considerable mortality occurred) for two months holding of clams by Hughes (1970), heavy mortalities in our case would not be attributed to overcrowding; what caused the maintenance conditions to be lethal is unknown, and only anoxia observed in Plym tanks for two weeks is suspected to account for some deaths. Water regime in tanks (empty for about 9 h per day) was designed to facilitate alternation of clam behaviour from filter- to deposit-feeding. Siphons often observed to be extended into overlaying water during high water and sampling surficial sediment around the entrance of their burrows during low water indeed suggest that both natural feeding strategies (Hughes, 1969) occurred in tanks. However, whatever was ingested (added cornflour and/or organic matter in sediment and water), it did not result in any obvious gonad improvement at the end of the 4 month period. It is possible, though, that better gonad condition had been achieved sometime in some clams, but it certainly did not last till the end of September. Corn starch supplemented to circulating water has been reported to increase meat weight of laboratory reared oysters (Haven, 1965); its usage during the present experiment in the conditions described in section 3.2.1 does not appear to have been successful, but more detailed investigations would be required to assess its nutritional values and adequacy for feeding *S. plana*.

Finally, holding temperature is most likely to have not helped in ripening of gonads. Although not monitored throughout the 4 months, the sediment temperatures were below the constant 21 ^{o}C needed to condition other clam species (see for instance Utting and Spencer, 1991) due to lack of appropriate facilities.

3.4.2. Reproduction of *S. plana.*

Although fertilization and culture techniques pioneered by Loosanoff and Davis (1963) made feasible bivalve reproduction in laboratory, achieving this in the case of *S. plana* has proved once again a difficult task. Since procurement of spawned ova is frequently assured by application of NH_4OH to ≈ 6 females as described by Frenkiel and Mouëza (1979), and sperm has been shown to be usable either if stripped off the gonad or spawned as a reaction to both NH_4OH and serotonin, the main factor limiting the reproduction of *S. plana* in laboratory is the quality of gametes (mainly ova). Reproductive condition of adults seems to determine 10-14% as the top proportion of successful development from embryo to D-larvae, and to which extent this is imposed by the possibly hampering action of NH_4OH is not known since no comparisons can be made either with other laboratory techniques or with the %SLD of natural fertilizations in the field.

Since gonadal development in *S. plana* is primarily determined by the sediment temperature (Hughes, 1971) and this is variable at a given site depending on particular years, it is somewhat surprising that the present observations confirm those of Frenkiel and Mouëza (1979) on a North Wales population about August being a constant period of reproduction in *S. plana.* This month is also the only one in which Plym clams achieved the complete maturation (gametogenic, meiotic and vitellogenic, see for instance Padilla and Olivares, 1986) needed for successful larval development, but in sites with heterogeneous sediment such as the Torridge patches of clams at diverse reproductive condition seem to be distributed throughout the intertidal flats and natural spawning can occur in July. As a result, only through intensive monitoring of populations can one expect some positive outcome in obtaining laboratory reared *S. plana* D-larvae unless a suitable conditioning effort, including appropriate food and temperature regimes, is undertaken.

3.4.3 Dynamics of TBT.

The total organotin content of Plym *S. plana* during the summer of 1990 (figure 3.1.b) was relatively constant and moderate as could be expected in an area lacking

important boating activity and the associated inputs due to leachates from antifouling paints; thus, in a first instance, clams would be presumed to be at that state of equilibrium between uptake and loss processes mediated by metabolism of the parent compound TBT to its degradation product DBT (and further to monobutyltin) reported by Langston and Burt (1991). However, the considerable variation of the proportions of both TBT and DBT relative to the total organotin in whole tissues argues for such a balance to be greatly disrupted by gonadal development throughout the reproduction season. The DBT pool existing on 5.6.90 was almost entirely eliminated by 21.8.90 and, assuming that all DBT is originated from TBT breakdown and that the degradation rate of DBT was kept constant, it can be concluded that no new DBT was produced and added to this pool during 11 weeks. On the contrary, %TBT during that period increased from 53% to 97%, probably as a result of the continued regular input to the clam combined, most likely, with a blockage of TBT catabolism. This stoppage seems to be somehow associated with the developing gonad, and since in post-reproduction season spent clams (17.9.90) the % of organotin species has returned to early season levels, it also seems to be cleared by spawning.

It appears that over the reproduction season the metabolism of the clam is mostly devoted to gametogenesis, and all TBT assimilated is accumulated to be later degradated when the reproductive cycle has been completed; it is suggested that, as a consequence of the lipophilic nature of TBT (see for instance Laughlin *et al.*, 1986) TBT would possibly be accumulated in the vitellus-rich gonadal follicles. Furthermore, the winter proportions of organotin species in the relatively TBT-unaffected Torridge clams (figure. 3.1.a) shows that, in time, the TBT-DBT dynamic balance can be greatly inclined to DBT; however, the levels of organotin species in *S. plana* around Southern England in various months (Langston and Burt, 1991) shows that this is not so for clams from many other estuaries where TBT contamination is more acute. For the want of more information regarding these matters, it can be concluded that reproductive stage affects the specific composition of the organotin content in some *S. plana* populations, and that this influence may depend on the level of contamination as suggested by Langston and Burt (1991) for the metabolism of the parent compound.

3.4.4 Effects of TBT.

Whatever the mechanisms involved in organotin storage and metabolism during the reproduction season of *S. plana*, it is clear that at the time of spawning Plym clams are dealing with a maximum body concentration of TBT (≈ 0.5 µg Sn/g), the most toxic of butyltin species; the site of this TBT accumulation is not known but it does not seem to be transferred onto ova since levels of total organotin in spent animals are nearly the same than those in ripe specimens. From the results in Table 3.2, these top levels of TBT in whole clam tissues does not appear to have any deleterious effect on their ova and subsequent embryonic development.

Although Loosanoff and Davis (1963) concluded that bivalve ovarian eggs are more resistant to water quality than spawned eggs and early cleaving embryos, this tolerance should have a limit and, thus, Zaroogian and Morrison (1981) found that treatment for 33 weeks of adult *Crassostrea virginica* with sea-water containing 15 µgCd/kg (sic) created a stress on gametes severe enough to effectively reduced embryonic development; similarly, Stiles *et al.* (1991) reported embryos of clams *Mercenaria mercenaria* from the most industrialized area within Long Island Sound to exhibit more irregularity in chromosome numbers and greater larval abnormality (possible indicators of long-term sublethal effects) than embryos of clams from cleaner sites. As for organotin, however, no such findings have been described for bivalves, and His and Robert (1987) reported that paints containing enough TBT to induce shell thickening and other noxious effects on adult *Crassostrea gigas* did not affect the viability of embryos and larvae of exposed oysters; they concluded that, although larval growth was somehow retarded, lack of larval recruitment in the bay of Arcachon could not be explained by a stress due to the transport of TBT by adult oysters to their gametes. Since no difference exists between the %SLD obtained from rearing ova of either Plym or Torridge clams under standard culture conditions, this seems also to be valid for the present case; however, whether it would still apply to more severely TBT-affected *S. plana* populations and to the growth and development rate of their larvae is still unknown, and only specific research can elucidate that possibility.

Table 3.1: Summary of work carried out during the years 1990 and 1991 on the reproduction of *Scrobicularia plana*, including: Site and date of sampling, and number of individuals collected (females, males and spent); percentage of females and males (%sf and %sm) spawning after injection of chemical (ammonium hydroxide except *: serotonin); number of batches on which procurement of gametes was attempted, number of occasions in which fertilization and culture of eggs (F+C) was performed and number of these occasions which rendered some success.

		Number of						Number of		
SITE	**Date**	**Ind.**	**Fem.**	**Mal.**	**Spent**	**%sf**	**%sm**	**Bat.**	**F+C**	**Suc.**
Plym	5.6.90	61	25	33	3	28	12	5	3	0
	11.7.90	74	46	28	0	63	64	6	3	0
	7.8.90	14	9	5	0	100	0	1	1	1
	8.8.90	47	20	27	0	100	100	3	3	3
	21.8.90	36	21	15	0	100	29	2	2	0
	17.9.90	24	0	0	24	-	-	0	-	-
	Total	256	121	108	27	69	48	17	12	4
Torridge	25.6.90	56	20	36	0	40	14	5	5	0
	12.7.90	95	53	41	1	65	43	7	3	0
	6.8.90	100	8	51	41	43	100	2	1	1
	20.8.90	50	0	0	50	-	-	0	-	-
	Total	301	81	128	92	57	38	14	9	1
Torridge	1.7.91	88	47	41	0	71	75*	4	3	0
	24.7.91	79	40	33	6	75	75*	4	3	0
	3.8.91	97	22	27	48	73	19*	4	4	2
	15.8.91	29	3	8	18	0	-	1	-	0
	4.9.91	27	0	0	27	-	-	0	-	-
	Total	320	112	109	99	70	43*	13	10	2

Table 3.2: Summary of successful fertilizations carried out with the gametes of *Scrobicularia plana*, including: site and date of collection of broodstock; number of individuals of each sex involved in the fertilization (females x males), and millions of ova spawned by females selected; culture conditions (density -ova/ml- and container -200 ml bowl or 400 ml beaker-); number of normal (and abnormal, in brackets) larvae found in vials (live, dead and total, see section 3.2.5. for further explanation); % of successful larval development (%SLD = ((no. of D-larvae hatched/no. of ova seeded) x100), all vessels pooled), and percentage of abnormality in live vials (%Abn.: (no. abnormal larvae/total larvae) x100); size of D-larvae (µm) as measured in n larvae which amounted to a % of total; * : larvae not preserved but further reared (see chapter 5). Shaded cultures were controls in embryo toxicity tests (see chapter 4).

Site	Plym								Torridge			
Date	7.8.90			8.8.90					6.8.90		3.8.91	
FxM	2x2			2x2			2x2	2x2	1x2		1x1	3x2
Ova	1.50			1			1.16	1.10	0.08		0.24	0.45
Density	25	25	125	25	25	125	25	25	25	25	25	25
Vessel	bowl	beaker	bowl	bowl	beaker	beaker	bowl	bowl	bowl	beaker	beak.	bowl
live	16 (0)	25 (0)	63 (1)	101 (1)	268 (3)	1321 (23)	8 (0)	43 (0)	12 (0)	21 (1)	*	28 (0)
dead	88 (1)	136 (1)	409 (2)	502 (7)	1142 (7)	5933 (45)	43 (2)	125 (1)	45 (1)	83 (1)	*	91 (1)
total	110 (2)	181 (1)	586 (4)	667 (6)	-	-	54 (3)	216 (4)	52 (0)	95 (1)	*	109 (2)
%SLD		1.85	2.12		13.40	14.51	1.05	3.84		1.03	> 10	2.28
%Abn.		0.00	1.59		1.08	1.74	0.00	0.00		3.03	*	0.00
size		90	90		90	90	90	90		90	*	90
n		55	110		130	350	10	20		30	*	50
%		10	10		5	5	10	5		10	*	22

Table 3.3: Levels of heavy metals (µg/g dry wt, Fe %) in *Scrobicularia plana* and sediments (Total: nitric extracted; Bioavailable: hydrochloric extracted) collected in August 1990 at Torridge and Plym sites, including size (mm, Avg of 6 specimens and SD in brackets) of clams and dry wt (g) of samples analysed.

	Size	Dry wt.	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Clams												
Torridge	39 (0.7)	1.1	3.5	1.2	10	3	58	0.10	130	9	29	643
Plym	40 (1.7)	2.1	0.4	4.6	4	3	24	0.08	33	3	27	1214
Sediment												
Total												
Torridge		1.9	0.5	0.8	10	21	20	2.66	535	24	32	148
Plym		1.6	2.4	2.5	3	16	84	1.13	130	11	61	180
Bioavailable												
Torridge		1	0.4	1.4	17	4	7	1.18	33	12	17	333
Plym		0.8	2.1	0.9	1	7	46	0.64	33	4	40	195

Table 3.4: Organotin (ngSn/g dry wt) in tissues of *Scrobicularia plana* and sediments collected at Torridge and Plym sites during the summer of 1990 (except *), including size (mm, Avg of 6 specimens except ¤, only 4, and SD in brackets) of clams and dry wt (g) of samples analysed: n.d.: not detectable. TBT: Tributyltin, DBT: Dibutyltin, Total: TBT + DBT.

	Size	Dry wt.	TBT	DBT	Total	%TBT
Clams						
Torridge						
25 Jun.	39 (0.8)	0.04	14	4	18	77
12 Jul.	39 (2.1)	0.03	15	6	21	71
6 Aug.	39 (0.8)	0.03	23	2	25	93
Feb. 91 *	40 (1.4)	0.04	12	36	48	26
Plym						
5 Jun.	42 (1.2)	0.04	256	224	480	53
11Jul.	41 (0.8)	0.04	417	208	625	67
8 Aug.	40 (1.9)	0.04	485	111	596	81
21 Aug.¤	42 (1.5)	0.02	526	17	543	97
17 Sep.	42 (1.5)	0.04	322	201	523	62

	Dry wt.	TBT	DBT	Total	%TBT
Sediments					
Torridge					
25 Jun.	0.55	n.d.	n.d.	n.d.	-
12 Jul.	0.52	n.d.	n.d.	n.d.	-
6 Aug.	0.41	n.d.	n.d.	n.d.	-
-	-	-	-	-	-
Plym					
5 Jun.	0.37	19	7	26	72
11Jul.	0.40	15	21	36	42
3 Aug.	0.38	4	14	18	23
21 Aug.	0.39	17	15	32	52
17 Sep.	0.34	3	8	11	29

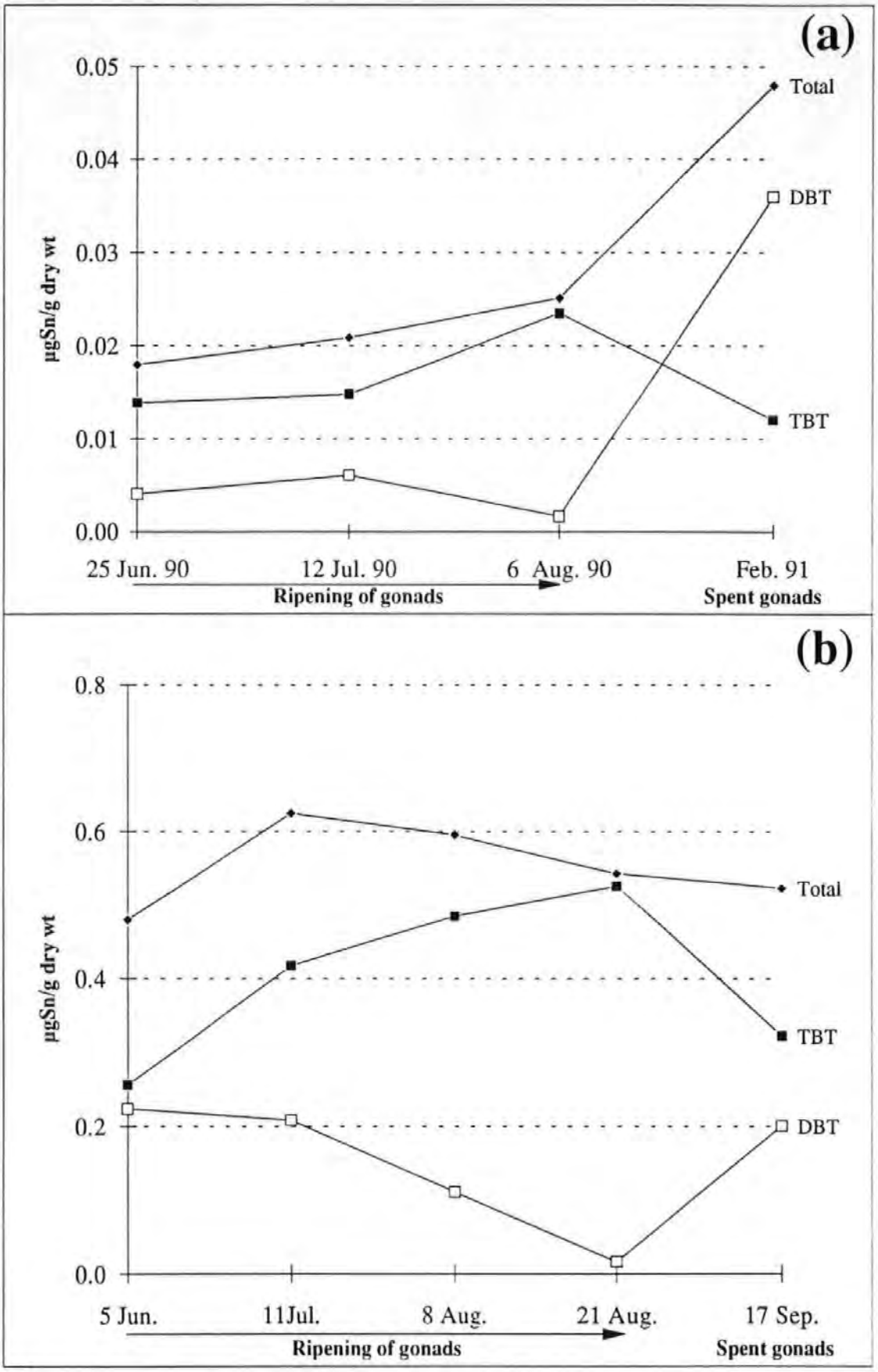


Figure 3.1: *Scrobicularia plana*. Variations of the organotin content in tissues of clams from the Torridge (a) and Plym (b) estuaries during the reproduction season of 1990. TBT: Tributyltin. DBT: Dibutyltin. Total: TBT + DBT.

Chapter 4: Effects of TBT and copper exposure on the 48 h embryonic development of *Scrobicularia plana.*

4.1. Introduction.

The early life stages of organisms are generally the most sensitive to environmental conditions of the entire life cycle. Embryos of marine bivalve species in which fertilization occurs externally are exposed to many physicochemical and biological hazards, not the least being toxic pollutants. The effects of the latter on planktonic bivalve embryos developing in the field cannot, thus far, be ascertained; however, the study of laboratory reared specimens facilitates a most useful approach to this end. After Loosanoff and Davis (1963) made available a set of relatively simple techniques to obtain and culture embryos of several bivalves, they have been widely used to assess acute and chronic effects of both natural and anthropogenic factors (see for instance Woelke, 1972; ASTM, 1989). Furthermore, they have also been recently used as bioassay organisms to determine water and sediment quality (Chapman and Morgan 1983; Williams *et al.*, 1986; Chapman *et al.*, 1987; Johnson, 1988; Long and Buchman, 1989; Thain, 1991) and, since bivalve embryo bioassays are quick, cost-effective, sensitive and of high environmental significance, they can be presumed to be of even wider application in the future.

The rate at which brood of *Scrobicularia plana* (and any other species) successfully complete their embryonic development will determine the final recruitment into the clam population. The work described herein was aimed at determining whether or not environmentally realistic concentrations of TBT and copper could diminish that rate in laboratory standard conditions devised to follow, as far as possible, those commonly used for static bivalve embryo toxicity testing.

4.2. Material and methods.

4.2.1. Standard test conditions.

Details on holding of broodstock, procurement of gametes, fertilization and seeding procedure, culture of embryos and recovery and examination of larvae have been

given in section 3.2. Conditions applied to every embryo toxicity test (3 run in August 1990 on Plym gametes, 1 run in August 1991 on Torridge material, see also Table 3.2) can be summarised as follows: 200 ml of filtered (0.45 µm) 24 ± 2 ppt sea water (FSW) were placed in Pyrex finger bowls (9 cm Ø) and 5000 recently fertilized eggs seeded into each; bowls had been soaked for several hours in detergent, acid (≈ 1 N HCl) and twice rinsed in distilled water before each test. Rearing vessels were then left undisturbed for 48 h at 20.5 ± 1 °C except for the introduction at ≈ 4 h after fertilization of a small volume of chemical solution (see 4.2.2 below) and immediate gentle agitation. After 2 days all D-larvae in duplicated treatments were recovered and transferred to vials (labelled live, dead and total) for later examination under the microscope; this investigated shell morphology and determined the length of a sample of D-larvae (minimum 5% of total) produced in every treatment.

4.2.2. Toxicant test concentrations and their analyses.

Nominal toxicant doses varied from test to test (see Table 4.1 for dates of termination of trials and levels tested); a range-finding trial included TBT concentrations from 25 to 1000 ngSn/l, and other screened effects of up to 50 µgCu/l. However, statistical analyses were only applied on the following dosages: 125, 250 and 500 ngSn/l (tested in 4 different occasions on different material) and 2.5, 5, 10 and 20 µgCu/l (only 1 test). Two stock solutions of TBT oxide dissolved in ethanol at 1 and 10 µgSn/ml ($[TBT^+] \approx 2.5\ [Sn]$) were prepared from standard TBT (bis tri-n-butyltin oxide, 97%, Ventron GMBH); the appropriate volumes of stock solution (up to 25 µl) were spiked into rearing vessels by means of a precision micropipette or a microsyringe. A stock Cu solution in distiled water at 100 µg/ml was made from standard cupric nitrate (1 ml ≈ 1 mgCu, Spectrosol, BDH), and the specific amounts of this stock (up to 100 µl) spiked into rearing solutions as for TBT. All treatments were run in duplicate and tested concurrently with water (WC) and ethanol (OC) controls; solvent was added to OC at the maximum concentration reached in TBT bowls (i.e. 125 µl EtOH/l).

Since TBT concentrations in solution are likely to be considerably depleted with time by adsorption onto the walls of the test container, it was deemed necessary to have

an estimation of the actual levels of TBT in exposure solutions freshly prepared (0 h) and at the end of the test (48 h). Thus, 48 h old solutions used in the 16.8.90 test were pooled per treatment after their contents had been retained by a 64 µm pore mesh, and 0 h solutions in FSW made up; these volumes of ≈ 400 ml were kept acidified (5 ml concentrated HCl/l) and in the dark until extraction and analyses were performed as detailed in Bryan *et al.* (1986). In summary, test samples and 24 ± 2 ppt sea water plus standard additions were extracted with 5 ml of hexane by hand-shaking for 4 min; the hexane was analysed in the same way as the tissues extracts (section 3.2.6), rendering a detection limit of < 1 ngSn/l. No analyses were done for Cu since it is unlikely that a significant amount of metal ion is removed from solution in tests lasting up to 48 h (Portmann, 1970).

4.2.3. Statistical treatment of all toxicity tests data.

As it is widely recommended in toxicology (see for instance Gad and Weil, 1988), procedures of analysis of variance (ANOVA) have been used in this thesis for the comparison of experimental data. When applying these techniques it is mandatory to check beforehand for the variances to be homogeneous, and the Cochran's test would seem to be the most appropriate for routine use to this end (Underwood, 1981). Cochran's test statistic (C) is the largest variance divided by the sum of the variances of all treatments, and the resulting C is to be compared with the critical value at the selected level of significance (either $\alpha = 0.05$ or $\alpha = 0.01$). The parameters of the sampling distribution of this statistic are K, the number of treatments, and n-1, the degrees of freedom for each of the variances; tables of the 95th and 99th percentile points of the distribution of the C statistic are given in Winer (1971, Table C.8). When reporting the result of a Cochran's test, the following notation will be used: $C.P(K,n\text{-}1)$, where P is the percentile point and K and n-1 are the parameters described above.

The Student-Newman-Keuls test has been used as a *post hoc* method to analyze data after finding a significant result in an ANOVA; it has always been run at $\alpha = 0.05$ and for the rest of the thesis will be noted SNK.

4.3. Results.

4.3.1. Production of D-larvae as the effective end point.

After 48 h at 20.5 ± 1 °C rearing bowls contained the mixture of dead and live material described in section 3.3.3; the proportion of the components of that mixture varied between tests and also within them from treatment to treatment. The number of D-larvae and abnormal larvae obtained in every bowl is shown in Table 4.1; the term "abnormal larvae" is used in the broad sense defined in section 3.3.3 to designate non-D-larvae and includes larvae which shell was either incomplete and/or misshapen. This criterion was originally used by Woelke (1972) and has been applied many times thereafter to determine larvae which underwent embryogenesis successfully. Length (mean µm and standard deviation) of the D-larvae measured for each treatment is also given in Table 4.1.

The percentage of successful development from ova to D-larvae (%SLD, see section 3.3.3) for each fertilization with which a toxicity test was run is also given in Table 4.1; it is calculated as the mean of the duplicated water control and establishes the baseline permitting the discrimination between the failure of embryos due to natural and experimental factors from that induced by chemical regimens. Success in culturing embryos of *S. plana* in all four fertilizations (12.7%, 1.05%, 3.84% and 2.28%, respectively) is poor in comparison with that of commercial species (oysters, mussels, clams) for which culture techniques are highly advanced and standardised. Although results of tests using these bivalves are advised not to be considered valid if % of embryo mortality in controls after 48 h development is > 30-40% (ASTM, 1989), this recommendation does not seem pertinent to the present case since ≈ 14% success from ova to D-larva is the maximum ever yielded from rearing *S. plana* and more than 95% of larvae hatched in controls were correctly shelled. The causes provoking these relatively low %SLD have been discussed in the previous chapter.

The most obvious effect of TBT was to decrease the number of D-larvae hatched by means of arresting embryonic development at different stages; TBT bowls were not observed to contain any greater proportion of embryonic aberrations or larvae with a complete but malformed shell than the extremely low proportions found in controls; the

same applies for Cu bowls. Interestingly, though, Cu vessels at 5, 10 and 20 µg/l showed increasing numbers of apparently correctly-shelled D-larvae still rotating within chorion after 48 h culture; rotation of these non-free D-larvae was distinct from the uniform and continual rocking motion displayed by control and TBT D-larvae before hatching occurred at ≈ 30 h under the same standard rearing conditions. The proportion of these unhatched D-larvae turning convulsively 48 h after fertilization in 10 and 20 µgCu/l bowls was observed to be higher with respect to that of free D-larvae within the same vessel; however, at the time when vials were processed to determine numbers and size of larvae, only D-larvae looking free (i.e. not enclosed in chorion) were found. Physical abrasion during recovery procedure and/or storage in ≈ 3.5% buffered formalin must have caused disintegration of the chorions and resulted in all D-larvae in these Cu bowls looking as free as in any other vessel. No such incident was observed in the vials ensuing from other fertilizations, or from the same culture subject to different treatments. It is therefore presumed that physicochemical characteristics of recovery and storage have differentially affected these chorions because they were particularly delicate; perhaps the rotating action for several hours of fully-formed D-larvae inside eroded them to a condition close to that of natural breakage. Since no mean was available to distinguish between those unhatched and those free at 48 h post-fertilization, all D-larvae found were counted.

The percentage abnormality, as defined by the proportion of abnormal larvae in live vials (see section 3.3.3), is shown in Table 4.1 for every treatment. It is very low and consistent (< 5%) in controls of all tests, increasing with the level of either TBT or Cu applied to bowls up to a mean 85% at 500 ngSn/l. Since incompletely-formed shells will result in retarded development and likely reduced larval survival in the natural environment (ASTM, 1989), no further attention is given to the percentage of occurrence of abnormal larvae in bowls. Besides, only by using more sophisticated methods such as polarization microscopy to quantify the degree of shell calcification (Cherr *et al.*, 1990) could specific subtle criteria of bivalve larval response to toxicants be assessed.

As a means of adjusting the amount of D-larvae in treatments for the controls, the total number of D-larvae produced in every bowl was expressed as a percentage of the mean number yielded in the duplicated concurrent water control, which was necessarily set to 100%. The resulting values were pooled per treatment independently of the test from which they were obtained; this gave 8 values for WC, OC and 125, 250 and 500 ngSn/l for the 4 TBT tests. For Cu, the 23.8.90 trial is considered just a preliminary range-finding try, and only Cu values from the 29.8.90 test (2 for WC and 2.5, 5, 10 and 20 µgCu/l) are taken into account. Results are plotted in figure 4.1.

A 2-way ANOVA of the %D-larvae obtained in all 4 TBT tests (Table 4.2.a) showed that: i) there is no significant difference between the results obtained in each individual test; this allows for considering all tests as part of the same group, independently of the origin of broodstock and of the date upon which they were run. ii) TBT doses applied to bowls affected significantly the production of D-larvae; application of a SNK test showed that %D-larvae was maximum in water controls, lower in 125 ngSn/l and ethanol controls, even lower in 250 ngSn/l and minimum in 500 ngSn/l. iii) differences caused by the dose were not influenced by the characteristics of individual tests.

ANOVA and subsequent SNK test of the %D-larvae obtained in the Cu trial (Table 4.2.b) found that production of D-larvae was lower in 20 µgCu/l bowls than in any other treatment; results of lower Cu doses were not significantly different ($P > 0.05$) from each other and from water controls.

Following these results, lengths of measured D-larvae of control and toxicant bowls (figure 4.2) were pooled per treatment for statistical analyses, but values for water control were excluded because their mean length had no variance (all larvae fell into the category of 90 µm). The variances were found to be heterogeneous: C = 0.45 > C.99(4,144) for TBT (OC and 125, 250 and 500 ngSn/l) and C = 0.73 > C.99(4,144) for Cu (2.5, 5, 10 and 20 µgCu/l). Since none of the transformations performed on the length data (i.e. $\log_{10}$, arcsin) resulted in variances satisfying the Cochran's test, ANOVA was not applied. However, mean length of D-larvae produced in doses of 250 and 500

ngSn/l and 20 µgCu/l appears to be reduced with respect to that of controls and other toxicant treatments.

4.3.2. Nominal and actual TBT concentrations.

Results of analyses of initial and final experimental TBT solutions performed on 16.8.90 test material are plotted in figure 4.3. They show that actual concentrations yielded by preparation procedure were ≈ 80% of nominal, and that TBT levels after 48 h in culture conditions decreased to about 83% of initial values; TBT in control solutions was not detectable. It can then be calculated that the average concentrations the embryos were actually exposed to for the duration of the test are 82, 188 and 364 ngSn/l rather than the nominal 125, 250 and 500 ngSn/l, respectively.

4.4. Discussion.

Results of static tests demonstrate that both TBT and Cu are toxic to the embryonic development of *Scrobicularia plana*. This toxicity is acute in that the proportion of D-larvae produced is significantly decreased, and chronic in that the size of the D-larvae obtained in acutely toxic treatments seems to be reduced. Although smaller D-larvae cannot be definitely discarded as potential recruits, limited size is a handicap which would serve to prolong their pelagic life and, thus, increase their chance of loss through predation, disease and dispersion (Calabrese *et al.*, 1973).

The no-effect level (NOEL) for the parameters investigated appears to be 125 ngSn/l for TBT and 10 µg/l for Cu. D-larvae hatched in TBT treatments of 250 and 500 ngSn/l amounted to less than 50% and less than 10% of control values, respectively; concentrations of 20 µgCu/l also prevented successful development in ≈ 90% of embryos. No embryo was observed to survive 48 h exposure to either 1000 ngSn/l or 40 µgCu/l. Therefore, for the production of D-larvae, an EC50 (toxicant level that prevents half the experimental population from achieving an effective end point) of less than nominal 250 ngSn/l can be set for TBT (actual EC50 < 188 ngSn/l). No conclusions can be drawn pertaining to the fitness of D-larvae obtained in treatments of 125 ngSn/l and Cu doses of 5 and 10 µg/l; however, the observation that many of their shells were less

translucent than control shells (TBT case) or were enclosed within a chorion after 48 h culture (Cu case) may be a reflection of some sublethal action of toxicants which could result in a sub-optimal performance during their larval existence.

Toxic effects of TBT on *S. plana* embryos have also been shown to be independent of the origin of broodstock, and, therefore, differential sensitivity of embryos from variously TBT-affected clam populations cannot be implied (see also previous chapter). Investigations into the performance of embryos from heavily TBT-affected populations when subject of trials similar to those described above are considered desirable. The techniques have been shown to be feasible, but they would need to be developed further than the standard needed for conducting the present static toxicity tests. It would also be enlightening to carry out embryos tests with mixtures of TBT and other metallic compounds and/or, even more important, with actual field water and sediment elutriates. Unfortunately, however, it would seem that the relative ease of carrying out work on early stages of other bivalve species such as oysters and mussels (of which nowadays even cryopreserved embryos are commercially available) may tend to focus the interest of environmentalists on these standard species, regardless of their relevance to particular scenarios like that described in chapter 1.

The quality of the water employed in a particular toxicity test is a considerable source of variation that can interact with other physicochemical and biological factors involved and result in differences on the estimated LC50 of nearly one order of magnitude (see for instance Calabrese *et al.*, 1973, and MacInnes and Calabrese, 1978, on toxicity of Cu to the 48 h embryonic development of *Crassostrea virginica*). However, a comparison of results for different species is instructive. Thus, embryos of *S. plana* (48 h EC50 > 10 µgCu/l) would appear to be slightly more resistant to Cu than those of *C. gigas* (48 h EC50 ≈ 10 µg/l -Coglianese and Martin, 1981-; 48 h EC50 = 5.3 µg/l -Martin *et al.*, 1981-) and *Mytilus edulis* (48 h EC50 = 5.8 µg/l, Martin *et al.*, 1981). On the contrary, the values herein reported for TBT (48 h EC50 < 188 ngSn/l or ≈ 0.5 µgTBT/l as analysed in experimental solutions) would argue for *S. plana* embryonic development to be considered more sensitive than that of mussels *Mytilus galloprovincialis* (48 h LC50 > 1 µgTBT/l, Robert and His, 1981), oysters *C. gigas*

(48 h LC50 > 1 µgTBT/l, Robert and His, 1981) and *C. virginica* (48 h LC50 = 1.3 µgTBT/l, Roberts, 1987), and clams *Mercenaria mercenaria* (48 h LC50 = 1.13 µgTBT/l, Roberts, 1987). It could be speculated that, if the chorion enclosing the embryos of *S. plana* was shown to be a protection against physical and chemical factors (Frenkiel and Mouëza, 1979), it would also seem to constitute some barrier to the action of Cu but not to TBT; this observation contrasts with the work of Fent (1991) indicating that chorions in minnows had a protecting property by limiting TBT incorporation into the embryo.

4.5. Conclusions.

Results constitute evidence of deleterious effects of TBT and Cu on *S. plana* embryonic development. While the effective Cu concentration (20 µg/l) is rarely found in estuarine waters (Mance *et al.*, 1984), noxious TBT levels (around 0.5 µgTBT/l) are known to have occurred during the 1980s at several European sites (Waldock *et al.*, 1987; Quevauviller and Donard, 1990; Ritsema *et al.*, 1991); TBT ecotoxicity is aggravated by the fact that: i) organotin levels are enhanced in the water surface microlayer (Cleary and Stebbing, 1987) where embryos of invertebrates may spend some part of their existence (Hardy *et al.*, 1987), and ii) the reproductive season of the clam and consequent occurrence of embryos throughout Europe coincides with summer peaks of TBT. It is therefore concluded that the action of TBT on its own may well have reduced recruitment into some *Scrobicularia plana* populations by preventing the successful development of a significant proportion of their embryos.

Table 4.1: *Scrobicularia plana.* Number of D-larvae (and abnormal larvae, in brackets) obtained from culturing 5000 ova in static toxicity tests, including % of successful larval development in WC for each fertilization (%SLD), % of abnormal larvae in live vials (%Abn) and length (µm) of the sized D-larvae (n specimens) for each treatment. Shaded cell indicates accidental partial spillage of sample.

Origin	Controls		%SLD	TBT treatments (ngSn/l)					
Plym, 8.8.90.	WC	OC		25	50	125	250	500	1000
Test 16.8.90.			12.7						
Dup. A: live	101 (1)	88 (1)		106 (0)	83 (3)	88 (4)	56 (98)	23 (72)	0 (0)
Dup. A: dead	502 (7)	393 (4)		509 (9)	359 (10)	359 (17)	182 (117)	42 (115)	0 (0)
Dup. A: Total	603 (8)	481 (5)		615 (9)	442 (13)	447 (21)	238 (215)	65 (187)	0 (0)
Dup. B: Total	667 (6)	522 (6)		230 (3)	508 (11)	471 (19)	207 (189)	58 (213)	0 (0)
Test 23.8.90.			1.05						
Dup. A: live	8 (0)	3 (0)				9 (1)	4 (6)	1 (5)	0 (0)
Dup. A: dead	43 (2)	37 (0)				50 (4)	22 (7)	2 (8)	0 (0)
Dup. A: Total	51 (2)	40 (0)				59 (5)	26 (13)	3 (13)	0 (0)
Dup. B: Total	54 (3)	41 (1)				43 (4)	23 (8)	1 (12)	0 (0)
Test 29.8.90.			3.84						
Dup. A: live	43 (0)	24 (0)				22 (4)	22 (10)	1 (16)	
Dup. A: dead	125 (1)	147 (2)				176 (4)	64 (18)	2 (30)	
Dup. A: Total	168 (1)	171 (2)				198 (8)	86 (28)	3 (46)	
Dup. B: Total	216 (4)	210 (5)				193 (4)	80 (23)	1 (52)	
Torridge, 3.8.91									
Test 13.8.91			2.28						
Dup. A: live	28 (0)	21 (1)				11 (1)	8 (4)	1 (6)	
Dup. A: dead	91 (1)	83 (1)				78 (3)	27 (7)	4 (11)	
Dup. A: Total	119 (1)	104 (2)				89 (4)	35 (11)	5 (17)	
Dup. B: Total	109 (2)	102 (2)				103 (4)	48 (12)	9 (26)	
%Abn: Avg (SD)	0 (0)	1 (2)				9 (4)	51 (12)	85 (7)	
length: Avg (SD)	90 (0)	90 (1.7)				89 (2.4)	79 (3.7)	78 (4.3)	
measured in n	105	105				105	219	95	

	Controls		%SLD	Cu treatments (µg/l)					
Plym, 8.8.90.	WC	OC		2.5	5	10	20	40	50
Test 23.8.90.			1.05						
Dup. A: live				9 (0)		7 (0)			0 (0)
Dup. A: dead				32 (0)		33 (1)			0 (0)
Dup. A: Total				41 (0)		40 (1)			0 (0)
Dup. B: Total				39 (0)		50 (0)			0 (0)
Test 29.8.90.			3.84						
Dup. A: live				30 (0)	44 (0)	45 (0)	13 (24)	0 (0)	
Dup. A: dead	as above			163 (2)	170 (4)	162 (4)	15 (35)	0 (0)	
Dup. A: Total				193 (2)	214 (4)	207 (4)	28 (59)	0 (0)	
Dup. B: Total				216 (5)	190 (3)	211 (4)	23 (67)	0 (0)	
%Abn: Avg (SD)				0	0	0	64.8		
length: Avg (SD)				90 (1.5)	90 (1.3)	90 (1.5)	76 (4.2)		
measured in n				30	20	30	51		

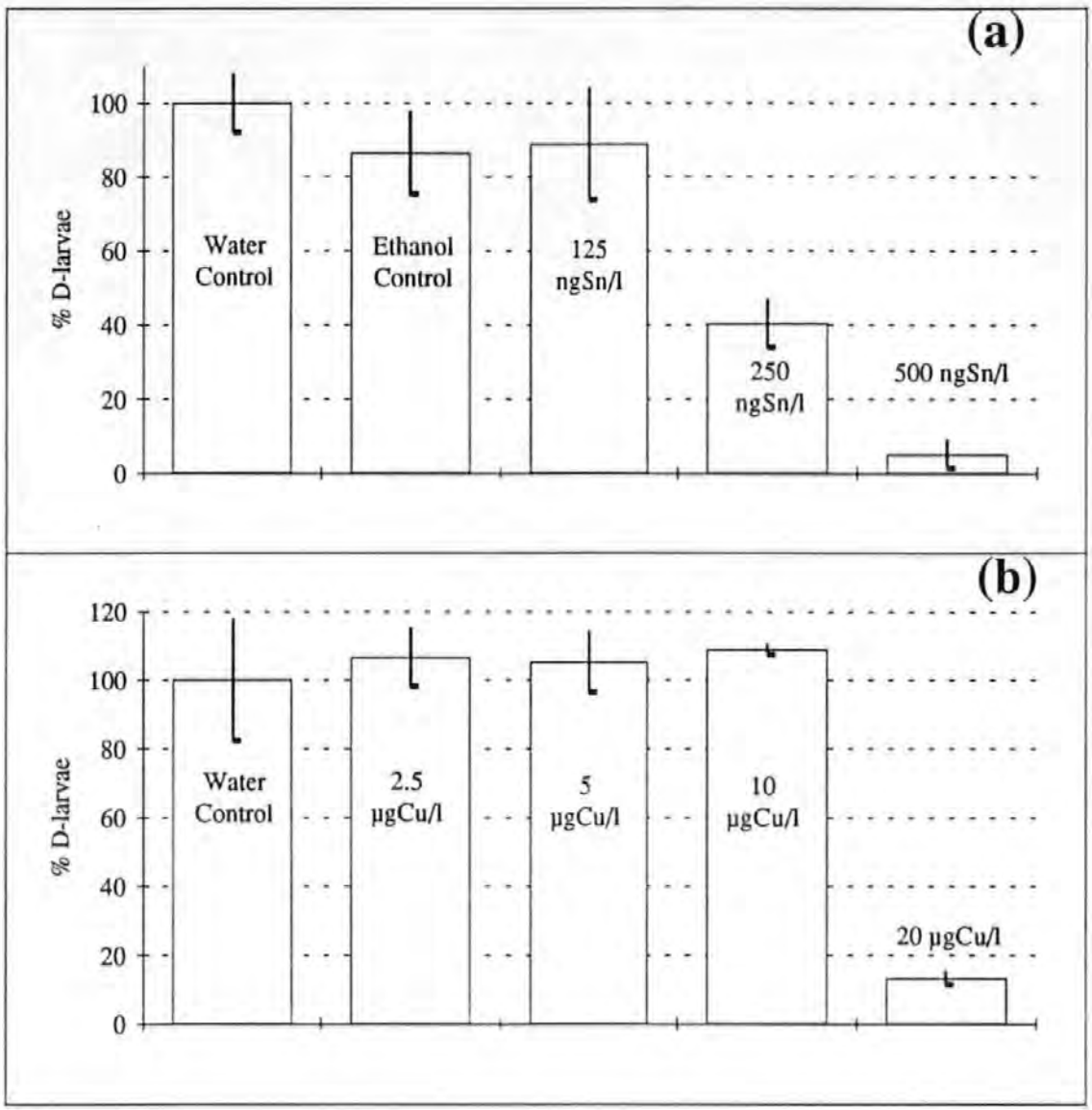


Figure 4.1: *Scrobicularia plana.* % of D-larvae (relative to water controls) obtained in controls and toxicant treatments (a: TBT; b: Cu) after 48 h embryonic development. Results for TBT and Cu are mean ± SD of duplicates in 4 and 1 test, respectively (see table 4.1).

Table 4.2: *Scrobicularia plana.* (a): 2-way ANOVA of % D-larvae produced in all 4 tests in bowls treated with TBT doses (ngSn/l) and in water (WC) and ethanol (OC) controls. (b): ANOVA of % D-larvae produced in bowls treated with Cu doses (µg/l) and in concurrent water control (WC).
The variances were homogeneous: (a) C < C.99(5,7), (b) C < C.95(5,1).

	Source	df	MS	*F*	Pr > F
(a): **TBT**	Test	3	167	2.54 NS	0.0856
	Dose	4	12879	196 ***	0.0001
	Test x Dose	12	123	1.87 NS	0.1046
	Error	20	66		
	SNK: WC > 125 = OC > 250 > 500				
(b): **Cu**	Dose	4	3396	36.3 ***	0.0007
	Error	5	94		
	SNK: 10 = 2.5 = 5 = WC > 20				

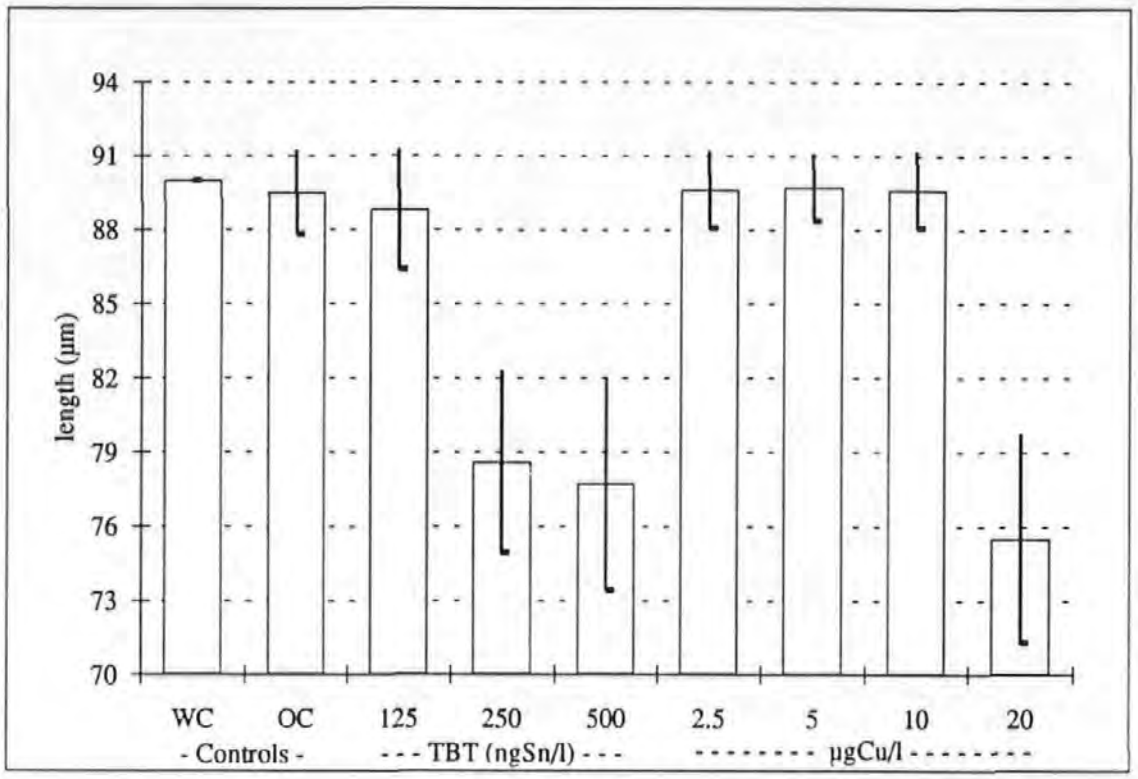


Figure 4.2: *Scrobicularia plana.* Length of D-larvae produced in control (WC, OC: water and ethanol) and toxicant treatments. Results are mean ± SD of all specimens measured (see table 4.1).

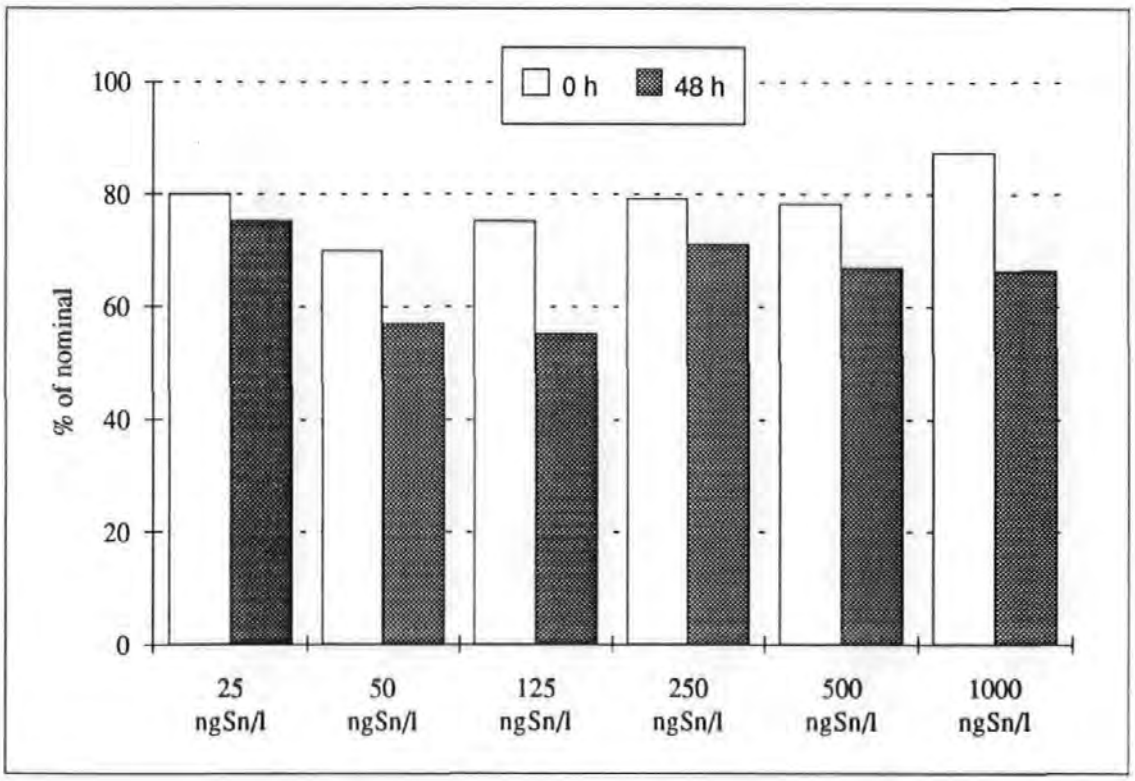


Figure 4.3: Initial (0 h) and final (48 h) tin concentrations analysed in TBT treatments (test 16.8.90) expressed as percentage of nominals. TBT in controls was not detectable.

Chapter 5: Effects of TBT exposure on the larval development of *Scrobicularia plana*.

5.1. Introduction

Larvae imported by currents from less-polluted areas have been suggested to account for the occurrence of some spat of *Scrobicularia plana* at TBT-contaminated sites where populations of the clam have virtually disappeared (Langston *et al.*, 1990). At 18 °C, recently-hatched *S. plana* larvae take some 30 days to attain the settling pediveliger stage, and several further weeks to complete metamorphosis and become juveniles (Frenkiel and Mouëza, 1979). Alike any other benthic species, it is during this pelagic larval stage that the clam, conditions permitting, is more likely to be dispersed and therefore colonize polluted areas where indigenous offspring are unable to develop.

The following experiments were aimed to test TBT effects on *S. plana* larval development. Two well-defined stages were identified: i) from hatching to pediveliger larvae (length ≈ 240 µm), individuals dwelling in a totally pelagic environment, and ii) from settlement to the completion of metamorphosis (length ≈ 900 µm), i.e. spat mostly buried into the top few mm layer of the sediment but, probably, frequently resuspended.

5.2. Materials and methods

5.2.1. Biological material.

Adult specimens of *S. plana* (≈ 4 cm long) were collected (3-8-91) from an intertidal mudflat at Appledore (O.S. Grid reference SS453308), sexed following a method modified from Gibbons and Castagna (1985) and then held in separate trays for 3 days in aerated sea water (24 ± 2 ppt) at outdoor air temperature. Methods to obtain fertile gametes and the embryos are described in sections 3.2.2 to 3.2.4, and will only be summarised below. On this occasion, 6 females and 4 males were induced to spawn but only the products of the best couple were mixed (≈ 5 spermatozoids per egg) in filtered (0.45 µm) 24 ± 2 ppt sea water (all subsequent references to FSW represent a salinity of 24 ± 2 ppt). Some 120,000 ova were seeded at ≈ 25 egg/ml in rearing beakers (400 ml) with fresh FSW and then left undisturbed for 2 days at 22.5 ± 1 °C in a water bath fitted with a thermostat-heater and a circulating pump. At 30 h post-

fertilization, many D-larvae were observed to be already swimming free in beakers and, as a result of overcrowding, bumping occasionally into each other; it was decided then to terminate the culture of embryos. The overlying water in rearing beakers was sieved through a 64 µm pore mesh, taking care not to include the bottom water containing mainly undeveloped embryos and some less advanced, unhatched larvae. The vigorous, straight-hinge larvae retained by sieve were concentrated in a 500 ml cylinder and, after gentle mixing with a perforated plastic plunger, 2 aliquots of 1 ml were taken to a Sedgewick-Rafter counting cell to assess the density of D-larvae. Mean density was estimated at 24 larva/ml so, considering that some more larvae would have hatched until completion of the 48 h usual embryonic incubation, the percentage of successful development (%SLD, see chapter 3) for this fertilization could be calculated to have been > 10%; no abnormal larvae (see section 3.3.3) were observed among those swimming freely in beakers.

Individuals for the immediate veliger experiment were removed from the cylinder (see section 5.2.2 below); the rest of them were cultured in a common bowl under the following conditions: 500 ml FSW; 22.5 ± 1 °C; ≈ 3 larva/ml; 40 cells/µl of algae *Isochrysis galbana*, Tahiti strain (T-*Iso*); 2 day static renewal. By the 10th day after hatching, larvae in this culture parallel to the veliger toxicity test were observed to alternate periods of active swimming (facilitated by their still-functional velum) with phases when they probed the bottom of the glass bowl, crawling or standing vertically on their strengthened foot. Larvae were then considered competent for settlement and ready to be used for the pediveliger experiment (see section 5.2.3 below).

5.2.2. Experimental procedure of veliger experiment.

Aliquots from the 500 ml cylinder where larvae were being held (see above) were successively taken with an automatic micropipette so that volumes estimated to total ≈ 150 recently hatched larvae were randomly inoculated into each of 61 glass cylindrical vessels (3.5 cm ∅); all glassware used in this work had been previously washed in detergent, acid (≈ 1 N HCl) and finally twice rinsed with distilled water. Volume was eventually brought to 50 ml with FSW in every experimental cylinder. Small quantities of

a dense culture of T-*Iso* were added as food to render a concentration of 40 cells/µl in vessels, and all of them but one (labelled "initial") maintained at 22.5 ± 1 °C in a water bath for the duration of the experiment. The "initial" cylinder was taken out of the bath when the other 60 vessels (10 per each of 6 duplicated treatments) were about to be spiked; its contents were then passed through a mesh (64 µm pore), washed into a vial and kept in ≈ 4% buffered formalin to estimate the lengths of veligers at the initiation of the test.

Due to an accident with the equipment, addition of toxicants had to be delayed until ≈ 50 h post-fertilization. Then, vessels were spiked by means of a microsyringe with 0.25 µl of stock TBT solutions (10, 25, 50 and 100 mgSn/l) freshly prepared in ethanol from a standard of bis TBT oxide (GMBH). This gave nominal concentrations of 50, 125, 250 and 500 ngSn/l of TBT in FSW; water (WC) and ethanol controls (OC, EtOH at the same concentration reached in TBT solutions, i.e. 5 µl/l) were tested concurrently. All treatments were run in duplicate for 10 days following a 2 day static renewal protocol. This consisted of sieving the contents of each vessel through a 64 µm pore mesh to quickly transfer them to a clean cylinder into which appropriate volumes of FSW, food and spike were introduced. To assess survival and growth every 48 h (days 2, 4, 6, 8 and 10), there were 5 replicated vessels per duplicate; every other day the entire contents of one of these 5 replicates were sieved as above and veligers concentrated in a small dish with 10 ml FSW. Careful visual observations were made at 50 x magnification with an stereo microscope, and dead or moribund larvae (i.e. showing no cilia beating) laying on the bottom of the dish counted and switched to a vial labelled "dead"; then 0.5 ml 40% formalin were added and, after 1 min allowed for the newly-killed larvae to sink, the dish searched for larvae which were counted and transferred to a vial labelled "live". A solution of buffered formalin (≈ 4%) was made in all vials and these then stored for further measurements. Veligers in duplicated "live" vials were pooled per treatment-day sample, and their shell morphology investigated at 100 x magnification with a compound microscope. Later, maximum valve length was determined in a random sample of 25 larvae at initiation of test, at days 2, 4 and 6 for every treatment, and also at days 8 and

10 for the water control; lengths were measured with an image analyser (Kontron Processing System) calibrated against a micrometer slide.

5.2.3. Pediveliger larvae experiment.

5.2.3.1. Experimental procedure.

Temperature in the water bath was reduced and maintained at 20.5 ± 1 °C during this test. All glassware involved had been previously washed (detergent and acid), twice rinsed with distilled water and then autoclaved; FSW employed on day 0, 2 and 4 was also autoclaved and allowed to cool for 3 h at 4 °C before use.

Since competent *S. plana* pediveligers showed a period of arrested growth in the absence of a proper substratum to settle in (Frenkiel and Mouëza, 1979), acid-washed sand (40-100 mesh, BDH) was ground down with a mortar and pestle to pass through a dry 100 µm pore mesh and then autoclaved; 1.3 g of this milled sand was placed into each of 12 experimental finger bowls to make a bottom layer about 3 mm thick. Because turbidity caused by the finer particles which do not readily deposit would impede clear visual observation and might stress bivalve larvae (Davis and Hidu, 1969), sand in each bowl was washed with FSW and, after 1 min, the overlying solution was discarded; this operation was performed twice. T-*Iso* at 40 cells/µl and 50 ml FSW were placed in bowls to receive the pediveligers. Only 10 days old larvae (≈ 240 µm in length) retained by a 150 µm pore mesh were used in this test; smaller specimens were reared in similar conditions but kept apart for additional information. Pediveligers were randomly shifted in groups of 10 to give 70 larvae in each of 12 temporary cylindrical vessels with FSW, keeping another set of 10 individuals in a vial with 4% buffered formalin to estimate initial length. Groups of 70 larvae held in temporary vessels were individually concentrated in a small dish to be checked, counted again and finally transferred into each of 12 experimental bowls; all handling was performed with aid of a fine Pasteur pipette.

Four nominal TBT concentrations (50, 125, 250 and 500 ngSn/l) were tested together with water (WC) and ethanol (OC) controls; all treatments were prepared as above and run in duplicate for 30 days according to a 2 day static renewal protocol.

Every other day contents of each bowl (including sand) were exhaustively searched and larvae switched with a pipette to a small dish; animals were carefully observed and dead or moribund ones (non-existent or inactive soft parts inside gaping valves) removed. Live larvae were then placed in clean bowls with the same sand and fresh experimental solutions (50 ml FSW, food and spike) made up. Dead larvae within a treatment were kept in a common vial with 4% buffered formalin; animals surviving until the last day were similarly preserved. However, post-larvae in one of the duplicates of both WC and OC controls were pooled together and with surplus specimens (see above) on day 30 to be reared longer for further observations. Twelve individuals of about 300-500 µm in length from these extra larvae were preserved in formalin independently to serve as reference models for normal shell growth assessment. The maximum valve length of 10 days old pediveligers (i.e. initial length) and of most of the post-larvae surviving to day 30 was determined with the image analyser as above.

5.2.3.2. TBT exposure concentrations and their analyses.

Analyses of exposure levels could not be done during the experiment because renewal procedure took full-time every 2nd day. Since an estimation of the actual TBT concentrations to which the larvae were exposed was desirable, TBT (50, 125, 250 and 500 ngSn/l) and ethanol control solutions were freshly made up according to the same protocol followed during test after its conclusion. Clean bowls (6 per treatment) were set with fine acid-washed sand and T-*Iso* (40 cells/µl) in 50 ml FSW; after spiking of bowls, 0 h solutions were pooled per treatment and the resulting volumes (300 ml each) were kept acidified (5 ml concentrated HCl/l) in a clean bottle and in the dark. Fresh exposure volumes (50 ml FSW + sand + T-Iso, 6 replicates per each of 5 treatments) were made again in bowls and these kept at 20.5 ± 1 °C for 48 h; then, they were similarly pooled and acidified. Extraction of solutions and TBT analyses were performed as detailed in Bryan *et al.* (1986) and summarised in section 4.2.2.

5.3. Results.

5.3.1. Veliger experiment.

5.3.1.1. Survival.

Total number of veligers (dead + live) found in experimental vessels (see Appendix 3) shows that volume aliquots supposed to have totalled ≈ 150 larvae generally contained a lower number of specimens (from 27 to 157, total mean = 77). Failure to repeat the mixing procedure while taking every aliquot dispensed is likely to explain this variability since, as opposed to inert ova, larvae swim actively and tend to disrupt their homogeneous distribution in suspension achieved momentarily by the action of the plunger; in addition, heavy mortalities occurring in some vessels (see below) resulted in empty shells and detached valves which may have been overlooked by the recovery procedure. This was performed as detailed in section 5.2.2 because results of some trials to dye only those live larvae attempted with several vital stains (Methyl Blue, Bengal Rose, Tripan Blue, Eosin Y, Brilliant Vital Red) were inconsistent.

The number of live veligers found in each rearing vessel is given in Appendix 3; data expressed as the mean percentage of survival throughout the 10 days experiment (plotted in figure 5.1) show that important mortalities occurred in every treatment. ANOVA and subsequent SNK test (Table 5.1) found that veliger survival in water controls at the end of the test (mean 70%) was higher than in any other treatment; no significant differences were displayed between mean % survival in ethanol controls and TBT doses. The small volumes of rearing solution used and the absence of antibiotics (feared to have produced secondary effects and interferences) probably created conditions appropriate for microbial infection; this was most severe in ethanol-spiked vessels (OC and TBT treatments) because organic solvents constitute an added source of organic carbon for fouling microorganisms.

5.3.1.2. Growth and development.

The valve length of all veliger larvae measured is compiled in Appendix 4, and their mean value (n = 25) for each treatment-day sample available is plotted in figure 5.2; widespread infection and subsequent mortalities in ethanol-spiked vessels prevented

examination of samples of sufficient size in days 8 and 10. Larvae surviving in ethanol controls quickly grew, from mean 100 µm on day 0 to mean 175 µm after 6 days; veligers reared in water controls experienced growth of a very similar rate, and by day 10 their valve length averaged 230 µm. On the contrary, growth rate of veligers in TBT treatments was much lower (see figure 5.2), their highest (in 50 ngSn/l) taking valve length of exposed larvae up to a mean of only 128 µm in day 6; growth rate at this dose is therefore estimated to be reduced by a factor of 2.7. Observations point out that TBT-treated veligers did not attain a valve length > 160 µm by the end of the test, nor did their shells appear to be deformed.

Length data were expressed as a percentage of the mean size of larvae at initiation of test, and then transformed ($\log_{10}$ [(length x 100) / mean initial length]) prior to statistical analyses to satisfy requirements that data be normally distributed and their variances homogeneous. ANOVA and SNK test applied to transformed data from ethanol-spiked vessels (Table 5.2.a, b and c) showed that growth in ethanol controls was greater than in any TBT dose for every day tested (days 2, 4 and 6, respectively); however, there was some significant growth in all treatments for every day considered singly. Even at the highest TBT dose (500 ngSn/l) veligers grew significantly every 2nd consecutive day (Table 5.2.d).

Development of *S. plana* veligers at experimental temperature (22.5 ± 1 °C) was some 3 times quicker than at 18 °C as reported by Frenkiel and Mouëza (1979); however, their measures of valve length at different developmental stages closely agree with those of the present study. Most surviving larvae in both control treatments of toxicity test, and also in the parallel culture, developed umbones in 4 days (valves ≈ 140 µm long); the foot first observed on day 7-8 (valves ≈ 190 µm long) was fully active and functional for crawling by day 10 (i.e. the pediveliger stage competent for settlement had been reached, valves 230-240 µm long). On the contrary, no larvae exposed to any TBT dose was observed to have developed a foot, but some reared at 50 ngSn/l showed reasonably clear-cut umbones at the end of the test. A few individuals surviving exposure to 125 ngSn/l seemed to have started developing their beak, but failed to form it to any

substantial extent. Finally, every larva in doses of 250 and 500 ngSn/l could be said to be straight-hinged still 10 days after hatching.

5.3.2. Pediveliger experiment.

5.3.2.1 Survival.

Recovery and handling of pediveligers for examination and renewal of exposure solutions every 2nd day proved a laborious and delicate matter due to: **i)** such small larvae had to be searched for all over the bowl, including sand (into which many buried and were not very distinct), overlying water (in which some were swimming, particularly during the first week) and the superficial water layer (in which some were found floating after having crawled up the walls of the bowl); **ii)** segregation of mucus by larval foot, notably in ethanol-spiked bowls, made larvae sticky so that most individuals had small sand grains attached to their shell; this produced more inconspicuous larvae, observed to have had difficulty moving freely sometimes. On a few occasions this mucus resulted in some specimens entangled with others, in such a way that masses consisting of several larvae in which each individual tried to move in different directions were noticed; these masses probably facilitated the spread of microbial infection and caused the group mortalities observed during the first week (see Appendix 5); **iii)** mucus also gave the larvae an exceptional ability to adhere to glass; thus, larvae drawn into the pipette were often not released without repeated attempts at ejection. Overall, it is not surprising that not every larva starting the test (70 in each of 12 vessels) could be repeatedly recovered. However, larvae (dead + live) found in containers ranged from 63 to 70, averaging 67; the number of corpses + living larvae effectively recovered is the figure taken as the initial one for each bowl (see Appendix 5).

Mortalities occurring during test in vessels are listed in Appendix 5, and the mean percentage of post-larvae survival throughout exposure for all treatments is plotted in figure 5.3. When data were analysed by ANOVA and SNK test (Table 5.3), mean survival after 30 days in the water control (99%) was found to be significantly higher than in any other treatment; mean survival in the ethanol control (74%) was not statistically different ($\alpha = 0.05$) than in 50 ngSn/l (56%), but it was significantly greater

than in any other TBT dose; finally, exposure to 500 ngSn/l for one month was shown to have resulted in the most reduced mean survival of post-larvae (4%).

5.3.2.2. Growth, shell abnormalities and metamorphosis.

Definitive acceptance of sand as a settling substratum seemed to be delayed in control treatments for the first week of culture, during which larvae alternated periods of burrowing beneath the sand with others of calm swimming with the velum upwards or crawling up the walls of the vessel to reach the surface film of the water and then drift; on the contrary, pediveligers exposed to high doses of TBT spent most of this week grounded, not crawling so actively or swimming. Since groups of pediveligers were distributed in vessels at random, it is thought that high-TBT individuals were not, in fact, more competent for settlement than those in other treatments; rather, doses of 250 and 500 ngSn/l probably were narcotic and impaired activity of pediveligers immediately. After ≈ 10 days of culture, control pediveligers stayed buried for most of the test; if dug out by a gentle jet with a fine pipette, they quickly reburied. They were, therefore, difficult to locate in the sand for their recovery every 2nd day, and when temporary in small dish for examination they were crawling continuously; many drifted hanging from the surface water layer by means of fine byssus threads coming out of their umbones; they were very active, progressively bigger, and a green patch inside them was evident through the translucent valves. However, animals in the 250 and 500 ngSn/l treatments displayed very limited activity beyond day 6. Thereafter they were mostly found on the surface of the sand, on the same spot as they were placed 2 days before, sometimes waving the foot but unable to crawl or bury; the only evidence of life was commonly some cilia beating within their pale soft parts. Similar indications of debilitation were noticed in larvae reared in 125 ngSn/l from day 18 on, and also in those in 50 ngSn/l at the latest stages of test.

The valve length of all larvae measured is listed in Appendix 6; length of a small percentage (≈ 10%) of individuals in those TBT treatments with substantial survival was not determined because outline of larval shell was distorted due to the cemented sand grains; estimation of edges for measurement was therefore unreliable. Mean length of

post-larvae initially and after exposure to treatments is given is Appendix 6 and plotted in figure 5.4; shell length after 30 days exposure indicates that growth was negligible in doses of 125 and 250 ngSn/l (mean length 262 µm and 257 µm, respectively).

Length data from treatments resulting in substantial larval growth were transformed as described in section 5.3.1.2 for veligers; data were than tested using general linear model analysis of variance (see Table 5.4) because cell sizes were unequal (n = 67, 50 and 70, respectively for WC, OC and 50 ngSn/l). Larval growth in 50 ngSn/l (mean final shell length = 320 µm) was shown to be significantly reduced with respect to that in either control treatment, and individuals reared with 5 µl/l of EtOH were found to have grown larger (mean length = 639 µm) than in the concurrent water control (WC mean length = 540 µm). Growth rate at 20.5 ± 1 °C can be estimated in the nominal 50 ngSn/l treatment to be reduced by a factor of 3.7 with respect to that in WC.

Interestingly, shell growth in most larvae exposed to 50 and 125 ngSn/l of TBT was observed from day 20 to be abnormal when compared with similar-sized larvae reared in the parallel culture (model larvae, see section 5.2.3.1 and Appendix 6 for lengths) or indeed with any other larva from TBT-free bowls. In these malformed shells, the ventral edge of valves progressed perpendicularly to a sagittal plane rather than in parallel to it (inwards rather than downwards, see plate 5.1.4), occluding to some extent the gap left between open valves. This feature was not as marked around the posterior margin of the shell as it was in the anterior (plate 5.1.2), and switched the compressed global appearance of the larvae (*plana* is the Latin for flat) to an abnormally spherical shape (see plate 5.1.3 and 4); "balling" was more obvious in post-larvae displaying greater growth (i.e. those reared in 50 ngSn/l of TBT), and seemed to impair their normal locomotion.

Observed sizes of developmental stages of *S. plana* post-larvae in control treatments again agree with data reported by Frenkiel and Mouëza (1979). The exhalant (superior) siphon is the first to be formed, when larvae are 600-700 µm long; this was noticed in a number of specimens from the ethanol control (OC) and in just a few from the water control. The inhalant siphon was only observed in those 2 specimens in OC which were ≈ 800 µm long after 30 days rearing, but it did not appear to be extensible

yet. On the contrary, some individuals > 900 µm from the surplus parallel culture (originally smaller, see section 5.2.3.1) had both siphons fully developed and apparently functional by that time; this event that marks the completion of metamorphosis and the incorporation of individuals into the juvenile stage suggest that experimental handling every 2nd day may have stressed the larvae so that their growth rate was not optimal.

Once the toxicity test was finished (18.9.91), a number of specimens (65 from WC.A + 45 from OC.A + 63 from surplus culture) from 500 µm to 1 mm length were pooled in a 200 ml bowl with aeration and further held in sand for additional information; temperature and food composition was as in test, but after 2 months diet was changed to be ≈ 1 mg cornflour per 100 ml per week. By 17.1.92 only 11 individuals (from 1.1 to 2.3 mm long, mean 1.6 mm) were found alive and the culture was terminated.

5.3.2.3. Nominal and actual levels of TBT exposure.

Results of analyses of TBT solutions performed on volumes prepared on a day after the experiment but following the same protocol are plotted in figure 5.5. While actual concentrations yielded by preparation procedure amounted up to 63-96% of nominal values, TBT levels after 48 h in experimental conditions decreased to 46%, 36%, 32% and 44% of initial values, respectively for doses of 50, 125, 250 and 500 ngSn/l; TBT in control solutions was not detectable. The marked drop between initial and final values is due to TBT adsorption characteristics and also to the presence in vessels of microalgae (40 cells T-*Iso*/µl) which are known to bind appreciable amounts of organotin (Laughlin *et al.*, 1988). Assuming that solutions analysed are a good estimation of conditions throughout the test, it could be calculated that the average dissolved concentrations the pediveligers were actually exposed to are 23, 69, 157 and 321 ngSn/l rather than the nominal of 50, 125, 250 and 500 ngSn/l, respectively.

5.4. Discussion

5.4.1. Veliger larvae.

Recently hatched *Scrobicularia plana* D-larvae have been shown to develop to pediveligers in 10 days under control culture conditions. Levels of TBT at nominal 50

ngSn/l and above reduced veliger growth rate (significantly by day 2 and severely by day 4) and prevented all larvae from reaching the footed stage that is required for settlement during the experimental period; exposure to nominal 250 and 500 ngSn/l resulted in no D-larvae developing substantially (i.e. umbones not formed).

Effects of metallic compounds on bivalve larval development have been studied for long (see for instance Calabrese *et al.*, 1977), and it has been commonly found that larvae are more sensitive to pollutants than juveniles or adults of a given species; the case of the mussel *Mytilus edulis* and Cu seems to be one of the few exceptions to this general rule (Beaumont *et al.*, 1987). It has also been shown that manifestation of lethal effects of toxicants on veligers is dependent on the time of exposure; thus, short-term lethality has been reported at concentrations > 1 µgTBT/l (*M. edulis* 48 h LC50 = 2.3 µgTBT/l, *Crassostrea gigas* 48 h LC50 = 1.6 µgTBT/l (Thain, 1983); *C. virginica* 48 h LC50 = 3.96 µgTBT/l, *Mercenaria mercenaria* 48 h LC50 = 1.65 µgTBT/l (Roberts, 1987)), while prolonged exposure usually reduces the lethal threshold (*M. edulis* 15 day LC50 = 0.1 µgTBT/l (Beaumont and Budd, 1984); *M. mercenaria* 8 day LC50 < 0.6 µgTBT/l (Laughlin *et al.*, 1989)). For *S. plana* no statistical grounds for reduced survival of veligers during 10 days exposure were found, but most of the same TBT doses proved lethal to well developed pediveligers after 30 days; however, it is likely that heavy ethanol control mortality resulting from microbial infection contributed to the lack of significance of results in the first instance. It is in these longer-term (> 1 week) experiments where sublethal effects are usually demonstrated: reduced shell growth of *C. gigas* in 12 days at 0.05 µgTBT/l (His and Robert, 1985), reduced shell growth of *M. mercenaria* in 14 days at ≥ 10 ngTBT/l (Laughlin *et al.*, 1988); furthermore, the recent finding that weight is probably an indicator of adaptative response and stress more sensitive than shell size in veligers (Ringwood, 1992) offers a new insight for chronic toxicity assessment in bivalve populations.

The mode of action of toxicants and their accumulation and elimination pathways in veligers are unknown. For *M. mercenaria*, Laughlin *et al.* (1988) suggested that the primary route of TBT exposure was through consumption of phytoplankton rather than by partitioning of dissolved TBT from water because cumulative effects on growth were

not apparent until after several days; however, when un-fed larvae (both veligers and pediveligers) of *Isognomon californicum* were exposed for 24 h to 20 ppb Cd, accumulation began at the time of exposure (Ringwood, 1991). Since limiting effects on growth of *S. plana* veligers are significant from as early as day 2 even at the lowest dose (50 ngSn/l), it is argued that a rapid accumulation of TBT from water did take place, although important uptake via food of this highly lipophilic organotin (Laughlin *et al.*, 1986) by actively planktotrophic stages cannot be discarded. As for elimination, recovery of veligers after several days exposure to TBT or Cd has been reported to be, if any, slow (Laughlin *et al.*, 1988 and Ringwood, 1992, respectively); in the case of organotins, bivalves are among the animal groups more sensitive to TBT probably because of their low mixed-function oxygenase activity (Lee, 1991), and the possibility of the detoxifying mechanisms being more limited in their larval stages do not seem unlikely.

The ecological relevance of results is clear since retardation of growth will prolong the pelagic life of the larvae, thereby decreasing the chance for survival against predation, disease and dispersion, and resulting in a reduced recruitment into the population (Calabrese *et al.*, 1977); for *S. plana* veligers exposed to nominal 50 ngSn/l of TBT, and assuming that the growth rate displayed during the first 6 days can be maintained, the extension of the planktonic stage can be estimated at ≈ 3 fold. Because planktotrophic pelagic larvae usually display positive phototaxis, it is possible that they occasionally dwell in, or close to, the water surface microlayer (see Hardy *et al.*, 1987); this will worsen toxic effects as a consequence of exposure to enhanced TBT levels in that habitat (Cleary and Stebbing, 1987).

5.4.2. Pediveliger larvae.

Settling larvae of *S. plana* suffered significant mortalities when exposed for 30 days to nominal doses of TBT ≥ 125ngSn/l (69 ngSn/l as analysed); more importantly, shell growth was negligible at those TBT levels, while it was severely reduced and abnormal at the lowest concentration tested (nominal 50 ngSn/l, 23 ngSn/l as analysed). On the contrary, some control post-larvae were developing their siphons by the end of the test, i.e. they were about to complete metamorphosis.

The activity of control pediveligers during the first week in the experimental bowls indicates that the nature of the fine sand offered as a settling substratum may not have been optimal. However, there is no evidence that initially-settling bivalve larvae are selective between sediments with highly-contrasting organic content (Bachelet *et al.*, 1992), and the sand was considered convenient for the design of the recovery procedure; further, eventual acceptance of sediment proves that it was adequate for the definitive initial settlement of pediveligers and their development. What seems clear is that every 2nd day handling prevented experimental post-larvae from achieving the larger size shown by some individuals in the parallel culture; overall, though, the size of *S. plana* larvae reared in the laboratory for 40 days (10 as veligers + 30 as post-larvae) is comparable to that observed by Frenkiel and Mouëza (1979) in field collected spat from a clam population of North Wales (i.e. 450-900 µm) and estimated to be 1½ months old.

Survival in the ethanol control was significantly lower than in the water control, a characteristic which is not unusual in toxicity testing of organic compounds as a result of increased microbial infection due to the solvent used (see for instance Laughlin *et al.*, 1989). In the present work, ethanol had also the unexpected effect of raising the production of mucus (otherwise a normal feature of the molluscan pedal gland) and, subsequently, the formation of masses of individuals which were then more susceptible of infection. On the contrary, proliferating bacteria may have constituted a substantial source of organic matter for those larvae surviving to the later stages of culture (Mengus, 1978), and thus explain the larger final mean size attained in the ethanol control treatments; in addition, dissolved organic material containing amino acids can also be absorbed by bivalve larvae and then constitute a supplementary source of energy (Manahan and Crisp, 1982).

Abnormal shell growth of pediveligers reared at the lower TBT levels (more marked in 50 ngSn/l because of greater shell development) constitutes a rare sublethal effect of this organotin which, however, has unknown ecological relevance. Shell deformities of marine bivalves induced specifically by TBT is a well known phenomenon reported only for some oyster species (Waldock and Thain, 1983; Alzieu *et al.*, 1986; Batley *et al.*, 1992), but not for their larval stages. In *S. plana*, shells of adults do not

appear to develop neither thickening or any other abnormality (Bryan and Gibbs, 1991); as for their larvae, visual examination failed to reveal any malformation in veliger shells, and nothing resembling the pediveliger "balling" illustrated in plate 5.1 has been found in the literature for other bivalve species. It is speculated that some malfunctioning of calcification mechanisms induced by TBT (perhaps of the type reported by Alzieu *et al.*, 1982, and Machado *et al.*, 1989) resulted in newly deposited shell layers being somehow soft and plastic in a mechanical sense, as described for *C. virginica* by Galtsoff (1969), and also less rigid than the usually brittle bivalve shell. Consequently, burrowing and burying activity (displayed by pediveligers before they became debilitated by TBT) caused the ventral edges of the shells to bend perpendicularly to a sagittal plane as a consequence of forces resulting from friction of valves with sand while crawling. According to this hypothesis, the deformation was more severe around the anterior margin of the shell because, due to forward movement typical of burrowing bivalves (see for instance Trueman, 1983), this is the edge bearing the brunt of friction; other possible explanations should account for the fact of asymmetry of deformities. No malformation of the type described here would have been reported for laboratory reared larvae of other bivalves because the plasticity of valves was not tested by active burrowing on substrata.

It is also possible that abnormal growth is an artefact provoked by the hard texture of artificial sand used and it might not, therefore, occur in the field where settling pediveligers usually select soft fine sediments offering less resistance to burrowing. If, however, shell abnormalities do happen in nature, affected bivalve larvae would not have been collected and reported because, suffering such a feature, they do not seem likely to survive and develop much longer. Artefact or not, deformed valves prove that low TBT levels ($\geq$ 23 ngSn/l) affects normal shell development of pediveligers, in addition to reducing substantially their growth rate (by a factor of $\approx$ 4) and activity; it is quite possible that TBT induces these sublethal effects at lower concentrations.

Finally, it is generally accepted that initially settling bivalve larvae interact repeatedly with the sediment while swimming and/or drifting confined to the near-bottom layer (see for instance Butman, 1987; Jonsson, 1991); for the related species *Macoma balthica*, Günther (1991) showed that the period of redistribution of post-larvae on an

intertidal sandflat in the Waden Sea took around 21 days, during which modal length of transported recruits changed from 250-300 μm to 500-550 μm. On the other hand, release from contaminated muds results in TBT levels at the water/sediment interface being much elevated than in the whole water column (water depth ≈ 30 cm, Waldock *et al.*, 1987). It is therefore thought that the toxic effects of TBT shown above are of high environmental relevance to settling pediveligers of *S. plana* (and, possibly, other bivalve species) because of their likely continued exposure to enhanced TBT concentrations in near-bottom waters.

5.5. Conclusions.

It has been demonstrated that exposure to nominal TBT concentrations ≥ 50 ngSn/l for a few days diminishes the growth rate of *S. plana* recently-hatched larvae; levels ≥ 250 ngSn/l prevented veligers from any substantial development in 10 days. Non-exposed pediveligers settling in unpolluted sediment have been shown to suffer significant mortalities after 30-day exposure to dissolved TBT levels ≥ 69 ngSn/l (as analysed), and concentrations ≥ 23 ngSn/l severely reduced post-larvae growth rate and activity; these low levels also altered normal development of larval shells.

Due to TBT restrictions on growth rate (by a factor of ≈ 3), exposed *S. plana* veligers would take some 30 days at 22.5 ± 1 °C to became competent for settlement; at 18 °C, though, this totally pelagic development takes 30 days (Frenkiel and Mouëza, 1979), and hence settling could be delayed until about 90 days after hatching. Since in U.K. and North Atlantic waters the spawning of *S. plana* populations is concentrated in August (see chapter 3), and bearing in mind that 18 °C is more representative of the water temperature during this warmer month at these latitudes, planktonic larvae would still be developing in TBT contaminated waters (nominal ≥ 50 ngSn/l) by the beginning of November: achievement of settling pediveliger stage during these less favourable months is considered unlikely.

If, however, competent *S. plana* pediveligers formed elsewhere were transported by currents and imported into a TBT polluted mudflat, exposure to ≥ 69 ngSn/l for a few weeks would kill a significant proportion of them; in areas of moderate TBT

contamination, this levels of organotin can readily be reached in the water/sediment interface where the pediveligers mainly dwell. At concentrations ≥ 23 ngSn/l, severe reduction of growth rate induced by TBT would delay completion of larval metamorphosis in North Atlantic waters until December-January; it is concluded that adverse alimentary and temperature conditions will render all larvae suffering those TBT levels (moreover less fit and possibly malformed) unable to constitute any effective recruitment to the population juvenile stock.

Relatively low TBT levels (> 20 ngSn/l to ≈ 300 ngSn/l) now proved to be acutely and/or chronicly toxic to both veligers and pediveligers of *S. plana* in laboratory experiments were commonly and persistently found in subsurface open waters of European estuaries during the 1980s, usually coupled with moderate to high organotin concentrations in sediments (Waldock *et al.*, 1987; Quevauviller and Donard, 1990; Ritsema *et al.*, 1991), and still during 1990 (Dowson *et al.*, 1992) More specifically, these noxious TBT levels have been of concern for years in areas where *S. plana* populations have been observed to disappear as a result of lack of recruitment (Langston *et al.*, 1987, 1990; Langston and Burt, 1991); it is therefore concluded that dissolved TBT is extremely likely to have caused or contributed substantially to clam extermination in these and, possibly, other North Atlantic coastal environments (see Essink *et al.*, 1991). In addition, TBT accumulated in sediments poses considerable risk to larval development and settlement of benthic bivalves.

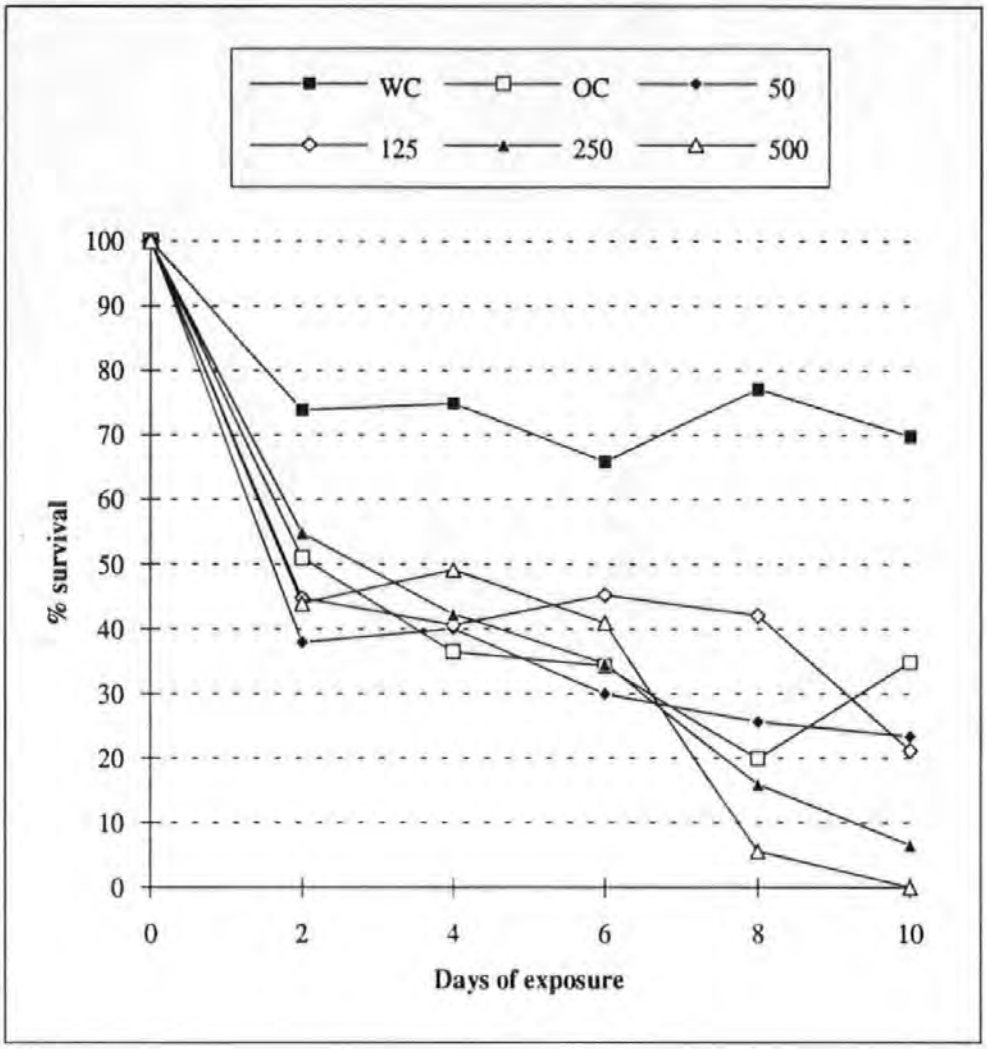


Figure 5.1: *Scrobicularia plana*. % survival of veligers (mean of duplicates) during exposure to TBT (50, 125, 250 and 500 ngSn/l) and water (WC) and ethanol (OC) control treatments for up to 10 days.

Table 5.1: *Scrobicularia plana*. ANOVA of % survival of veligers after 10 days exposure to TBT (50, 125, 250 and 500 ngSn/l) and water (WC) and ethanol control (OC) treatments. The variances were homogeneous: C < C.95(6,1).

Source	df	MS	*F*		Pr > F
Treatment	5	1232	7.45	*	0.0149
Error	6	165			
SNK: WC > OC = 50 = 125 = 250 = 500					

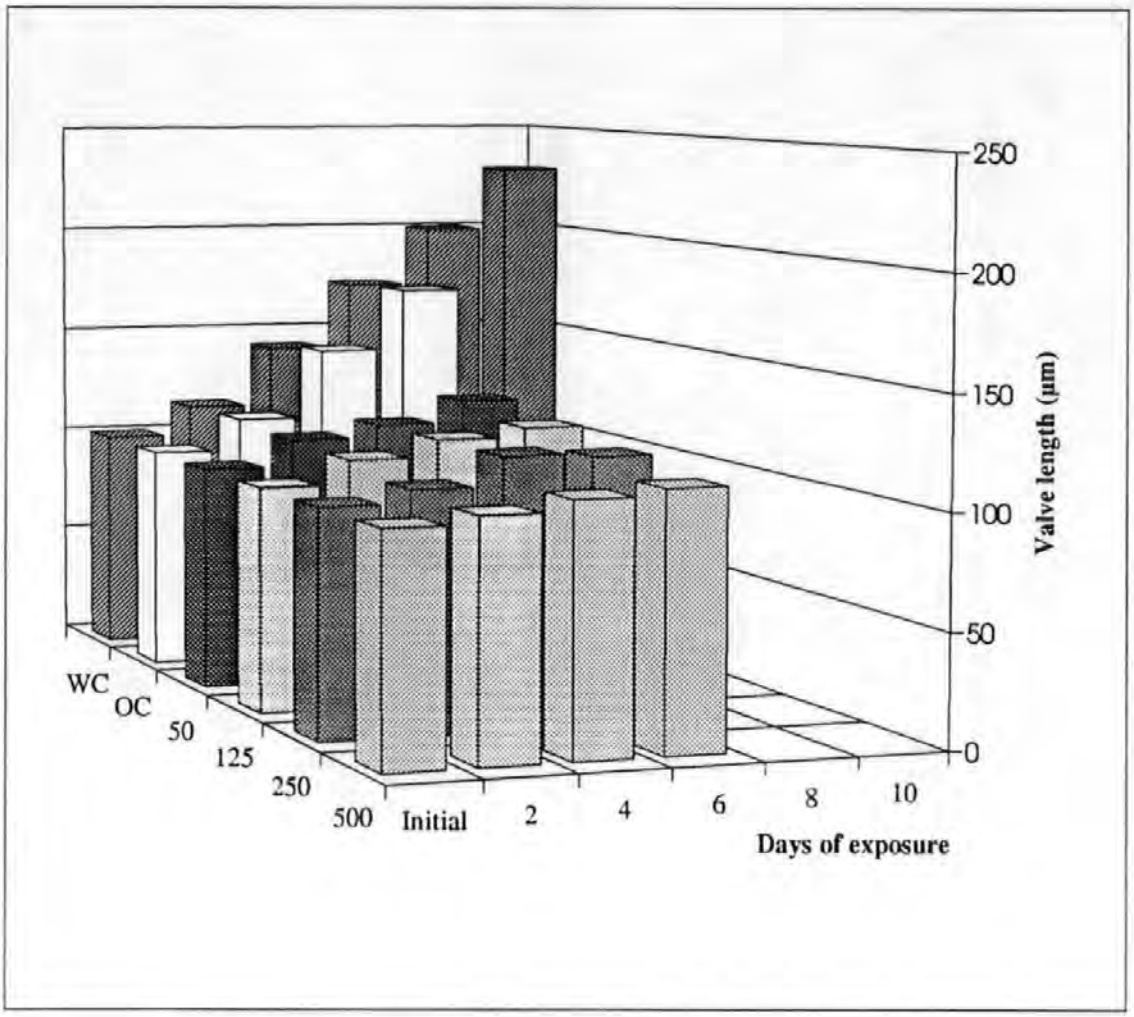


Figure 5.2: *Scrobicularia plana.* Mean length of larvae (n = 25 for every independent sample) after exposure to TBT (50, 125, 250 and 500 ngSn/l) and ethanol control (OC) treatments for 2, 4 and 6 days, and to water control (WC) for up to 10 days. Initial is length of day 0 D-larvae in separate sample.

Table 5.2: *Scrobicularia plana.* (a), (b), (c): ANOVA of larval growth (raw data transformed as decimal log [(length x 100) / mean initial length]) after exposure to TBT (50, 125, 250 and 500 ngSn/l) and ethanol control (OC) treatments for 2, 4 and 6 days, respectively; all variances were homogeneous: C < C.99(6,36). (d): ANOVA of veliger growth on consecutive days of exposure to 500 ngSn/l; the variances were homogeneous: C < C.95(4,36).

	Source	df	MS	*F*		Pr > F	
(a): **Day 2**	Dose	5	0.013	55	***	0.0001	SNK: OC > 50 = 125 > 250 > 500 > Initial
	Error	144	0.00024				
(b): **Day 4**	Dose	5	0.076	116	***	0.0001	SNK: OC > 250 = 125 = 50 > 500 > Initial
	Error	144	0.00066				
(c): **Day 6**	Dose	5	0.168	438	***	0.0001	SNK: OC > 50 > 125 > 250 > 500 > Initial
	Error	144	0.00038				
(d): **500**	Day	3	0.011	36	***	0.0001	SNK: Day 6 > Day 4 > Day 2 > Initial
	Error	96	0.00031				

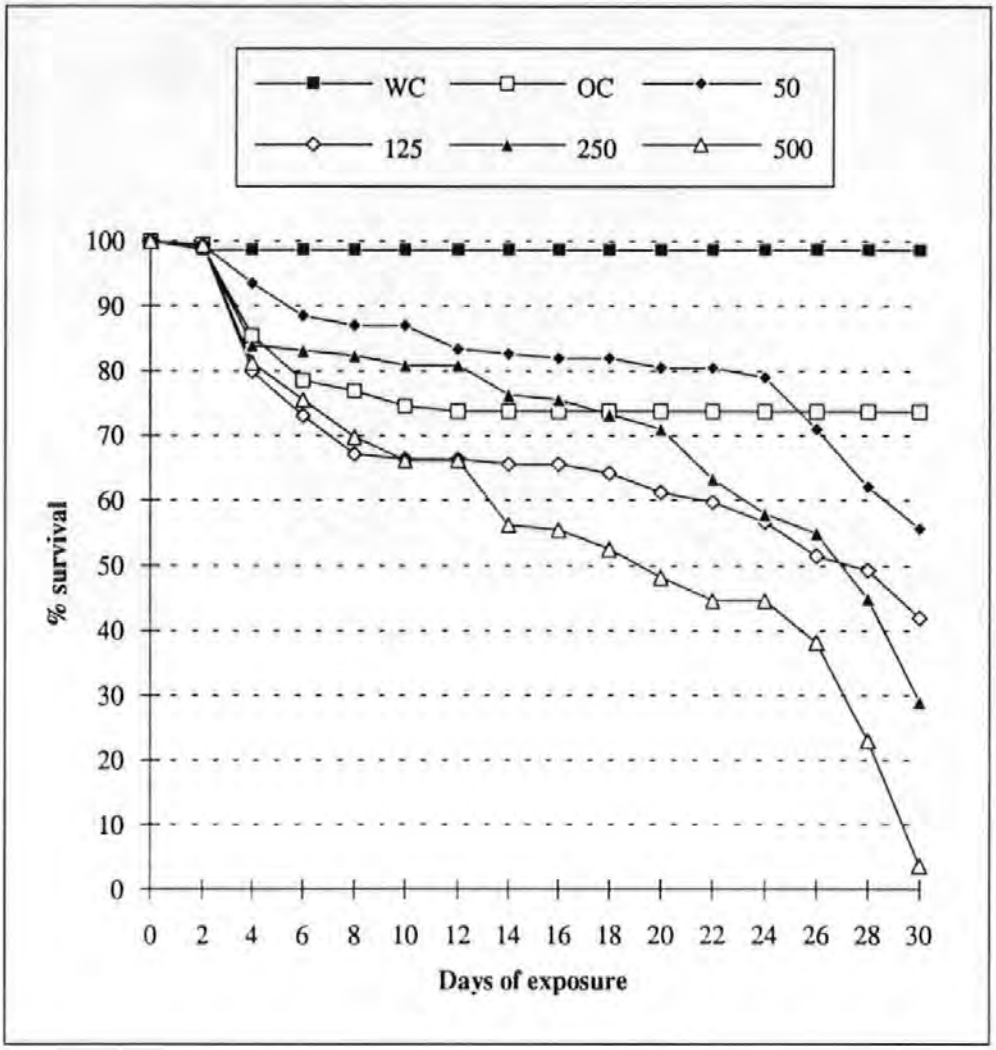


Figure 5.3: *Scrobicularia plana*. % survival of postlarvae (mean of duplicates) during exposure to TBT (50, 125, 250 and 500 ngSn/l) and water (WC) and ethanol (OC) control treatments for up to 30 days.

Table 5.3: *Scrobicularia plana*. ANOVA of % survival of postlarvae after 30 days exposure to TBT (50, 125, 250 and 500 ngSn/l) and water (WC) and ethanol control (OC) treatments. SNK: treatments not underlined by the same line are significantly different at $p < 0.05$.
The variances were homogeneous: C < C.95(6,1).

Source	df	MS	*F*		Pr > F
Treatment	5	2247	24	***	0.0007
Error	6	93			
SNK: WC OC 50 125 250 500					

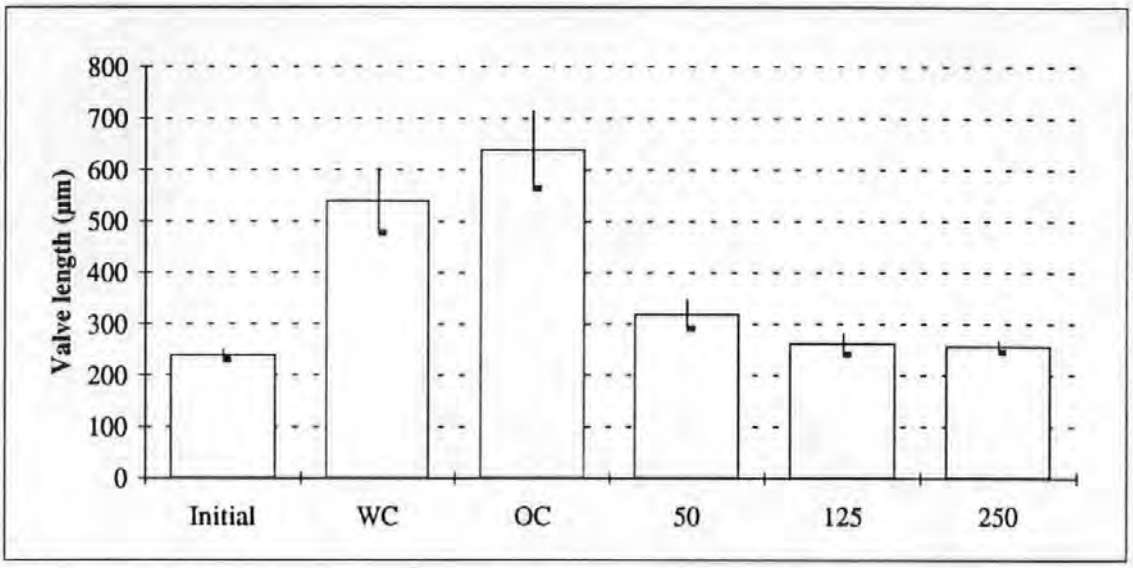


Figure 5.4: *Scrobicularia plana.* Length of postlarvae (mean ± SD) after exposure to TBT (50, 125 and 250 ngSn/l) and water (WC) and ethanol (OC) control treatments for 30 days; n = 70, 53, 33, 67 and 50, respectively. Initial length was assessed in 10 pediveligers ten days after hatching.

Table 5.4: *Scrobicularia plana.* ANOVA of post-larval growth (raw data transformed as decimal log [length x 100] / mean initial length]) after 30 days of exposure to TBT (50 ngSn/l, n = 70) and water (WC, n = 67) and ethanol control (OC, n = 50) treatments; initial length of sets was assessed as the mean of 10 pediveligers ten days after hatching. The variances were homogeneous: C < C.99(3,144).

Source	df	MS	*F*		Pr > F
Treatment	2	1.53	714	***	0.0001
Error	184	0.0021			
SNK: OC > WC > 50					

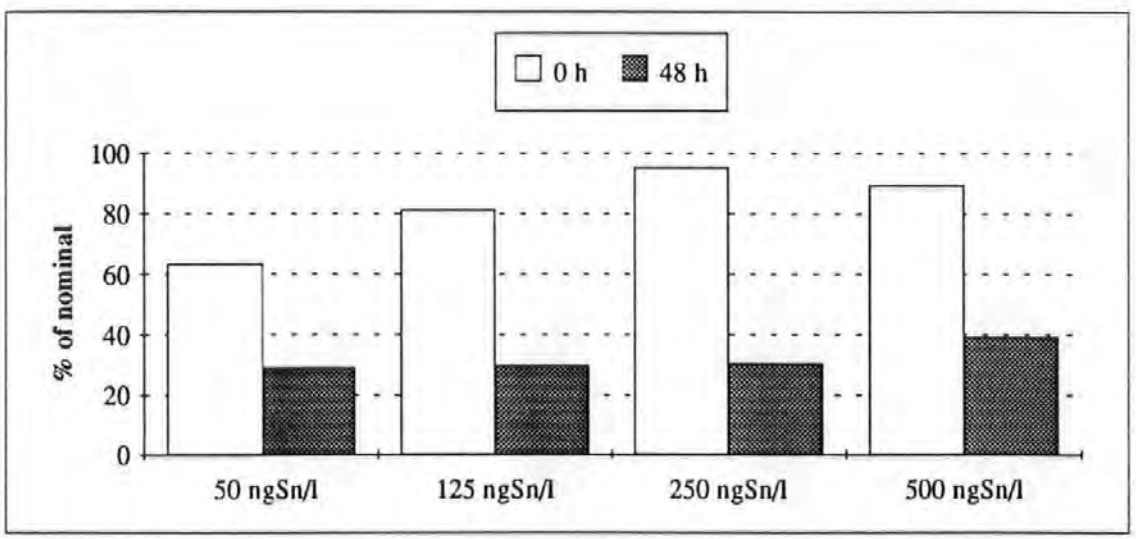


Figure 5.5: Initial (0 h) and final (48 h) tin concentrations analysed in larval treatment solutions (as prepared in day out of experiment) expressed as % of nominals. TBT in controls was not detectable.

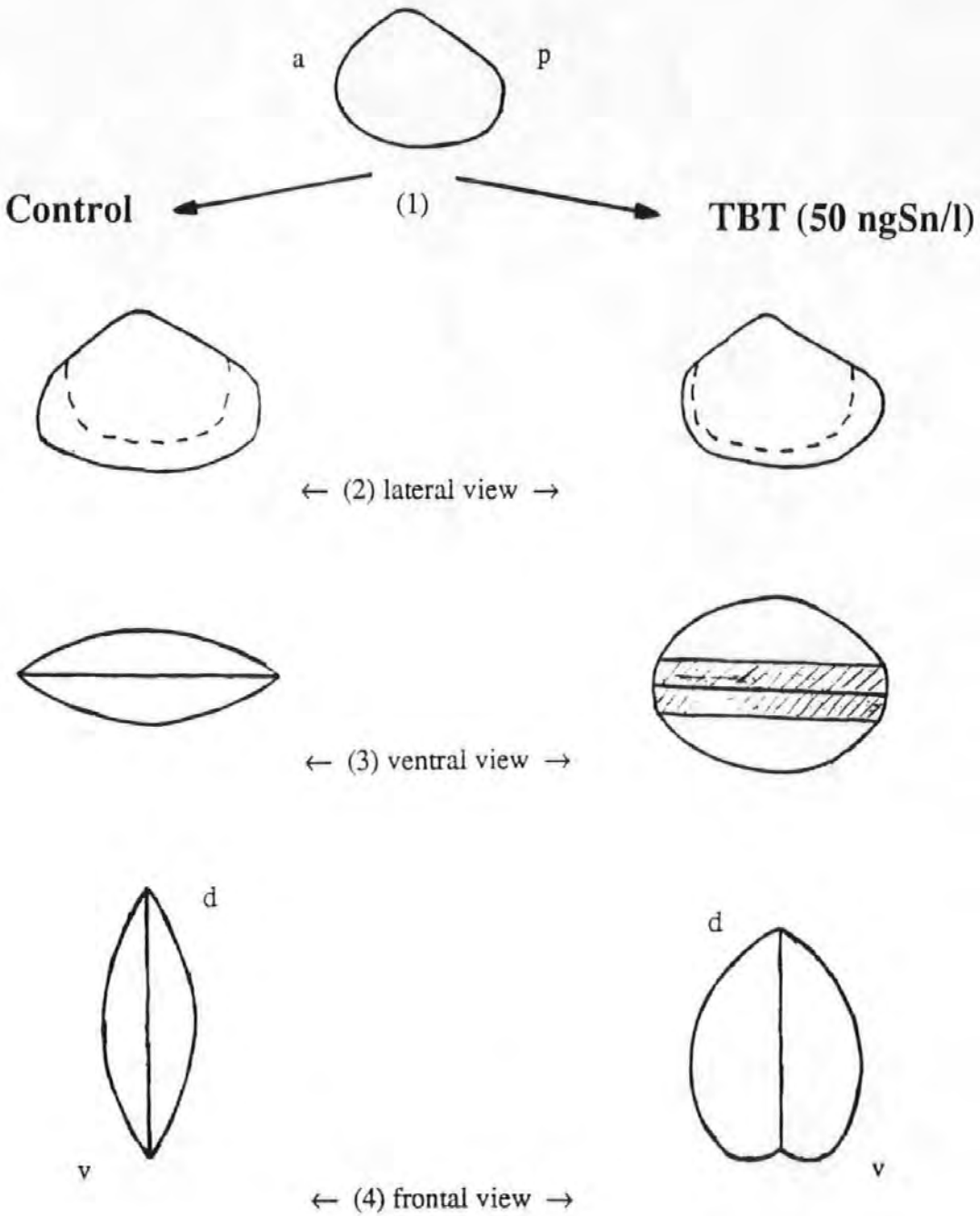


Plate 5.1: *Scrobicularia plana*. (1): shape of pediveligers from a lateral view at initiation of toxicity test. (2), (3), (4): different views of post-larvae after 30 days culture in TBT (50 ngSn/l) and control treatments: growth abnormalities were less developed in larvae reared at 125 ngSn/l. a, p, d and v: anterior, posterior, dorsal and ventral edges of shell, respectively.

Chapter 6: Effect of exposure to dissolved TBT and copper on survival, growth and burying activity of juvenile *Scrobicularia plana.*

6.1. Introduction.

The size frequency distributions of populations of *Scrobicularia plana* in clean estuaries are generally continuous, with modes reflecting successive recruitments. In contrast, size frequency histograms of TBT-affected populations show a perturbed, discontinued structure. Thus, although some settlement of spat may occur even at moderately contaminated sites such as Poole Harbour (possibly representing imports from less polluted areas), recruits do not reach maturity and they are presumed to die out as toxic burdens are accumulated in tissues (Langston *et al.*, 1990); short-lasting recruitment, whether resulting from primary (i.e. pediveligers metamorphosing *in situ*) or secondary (i.e. colonizing postlarvae) settlement, has also been observed during non-winter periods in several estuaries from European countries other than U.K. over the 1980s (Essink *et al.*, 1991, see figure 1.1).

Siphon size is one of the main factors determining the burying depth of benthic bivalves (Zwarts & Wanink, 1989), and therefore recently metamorphosed spat of *S. plana* (< 1 mm) are confined to the top few cm layer of the sediment for at least several months. Over that period, juveniles are exposed to a number of natural risks (cold winter, predators) to which they are particularly sensitive because of their shallow burial; this also results in likely accidental emergence caused by mechanical disturbances such as wave action in stormy weather (Hughes, 1970) and current scour (Palmer and Gust, 1985). Such events promote a prompt reburial as a behavioural response to reduce blatant exposure to predators and avoid being washed away from the selected substratum. Consequently, there is a very high mortality among juveniles, a significant percentage of which is due to predation of flatfishes, shrimps, crabs (see several references in Zwarts and Wanink, 1989) and birds (Hughes, 1970). Their most effective means of protection is to increase burial depth, and as a result, quick growth to increase

siphon mass seems to take priority over reproduction in benthic bivalves (Zwarts and Wanink, 1989). Thus, in addition to the directly fatal action of acute toxicity, the ecological significance of sublethal effects of pollutants reducing the normal growth rate of juveniles is also obvious. On the other hand, the applicability of behavioural measures in environmental stress assessment has long been recognized (Olla *et al.*, 1980), and the relevance of impairment or curtailment of burying activity induced by pollution for bivalve avoidance of crab predation has been explicitly proved (Pearson *et al.*, 1981).

Work aimed at determining dissolved TBT (and Cu) effects on survival, growth and burying activity of *S. plana* juveniles is hereafter presented; possible effects of sediment TBT will be dealt with in the next chapter.

6.2. Material and methods.

6.2.1. Experiment of 1991: high TBT and copper.

6.2.1.1. Animals, conditions and experimental procedure.

Surficial sediment from an intertidal mudflat in the Torridge estuary (North Devon, O.S. Grid reference SS453308) was sieved *in situ* through a 1 mm pore stainless steel mesh and brought to the laboratory in February 1991. About 900 juveniles of *Scrobicularia plana* were sorted out and sized to the nearest 166 µm using a stereo microscope and ocular graticule calibrated against a micrometer slide. This process took 3 days, during which time the clams were held at the experimental temperature (15 ± 1 °C in a constant temperature room illuminated 12 h per day) in sea water of 24 ± 2 ppt salinity. Only those 800 juvenile clams ranging from 1.67 to 3.50 mm in length (average 2.33) were used, and their size distribution is shown in figure 6.1. Twenty experimental groups (40 specimens each) were selected to be as similar in size as possible since differences of a few mm between juveniles of this size may result in increased variability (either in terms of growth and/or burying activity); size classes composition of experimental groups, their average length and standard deviation of the mean is shown in Table 6.1. Since the external morphology of the juvenile of *S. plana* resembles some other clams cohabitant of estuaries, notably *Abra tenuis* (Montagu), and can lead to error (Gibbs, 1984), some positive identification of the animals used was deemed necessary;

although no clam was sacrificed for this purpose, every juvenile that died in the course of the experiment was examined and species identity confirmed.

Pyrex cylindrical bowls (9 cm ∅) were used as containers of the experimental microcosm; they had been soaked for several hours in detergent, later in acid (≈ 1N HCl) and finally twice rinsed in distilled water. The bottom of each vessel was covered with a layer (≈ 7 mm thick) of 75 g of acid washed sand (40-100 mesh, BDH) into which juvenile clams could promptly bury themselves. Filtered (0.45 µm) 24 ± 2 ppt sea water (FSW) was poured at 200 ml per bowl and, after levelling up the layer of sand with some hand-tapping, a set of 40 animals was gently introduced; the flagellate *Isochrysis galbana* was added as food to achieve a concentration in bowls of 15 cells/µl. Four TBT doses (0.5, 1, 2 and 4 µgSn/l) and four Cu doses (10, 20, 40 and 80 µg/l) were then made up in vessels and tested together with water (WC) and ethanol (OC) controls (see below); all treatments were run in duplicated bowls containing sets of clams chosen at random.

The experiment was designed as a 30-day static renewal test. Every other day the entire contents of each bowl were sieved through a tea strainer (1 mm pore) in which clams were retained; they were carefully observed under a microscope and dead animals (i.e. showing wide opened valves and not reacting to gentle probe) removed for their later identification and measurement. After decanting the two-day-old water, fresh FSW was added to the original bowls and the layer of sand rearranged. Finally, juveniles were introduced with care so that all of them deposited on the sand surface simultaneously, food added by pipette and treatment concentrations made up to; vessels were then left undisturbed for the next 48 h. A record of the number of clams found on the surface of the sand layer before each renewal was kept. As a burrowing assay conducted immediately after renewal of each bowl on day 6, 12, 18, 24 and 30, the number of clams failing to be totally buried was registered over a 1 h period, at 5 min intervals for the first 40 min, and every 10 min thereafter. McLachlan and Young (1982) and Donn and Els (1990) measured burying time of other bivalves as the time from initiation of digging by the foot until complete burial or until all burrowing activity stopped, and Phelps (1989) chose achievement of vertical position prior to digging as the behavioural end point

because the clam she was working on do not bury entirely. In the present case, time from deposition of juveniles on top of the sand until their complete burial was monitored for 1 hour because this lapse of total or partial exposure is the period of maximum risk for clams; in addition, all burying individuals (some others were observed to draw the shell erect but failed to progress inwards and fell laterally) dug their whole shells beneath the sand surface. Depth of burial was not measured. Figure 6.2 illustrates experimental conditions and procedure. After Day 30 tests, every animal was sieved out again; surviving clams (replicates pooled) were blotted dry for 2½ h, weighed and frozen. Samples of sand (pooled per treatment) were also frozen for subsequent analyses.

6.2.1.2. Toxicant test concentrations and their analyses.

As it is known that the carrier in which TBT is dissolved (ethanol in this case) may promote microbial growth in rearing vessels, efforts were made to reduce solvent levels to a minimum; 400 ml of TBT solution at 10 ngSn/ml in FSW were prepared from a 1000 ppm Sn stock TBT made up in ethanol from standard TBT (bis TBT oxide, GMBH). The appropriate volumes of this 10 ngSn/ml TBT solution (10, 20, 40 and 80 ml) were added to vessels with FSW (190, 180, 160 and 120 ml, respectively) so that nominal concentrations of 0.5, 1, 2 and 4 µgSn/l were achieved in the experimental bowls. OC were spiked with ethanol to be at the maximum concentration reached in TBT bowls (i.e. 4 µl EtOH/l). Small volumes (up to 16 µl) of standard cupric nitrate (BDH) were added with a micropipette or a microsyringe to render the desired concentrations of Cu in experimental vessels.

Given the characteristics of the experiment and the tendency of chemicals (particularly TBT) to get adsorbed onto the walls of the container therefore diminishing the actual concentration in solution, levels of TBT and Cu in experimental volumes were analysed at the beginning (0 h) and at the end (48 h) of the exposure period. Thus, on day 20 of experiment, 48 h TBT solutions were pooled per treatment in acid soaked bottles, acidified (5 ml concentrated HCl/l) and kept in the dark until analysed; 0 h volumes were freshly made up (FSW + stock TBT + algae) and stored accordingly. TBT solutions (≈ 400 ml) were extracted and analysed as detailed in Bryan *et al.* (1986) and summarised in section 4.2.2; Cu levels were directly determined on a few ml of acidified

sample in a Varian SpectrAA-300 Zeeman graphite furnace. Concentrations of tin and copper in sand were analysed as described for sediments in sections 3.2.6 and 2.2, respectively.

6.2.2. Experiment of 1992: low TBT.

6.2.2.1. Animals, conditions and experimental procedure.

On 22 January 1992 new material was collected at the same Torridge site and brought to the laboratory. During 3 days, about 1000 juveniles of *S. plana* were sorted out and sized as the previous year; however, only a small percentage of them buried themselves into the artificial acid-washed sand contained in holding bowls, and by the 5th day after collection most of them were still on the surface. It was suspected that the sand in that particular batch might be somewhat more acidic than usual and therefore unsuitable for the clams to bury. The sand in one bowl was then thoroughly washed with sea water for 3 times and unburied clams deposited on it; since many of them dug into the sand and stayed buried, it was concluded that sand would be an acceptable substratum after washing. Regrettably, though, juveniles had been exposed to un-washed sand for 5 days and were probably affected so that normal performance could not be expected. The whole lot had to be rejected and the experiment postponed.

A new field trip to the Torridge site was made on 18 February 1992, and material within surficial sediment retained by a 1 mm sieve brought to the laboratory. The day after, ≈ 1200 juvenile clams were sorted out and placed in several holding bowls with artificial sand (40-100 mesh, BDH) which had been rinsed repeatedly with sea water; 3 hours later 87 animals had not buried and were discarded. One day later another 120 clams found on the surface of the sand were also rejected; the rest were sized to the nearest 166 µm as above and 800 of those juveniles from 1.83 to 2.50 mm long distributed in 20 bowls so that each set of 40 clams had identical size classes composition (see figure 6.3). Of the 20 sets, 8 were used in the sediment toxicity bioassay described in chapter 7, 10 in the present experiment and 2 utilised to estimate initial values of burrowing activity and weight. For this purpose, each one of the 2 initial clam sets were deposited on sand in individual bowls and observed for 1 h as described in section

6.2.1.1; sets of animals were then dug out, blotted dry for 2½ hours, weighed and frozen. Spare clams 1.83-2.50 mm in length, other than the 800 used in burying trials, were pooled per size class and similarly weighed to estimate dry weight of individuals of a certain size. A, further 127 clams 2.67-3.17 mm long were not used as above but sacrificed for identification purposes. The 10 sets of 40 animals considered here were subject of an experiment very much like that of 1991 and also run at 15 ± 1 °C (see section 6.2.1.1); groups of clams were deposited on top a sand layer ≈ 7 mm thick contained in a bowl to which 200 ml FSW and algae *I. galbana* at 15 cells/µl had been added. This time duplicated treatments consisted of 4 lower TBT doses (50, 125, 250 and 500 ngSn/l) and the indispensable ethanol or solvent control (OC).

Experimental solutions were renewed every 2nd day and dead animals separated and identified. Effects of lower TBT doses were expected to be more subtle and response of clams less marked than the previous year; therefore, 1 h burrowing activity (see above) was only observed on days 6, 18 and 30. After the burrowing assay of day 30, animals were sieved out, blotted dry for 2½ hours and individual sets weighed and frozen.

6.2.2.2. Toxicant test concentrations and their analyses.

The method followed for preparing test concentrations in 1992 was different than that described for 1991 in section 6.2.1.2. Stock solutions at 10, 25, 50 and 100 µgSn/ml in ethanol were prepared from standard TBT, and spiking of 200 ml bowls with 1 µl of these stocks rendered nominal concentrations of 50, 125, 250 and 500 ngSn/l, respectively. OC bowls were added 1 µl ethanol so that every bowl had the same solvent concentration (i.e. 5 µl EtOH/l). On day 26 of the experiment, 48 h old solutions were kept, 0 h solutions made up and ≈ 400 ml volumes extracted and later analysed as usual.

6.3. Results.

6.3.1. Experiment of 1991: high TBT and copper.

6.3.1.1. Effects on survival.

Behaviour of clams once in bowls was uniform and the great majority of them readily accepted the sand as a burying substratum; only a few of them found it

unacceptable and crawled up the walls of the container to reach the water surface and drift. However, this tendency was only observed during the first few days, and after a week every clam either buried or stayed on surface of the sand.

Of a total of 163 clams dead in course of the experiment (see Appendix 7), up to 15 were accidentally killed by experimental handling, particularly on day 0 and 30; since every one of the 163 was positively identified as juveniles of *Scrobicularia plana*, it is assumed that all animals used belonged to such species. The number of survivors as assessed in each bowl every 2nd day was transformed to be expressed as a percentage of the original 40, and the mean result for every duplicated TBT and control treatment are plotted in figure 6.4. When transformed data were analysed by ANOVA (Table 6.2) this showed that survival of juveniles was significantly reduced by day 12 in bowls exposed to the highest TBT dose (4 µgSn/l) and by day 24 also in bowls at 2 µgSn/l. At the end of the experiment, survivors in these 2 high TBT doses amounted only to ≈ 10% and ≈ 40% of initial numbers, respectively. No other treatment caused any mortality significantly different than that in controls (≈ 10%).

6.3.1.2. Effects on burying activity.

Appendixes 8.A and 9.A compile the number of clams not totally buried in every bowl as observed during 1 h every 6th day of experiment. Data were transformed and the number of juveniles buried expressed as a % of the total participating in a particular assay (i.e. total surviving to that moment); the results (mean of duplicates) are plotted in figure 6.5 (values for ethanol control and TBT treatments except in days when survival was significantly decreased) and figure 6.6 (water control and copper doses). It seems from these charts that juveniles in both control treatments maintained a consistent and relatively quick (100% dug in by 35-40 min) burying performance throughout the duration of the experiment. On the contrary, clams in every toxicant treatment (except 10 µgCu/l) apparently deviated from control activity in all (TBT) or some (Cu) of the 5 every-6th-day burying trials. This is reflected in a reduction of the slope (i.e. burying speed) of the curve defined by the data points, and also in a decrease of the asymptote reached by the 1 h curve (i.e. number achieving complete burial) from 100% in control and some toxicant treatments down to ≈ 12% on day 30 for clams exposed to 80 µgCu/l.

Furthermore, the numbers still unburied 2 days after burrowing trial of days 6, 12, 18 and 24 (as stated in the record of clams found on surface before every 2nd day renewal, see Appendix 7) is many times equal or greater than the number left unburied in 1 h, suggesting that animals which did not achieve complete burial in 1 h could not dig themselves in during the following 48 h; this would argue for a permanent inability to bury in those circumstances. However, this possibility cannot be confirmed since individuals on surface 48 h after observation may include some clams which got buried at some stage but dug themselves out later on; some monitoring technique such as continuous video recording would have been required to ascertain juveniles permanently on surface.

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

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

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

Appendices 8.B and 9.B compile the every-6th-day burying time of juveniles (mean and standard deviation, in minutes) in every treatment as estimated by the above transformation. Figure 6.7 plots results for clams in TBT and control treatments; when data were subject of ANOVA and subsequent SNK (Table 6.3), differences were shown

to be significant in every one of the 5 trials considered: day 6 (all treatments), day 12 and 18 (4 µgSn/l not included because survival was already affected) and day 24 and 30 (2 µgSn/l also excluded for same reason). SNK test included both controls in the same group every time; also, it always considered results for every TBT doses as different from each other and separated of control group. Lack of independence of samples (same clams throughout) precludes use of ANOVA to test effects of one particular treatment over time; however, there is no significant difference between the burying time of water controls in day 6 and that of ethanol controls in day 30, and vice versa. Although there is no estimation of initial burying time of clam sets, it can be assumed that it was similar for every set and it was not significantly different than that maintained by control groups for the duration of the experiment (5-6 min overall). It can surely be concluded that exposure to every TBT dose considered resulted in a progressive increase of the time taken by juveniles to rebury, significant from as early as day 6.

Figure 6.8 plots the estimated burying time of juveniles in copper and water control treatments, and Table 6.4 compiles results of ANOVA and SNK tests performed on them. No significant differences between treatments are displayed for day 6. However, differences are shown to be significant in every trial thereafter, and by day 30 burying time of clams in all copper doses except 10 µg/l was augmented with respect to that of controls; effect of Cu was also progressive over time and its severity became more acute with increasing levels of exposure.

Although no attempt was made to measure depth of burial, the sand surface of each bowl was closely observed at time of counting unburied individuals before renewal every 2nd day; while this could not detect exact position of clams within control bowls (except if there were some activity like siphons movement), location of individuals in most of the toxicant bowls was often possible because the distinct coloration of shells showing through the thin covering layer of sand. This is a clear indication that many individuals which achieved complete burial in toxicant treatments did not buried as deep as control juveniles.

6.3.1.3. Organotin and copper concentrations in solution and sand.

Results of analyses of TBT solutions performed on test volumes freshly made up and those used on day 20 of experiment are plotted in figure 6.9. While actual concentrations yielded by preparation procedure amounted up to around 80% of nominal values, TBT levels after 48 h in experimental conditions decreased to 40%, 53%, 70% and 67% of initial values, respectively for doses of 0.5, 1, 2 and 4 µgSn/l; TBT in control solutions was not detectable. The marked drop between initial and final values is due to TBT adsorption characteristics and also to the presence in bowls of microalgae (15 cells *I. galbana*/µl) which are known to bind appreciable amounts of organotin (Laughlin *et al.*, 1988). Assuming that solutions used on day 20 are representative of conditions throughout the test, it could be calculated that the average concentrations the juveniles were actually exposed to are 0.255, 0.665, 1.55 and 3.13 µgSn/l rather than the nominal of 0.5, 1, 2 and 4 µgSn/l, respectively. Levels of TBT in solution resulted in no detectable concentration of organotin in sand from any treatment.

In view of the results of analyses carried out on initial and final copper solutions (figure 6.10), levels of metal during the 48 h between renewals were consistent and close to nominal figures; deviations from this general rule may be due to interaction of spikes with Cu present at detectable concentrations in supply water. Levels of copper as analysed in sand exposed for 30 days to solution concentrations were 0.4, 0.7, 0.6, 0.8 and 1.3 ppm dry weight, respectively for water controls and 10, 20, 40 and 80 µgCu/l treatments.

6.3.2. Experiment of 1992: low TBT.

6.3.2.1. Effects on burying activity.

Only 4 of the 400 clams considered herein died during the course of the experiment; all of them, together with the 127 specimens sacrificed beforehand, were positively identified as juveniles of *S. plana*. Appendix 11 shows days of occurrence of deaths and length of dead animals, and also number of individuals found unburied in each bowl at time of renewal. Appendix 13.A displays the burying behaviour of each lot of clams (initial sets and experimental treatments) during 1 h assays; results are plotted in

figure 6.11 after transformation of data as % of juveniles buried. No decrease of the asymptote reached by the resulting curves (i.e. % buried in 1 h) is obvious, but reductions over time of slopes (i.e. burying speed) with respect to that of initial sets becomes apparent; this is more marked with increasing TBT doses, but it also seems to happen in ethanol control on day 18.

Using the formula described in section 6.3.1.2 above, original data were transformed and the average burying time calculated for each individual observation; results are gathered in Appendix 13.B and plotted in figure 6.12. Considering the mean burying time of initial sets as an acceptable and independent estimation of the burying time of every group before exposure, data were analysed by ANOVA and *post hoc* SNK test (Table 6.5). Significant differences were displayed in every assay (days 6, 18 and 30) between mean burying times in the treatment of 500 ngSn/l and those in initial sets, but never between these and ethanol control values; lower TBT doses were also shown to have resulted in burying times higher than initial on day 18, but not on days 6 and 30. Indications of shallower burial of juveniles as described in section 6.3.1.2 were also observed in bowls treated with 250 and 500 ngSn/l from day 20 onwards.

6.3.2.2. Effects on growth.

Appendix 12.A shows the blotted dry weight (mg) on day 0 of initial sets and some spare clams not used for the experiment but of the same size classes that those exposed to treatments for 30 days; Appendix 12.B presents the dry weight of clams surviving in experimental sets. The net weight gain of sets where no mortality occurred ($n = 40$) was calculated as the weight of groups on day 30 minus the mean weight of initial sets; weight gain of sets with final $n < 40$ was corrected by subtracting from the mean initial weight the individual weight (as stated in Appendix 12.A) of specimens from the same size class as the dead animals. The net weight gained by animals in each individual bowl was then transformed as a percentage of the mean weight of initial sets, and the average result for all treatments are plotted in figure 6.13; when data were analysed by ANOVA and SNK test (Table 6.6), animals in control sets were shown to have gained significantly more weight than clams exposed to any dose of TBT.

6.3.2.3. Nominal and actual organotin concentrations in solution.

The actual concentrations yielded by direct spiking of FSW with stock TBT (figure 6.14) amounted on this occasion to 87-115% of nominal values; however, levels detected in 48 h solutions dropped to 27%, 23%, 23% and 29% of initial values, respectively for nominal treatments of 50, 125, 250 and 500 ngSn/l. This remarkable drop is due again to adsorption of TBT to the walls of vessels and the presence of microalgae in experimental volumes; while the reduction of TBT levels due to the former cause should be comparable to that which also occurred in the previous year (same type of bowls), the action of the latter was apparently more acute this time round on lower concentrations of TBT. On the average, exposure levels (as analysed) could be calculated to have been 28, 75, 177 and 297 ngSn/l rather than the nominal 50, 125, 250 and 500 ngSn/l.

6.4. Discussion.

6.4.1. Survival.

According to the data above, a 30-day LC50 (concentration killing 50% of the population considered) for *S. plana* ≈ 2.3 mm long can be set for TBT at < 1.55 µgSn/l (based on average of values analysed at 0 and 48 h). This would suggest that spat of *S. plana* are far more resistant to organotin than those of *Mytilus edulis* (14 day LC100 ≈ 1 µgSn/l or 2.6 µg/l TBT oxide as analysed, Thain and Waldock, 1985). However, proper comparisons between their work (including data on other bivalves) and the present one cannot be established because of methodological differences and, more importantly, weight of their experimental spat was 3 orders of magnitude higher than that of juveniles used here; this also applies to the work of Paul and Davies (1986) on juvenile scallops which, unfortunately, do not provide data on levels of TBT exposure. Since organotin concentrations shown to be lethal may have occurred only in areas adjacent to some boating centres, results probably bear little environmental relevance.

6.4.2. Growth.

Although shell length and weight gain may be monitored to assess growth of bivalves, only the latter was used here due to simplicity and accuracy of procedures; nevertheless, shells were measured initially to guarantee uniform distribution of size classes among experimental groups. As data in section 6.3.2.2 show, treatment of juveniles for 30 days with nominal doses from 50 to 500 ngSn/l (from 28 to 297 ngSn/l as analysed) resulted in weight gain (up to 35% of initial) being significantly reduced with respect to controls (≈ 60% of initial). Decreased growth rates induced by low concentrations of TBT have also been reported for juveniles of *Mytilus edulis* (as shell length, Strømgren and Bongard, 1987), *Crassostrea gigas* (as shell length, Lawler and Aldrich, 1987) and *Ostrea edulis* (as weight, Thain and Waldock, 1985). While in this latter work weight increase of control *O. edulis* (2-3 mm) after 20 days amounted up to 200%, *S. plana* control individuals (2.14 mm long) in this present study only gained 60% of initial weight after 30 days; apart from species-specificity of toxicants, this might be also due to composition of algal diet and differences in the filtration efficiency of the oyster (a suspension-feeder) and the clam (mainly a deposit-feeder). Although data in Appendix 10 are not conclusive, a dose of 80 µgCu/l would also have impaired normal weight gain of *S. plana* spat.

6.4.3. Burying activity.

The act of burial is the result of a series of events (see Trueman, 1983) which demands considerable mechanical energy cost, particularly in bivalves where the entry into the sediment is achieved after drawing the shell vertically (Trueman and Brown, 1992). Even if the energy required for burying into artificial coarser sand was higher than the cost needed to dig in the natural finer deposits usually selected as a settling substratum by juveniles of *S. plana*, unaltered burying time of control individuals through both 1991 and 1992 tests shows that small juveniles (2-3 mm long) were fit enough to pass successfully every 2nd day burying trial after enforced surfacing; this was expected from animals inhabiting the less stable layers of the sediment.

Undetectable levels of organotin in sand at the end of the higher TBT experiment permit to adscribe increased burying time of exposed clams to gradual debilitation induced by dissolved toxicant. On the contrary, levels of Cu in sand (≈ 1 µg/g dry wt) may have activated some behavioural avoidance as a factor contributing with debilitation to the enhanced burying time of animals; however, these levels are much lower that those in the surficial sediments from which the clams were collected (≈ 20 ppm, see Table 3.3), and the possibility that 0.4 ppm Cu (maximum difference between sand in copper bowls and control sand) have added to observed burying time growing disparities seems unlikely.

The progressivity of clam debilitation can be seen particularly from figure 6.8 (copper test): burying time of juveniles exposed to 80 µgCu/l was not affected by day 6, but it was significantly higher than that of controls for every trial thereafter; exposure to 40 and 20 µgCu/l took 24 and 30 days, respectively, to induce significant deviations from control burying activity. Progression of reduced burying performance is also clear in figure 6.7 (high TBT experiment, 1991), where effects of organotin were significant from the first assay (day 6) and considerable by the second day of trial in 2 and 4 µgSn/l if attention is paid to the numbers found on surface before renewal (Appendix 7); these individuals are thought to have emerged after having dug in during the first hour of the experiment.

On the contrary, the effects of lower TBT levels on juvenile burying time are not gradual in the experiment of 1992 (figure 6.12); while linking of groups produced by SNK tests (Table 6.5) is probably due to the intervals of confidence of the respective means overlapping correlatively and could have therefore been avoided by including 3 or more replicates per treatment, no satisfactory argument can be put forward to explain why burying times peak on day 18 for every treatment including control. Whether quality of supply water used was particularly poor around day 18 or there were some exogenous or endogenous cycles resulting in these observations is not known; certainly, none of the latter can be noticed in the data of the previous experiment. It is possible that differential endurance of cohorts of consecutive years have interfered with experimental conditions and/or these with manifestation of subtle sublethal effects of TBT. Differences between

burying time of day 18 and those of other days within the same treatment are more marked in TBT-exposed clams, and as a consequence some of their burying times are significantly different from the baseline defined by the initial sets only in day 18; however, differences between control burying time for every assay (including day 18) and initial burying time are not significant, and juveniles exposed to 500 ngSn/l always buried significantly slower than initial sets. This is consistent with results of the previous year for the same dose, and variations could therefore be attributed to differences of degree; although failure to get buried is the main indication of reduced fitness, some index integrating this with depth of burial achieved could have been useful in explaining variability of the degree of affection should it had been used. Since curtailed burying performance was shown by animals which had actually gained weight with respect to initial sets, whether observed effects resulted from TBT-induced narcotization of animals (as oil was suggested to induce in polychates, Olla *et al.*, 1984) is a contentious question.

6.5. Conclusions.

It has been shown that exposure for a month to ≥ 28 ngSn/l (as analysed) of dissolved TBT significantly reduces the growth rate of juvenile *S. plana* (≈ 2.2 mm long); exposure to ≥ 297 ngSn/l or ≥ 20 µgCu/l also impairs the normal burying performance of spat in sand. The ecological implications of these findings are clear: diminished growth rate will result in prolonging the stay of recruits in shallower sediment layers and, subsequently, their incorporation to the reproductive stock of the population will be delayed. Extended juvenile life will also increase chances of effective loss through dispersal and particularly predation, since, in agreement with the optimal foraging theory, crabs consume more small sized than larger bivalves (Walne and Dean, 1972; Pearson *et al.*, 1981) as a result of increased vulnerability and reduce handling times (Boulding, 1984); the probability of crab predation will be increased further by restrictions on burying speed and depth (Pearson *et al.*, 1981; Blundon and Kennedy, 1982; Haddon *et al.*, 1987).

While the minimum effective copper concentration (20 µgCu/l) is rarely found in estuarine waters (Mance *et al.*, 1984), relatively low TBT levels (> 20 ngSn/l to < 300

ngSn/l) now proved to be sublethally toxic to juveniles of *S. plana* in laboratory experiments were commonly and persistently found in subsurface open waters of estuaries during the 1980s (Waldock *et al.*, 1987; Quevauviller and Donard, 1990; Ritsema *et al.*, 1991), and specifically at field sites where spat have been observed to have disappeared (Langston *et al.*, 1987, 1990); it is therefore concluded that dissolved TBT may well have caused, or contributed substantially to, this demise.

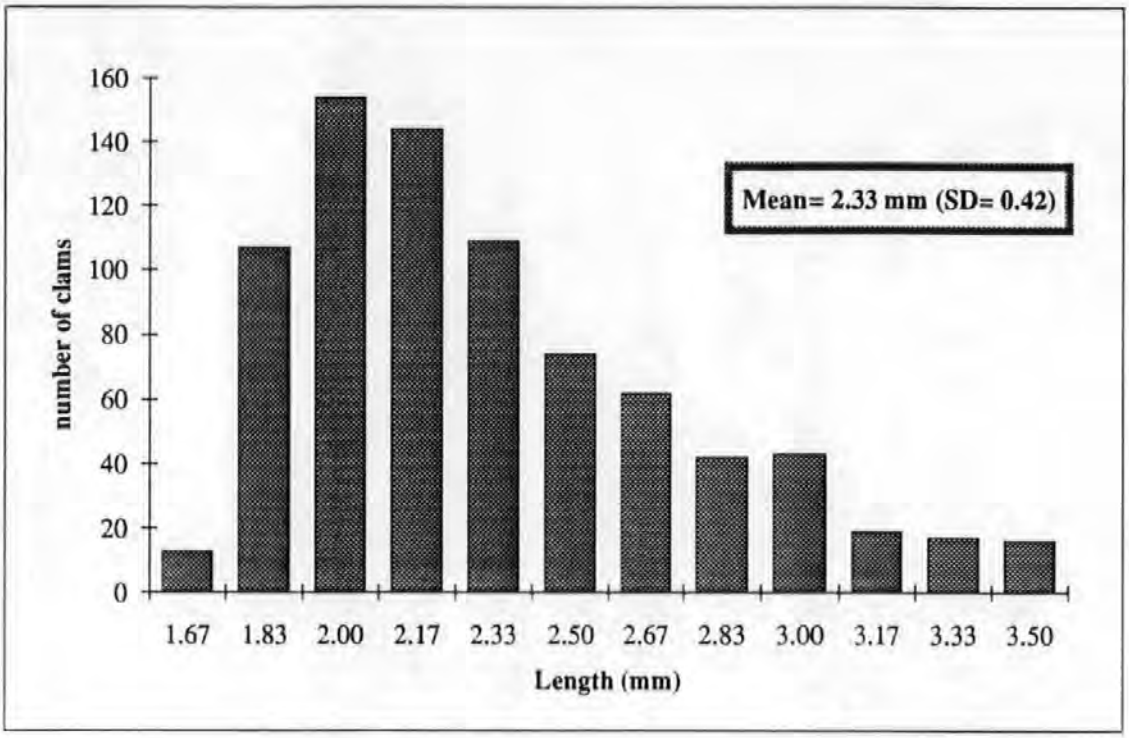


Figure 6.1: *Scrobicularia plana*. Distribution of the 800 juveniles used in the experiment of 1991 into the size classes defined (nearest 166 µm).

Table 6.1: *Scrobicularia plana*. Size classes composition, mean size and standard deviation (mm) of experimental sets of 40 juveniles, year 1991. WC, OC: water and ethanol controls; TBT doses: 0.5, 1, 2 and 4 µgSn/l; Cu doses: 10, 20, 40 and 80 µg/l. All treatments run in duplicate (a and b).

		Size classes													
		1.67	1.83	2.00	2.17	2.33	2.50	2.67	2.83	3.00	3.17	3.33	3.50	Mean	SD
WC	a	0	6	12	7	4	1	0	2	4	1	1	2	2.33	0.50
	b	1	5	7	8	6	3	3	2	2	1	1	1	2.33	0.44
OC	a	1	5	7	8	6	3	3	2	2	1	1	1	2.33	0.44
	b	1	5	7	8	6	3	3	2	2	1	1	1	2.33	0.44
0.5	a	1	5	7	7	6	5	3	2	2	0	1	1	2.33	0.42
	b	1	5	7	7	6	4	4	2	2	1	1	0	2.32	0.40
1	a	1	5	7	7	6	4	4	2	2	1	0	1	2.33	0.41
	b	1	5	7	7	6	4	3	3	2	1	0	1	2.33	0.42
2	a	1	5	7	7	6	4	3	3	2	1	0	1	2.33	0.42
	b	1	5	7	7	6	4	4	2	2	1	1	0	2.32	0.40
4	a	1	5	8	7	5	4	3	2	2	1	1	1	2.33	0.44
	b	1	5	8	7	5	4	3	2	2	1	1	1	2.33	0.44
10	a	1	5	8	7	5	4	3	2	2	1	1	1	2.33	0.44
	b	0	6	8	7	5	4	3	2	2	1	1	1	2.34	0.44
20	a	0	6	8	7	5	4	4	2	2	1	1	0	2.32	0.40
	b	0	6	8	7	5	4	3	2	2	1	1	1	2.34	0.44
40	a	0	6	8	7	5	4	4	2	2	1	1	0	2.32	0.40
	b	0	6	8	7	5	4	3	2	2	1	1	1	2.34	0.44
80	a	0	6	8	7	5	4	3	2	3	1	1	0	2.33	0.41
	b	1	5	7	8	6	3	3	2	2	1	1	1	2.33	0.44

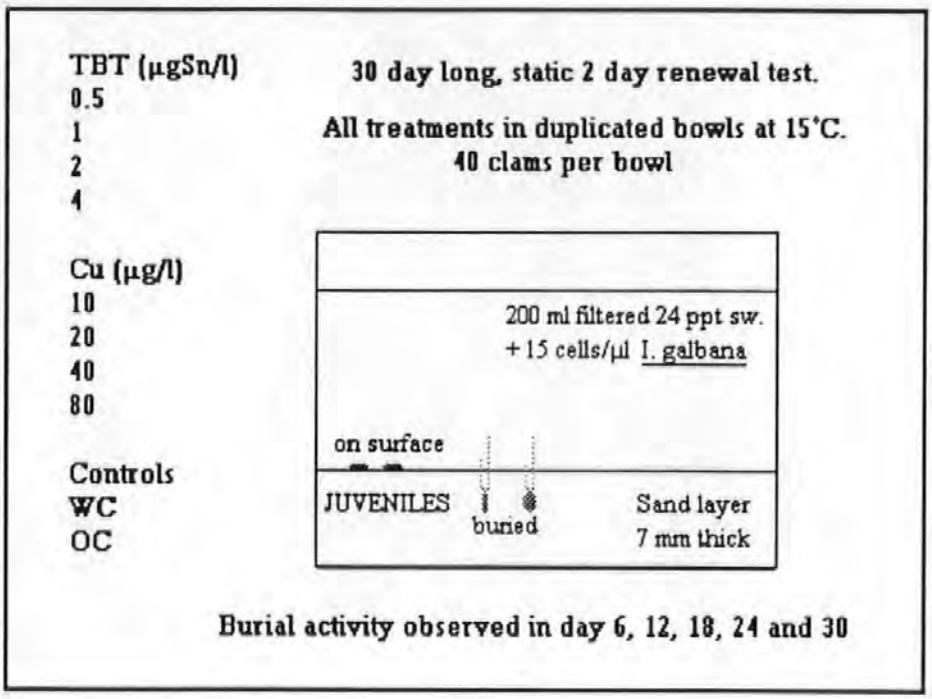


Figure 6.2: Diagram illustrating conditions and procedure applied to juveniles of *Scrobicularia plana* during the experiment of 1991.

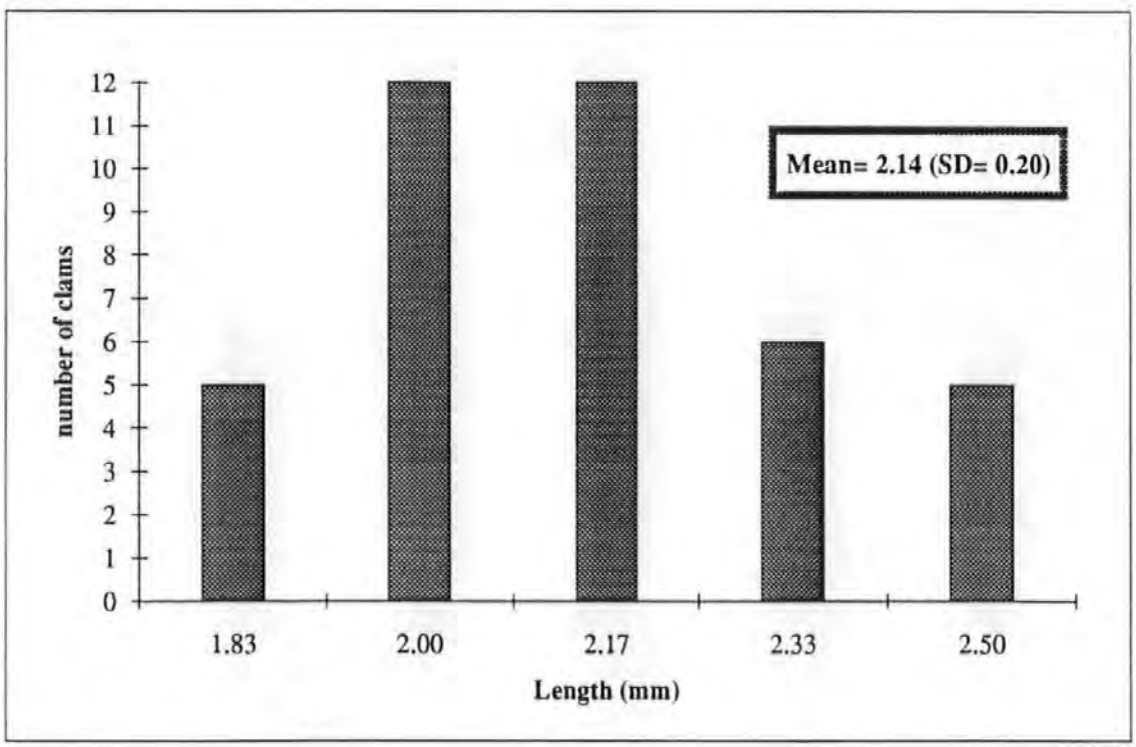


Figure 6.3: *Scrobicularia plana*. Size classes composition, mean size and standard deviation (mm) of experimental sets of 40 juveniles, year 1992. Every group out of 12 (2 to estimate initial parameters plus duplicated treatments of ethanol control and 50, 125, 250 and 500 ngSn/l) had the same composition. This also applies to the other 8 sets used in a sediment toxicity bioassay (see text).

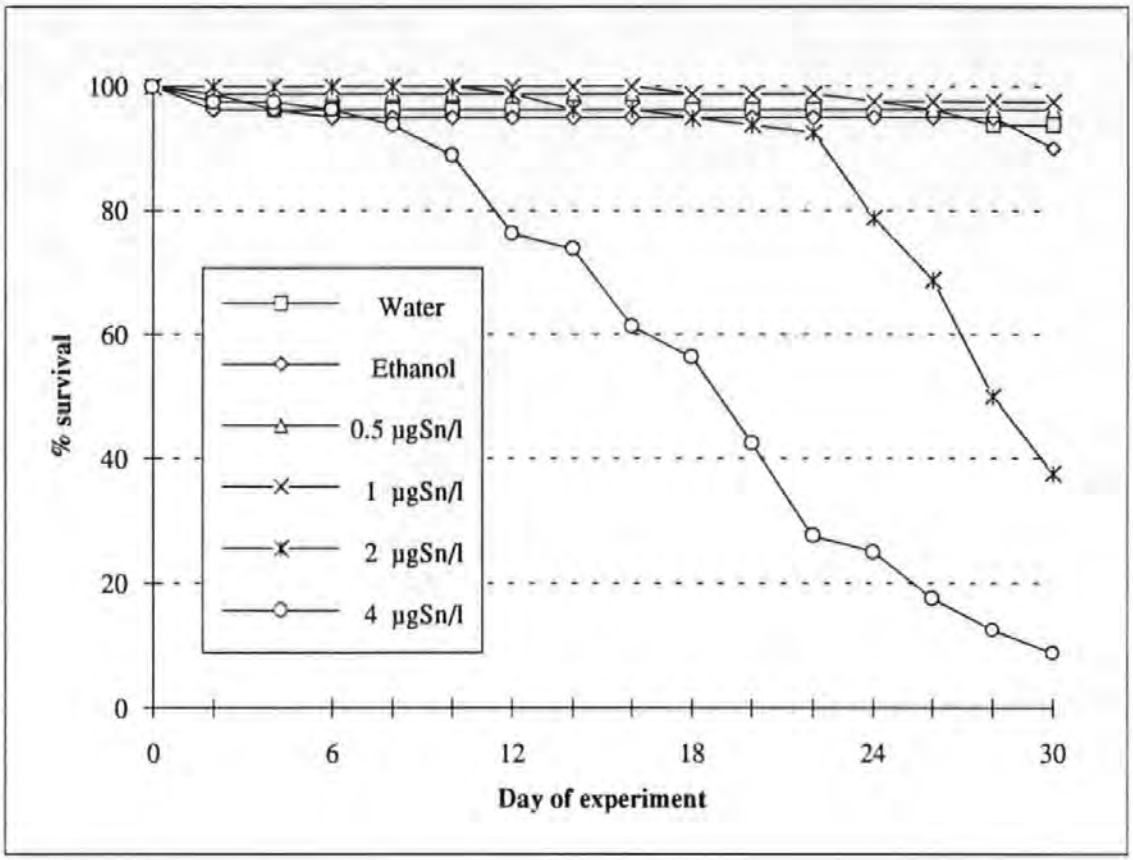


Figure 6.4: *Scrobicularia plana.* % survival (mean of duplicates) of juveniles in control (water and ethanol) and TBT treatments during the experiment of 1991.

Table 6.2: *Scrobicularia plana.* ANOVA of % survival of juveniles after exposure to TBT doses (0.5, 1, 2 and 4 µgSn/l) and water (WC) and ethanol (OC) controls treatments during (a): 12 days, (b): 24 days and (c): 30 days.
The variances were homogeneous: (a) C < C.95(6,1), (b) C < C.99(6,1), (c) C < C.95(6,1).

Source	df	MS	*F*	Pr > F
(a): Day 12				
Dose	5	160.8	19.3 **	0.0012
Error	6	8.3		
SNK: 1 = 2 = 0.5 = WC = OC > 4				
(b): Day 24				
Dose	5	1426	60 ***	0.0001
Error	6	24		
SNK: 0.5 = 1 = WC = OC > 2 > 4				
(c): Day 30				
Dose	5	2886	61 ***	0.0001
Error	6	47		
SNK: 1 = 0.5 = WC = OC > 2 > 4				

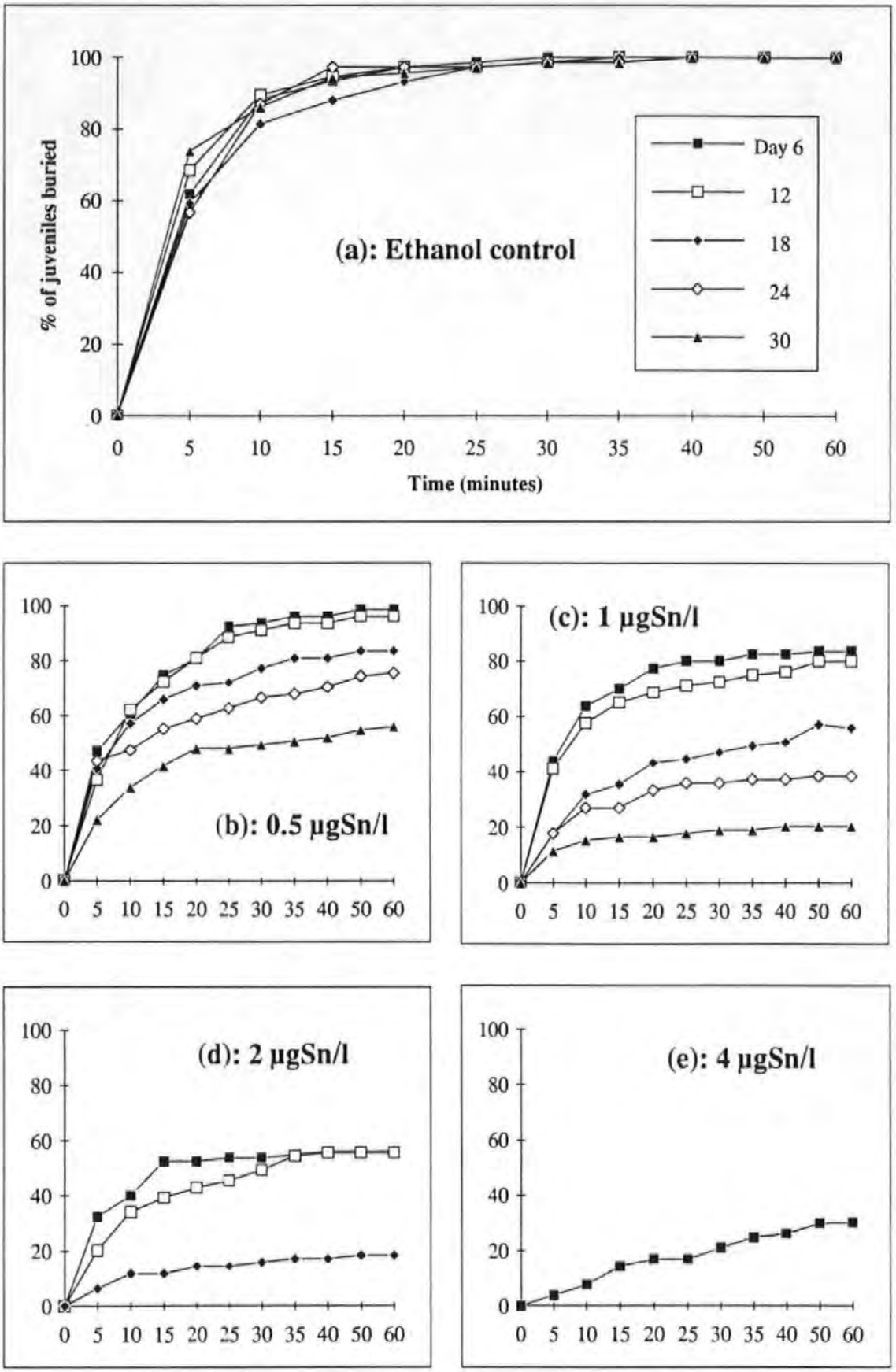


Figure 6.5: *Scrobicularia plana.* Every-6th-day reburying activity (mean of duplicates) displayed by juveniles exposed to TBT, expressed as ([no. buried/clams surviving to that time] . 100). Axes and symbols in (b), (c), (d) and (e) as in (a). No data are plotted for 2 and 4 µgSn/l after day 18 and 6, respectively, because survival was significantly affected.

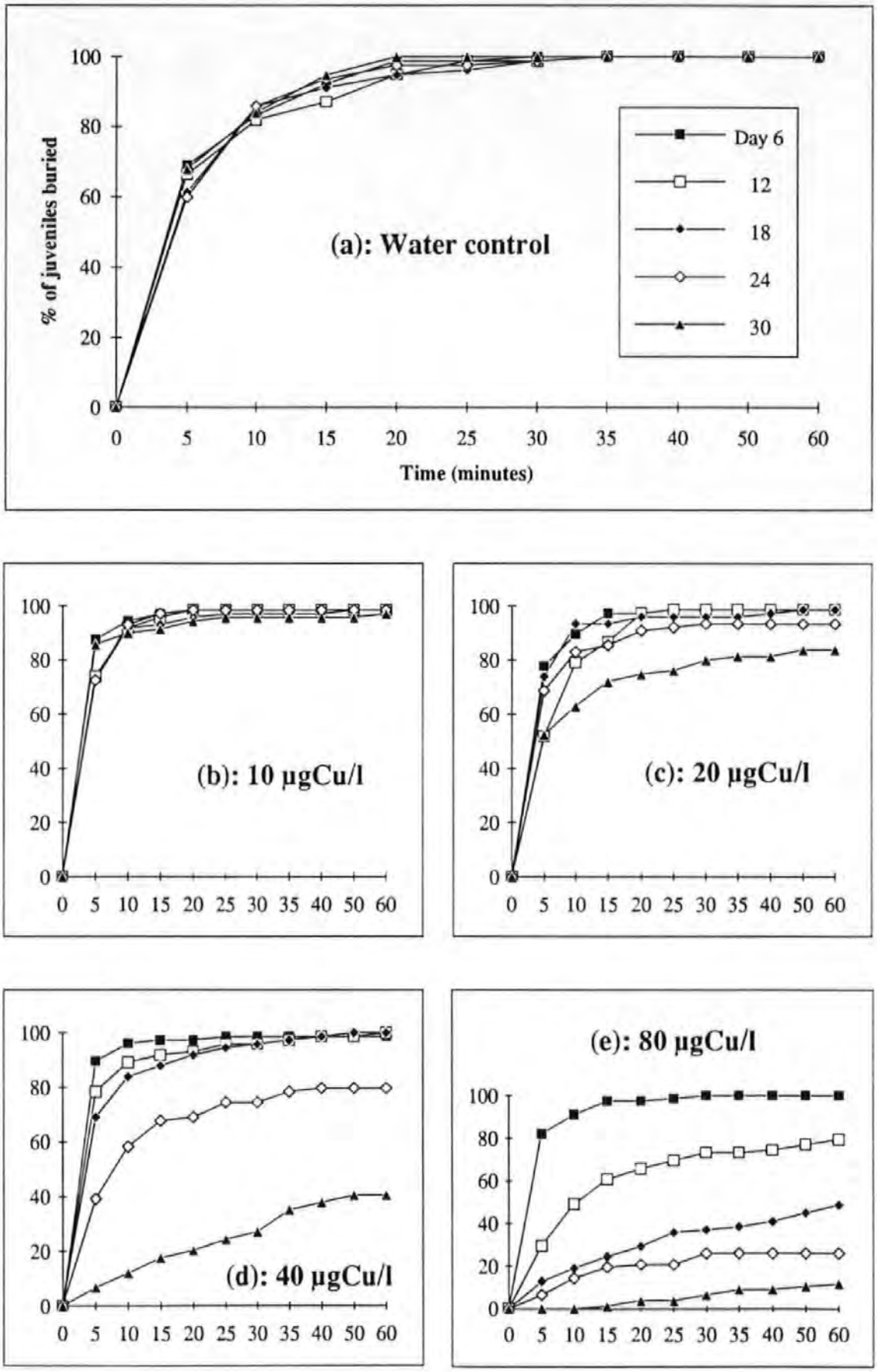


Figure 6.6: *Scrobicularia plana.* Every-6th-day reburying activity (mean of duplicates) displayed by juveniles exposed to copper, expressed as ([no. buried/clams surviving to that time].100). Axes and symbols in (b), (c), (d) and (e) as in (a).

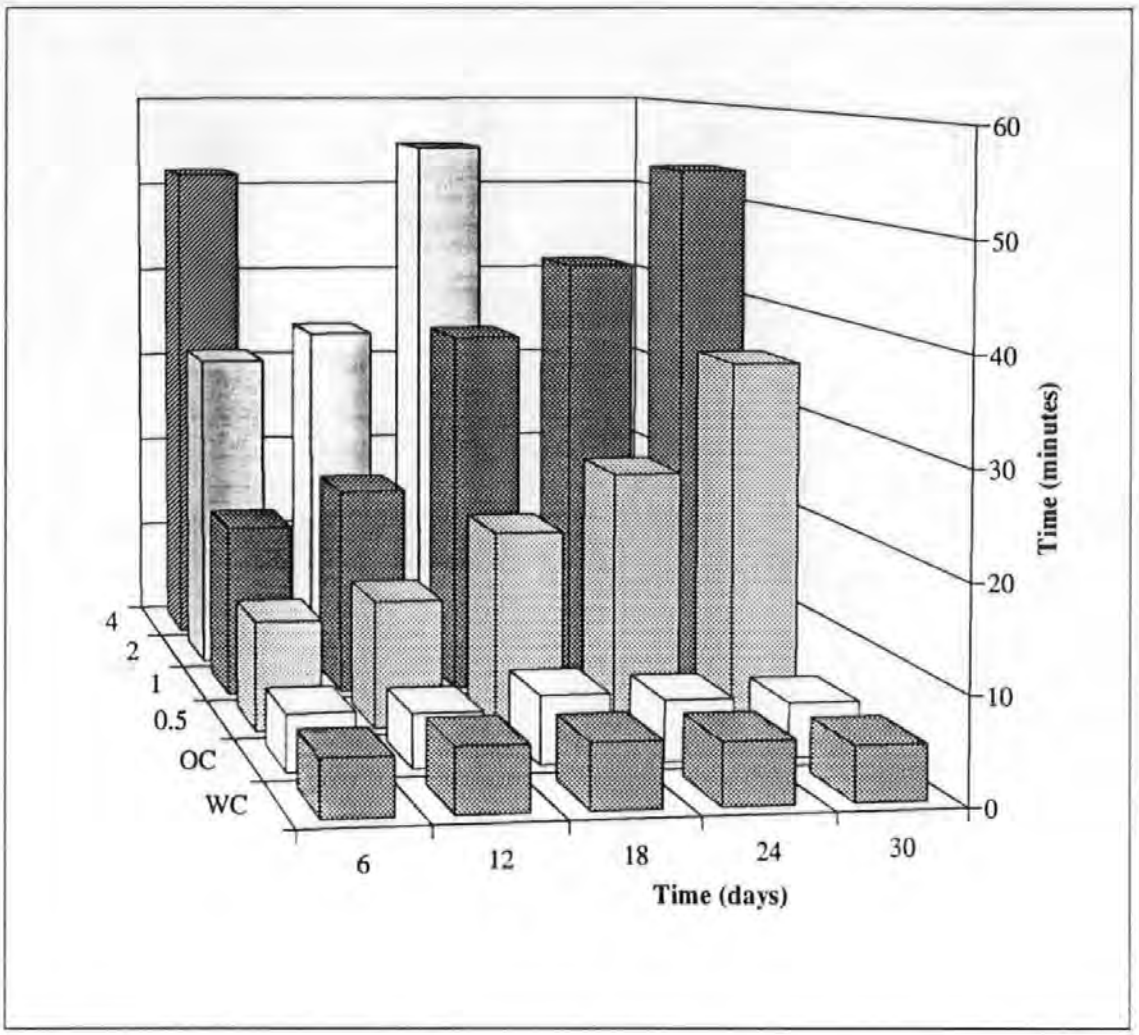


Figure 6.7: *Scrobicularia plana.* Estimated every-6th-day burying time (mean of duplicates) of juveniles after exposure to TBT (0.5, 1, 2 and 4 µgSn/l) and water (WC) and ethanol (OC) control treatments in the experiment of 1991. No data are plotted for 2 and 4 µgSn/l after day 18 and 6, respectively, because survival was significantly affected.

Table 6.3: *Scrobicularia plana.* ANOVA of burying time of juveniles after exposure to TBT doses (0.5, 1, 2 and 4 µgSn/l) and water (WC) and ethanol (OC) control treatments in the experiment of 1991 during 6, 12, 18, 24 and 30 days, respectively for (a), (b), (c), (d) and (e).
All variances were homogeneous: (a), (b), (c) and (d): C < C.95(6,1); (e): C < C.99(6,1).

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

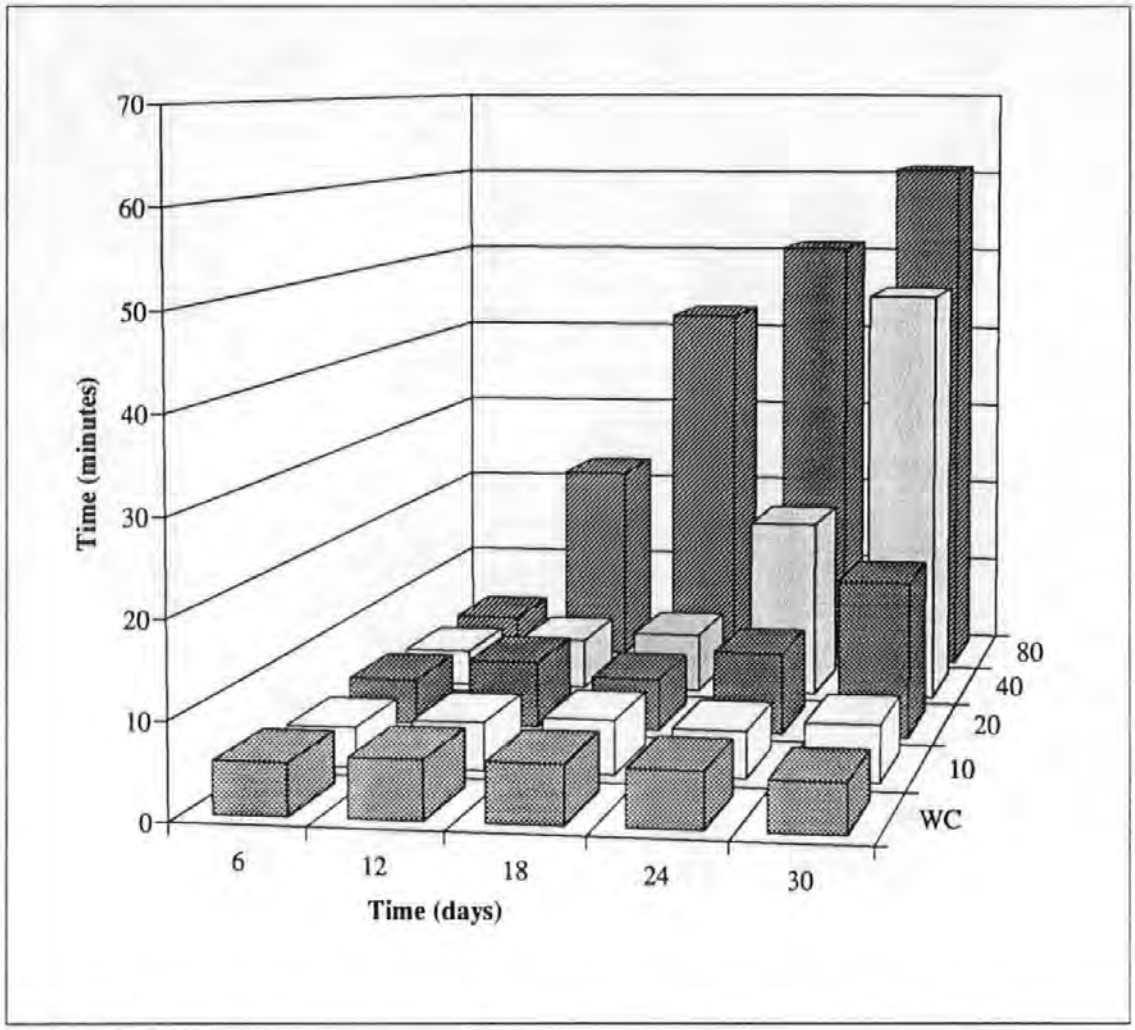


Figure 6.8: *Scrobicularia plana.* Estimated every-6th-day burying time (mean of duplicates) of juveniles after exposure to copper (10, 20, 40 and 80 µg/l) and water (WC) control treatments in the experiment of 1991.

Table 6.4: *Scrobicularia plana.* ANOVA of burying time of juveniles after exposure to copper doses (10, 20, 40 and 80 µg/l) and water (WC) control treatment in the experiment of 1991 during 6, 12, 18, 24 and 30 days, respectively for (a), (b), (c), (d) and (e).
All variances were homogeneous: (a), (c), (d) and (e): C < C.95(5,1); (b): C < C.99(5,1).

	Source	df	MS	*F*		Pr > F	
(a): Day 6	Dose	4	0.71	0.8	NS	0.5844	
	Error	5	0.92				
(b): Day 12	Dose	4	112	8.7	*	0.0180	SNK: 80 > 20 = WC = 40 = 10
	Error	5	13				
(c): Day 18	Dose	4	537	271	***	0.0001	SNK: 80 > 40 = WC = 10 = 20
	Error	5	1.98				
(d): Day 24	Dose	4	749	64	***	0.0002	SNK: 80 > 40 > 20 = WC = 10
	Error	5	11.7				
(e): Day 30	Dose	4	1277	245	***	0.0001	SNK: 80 > 40 > 20 > 10 = WC
	Error	5	5.21				

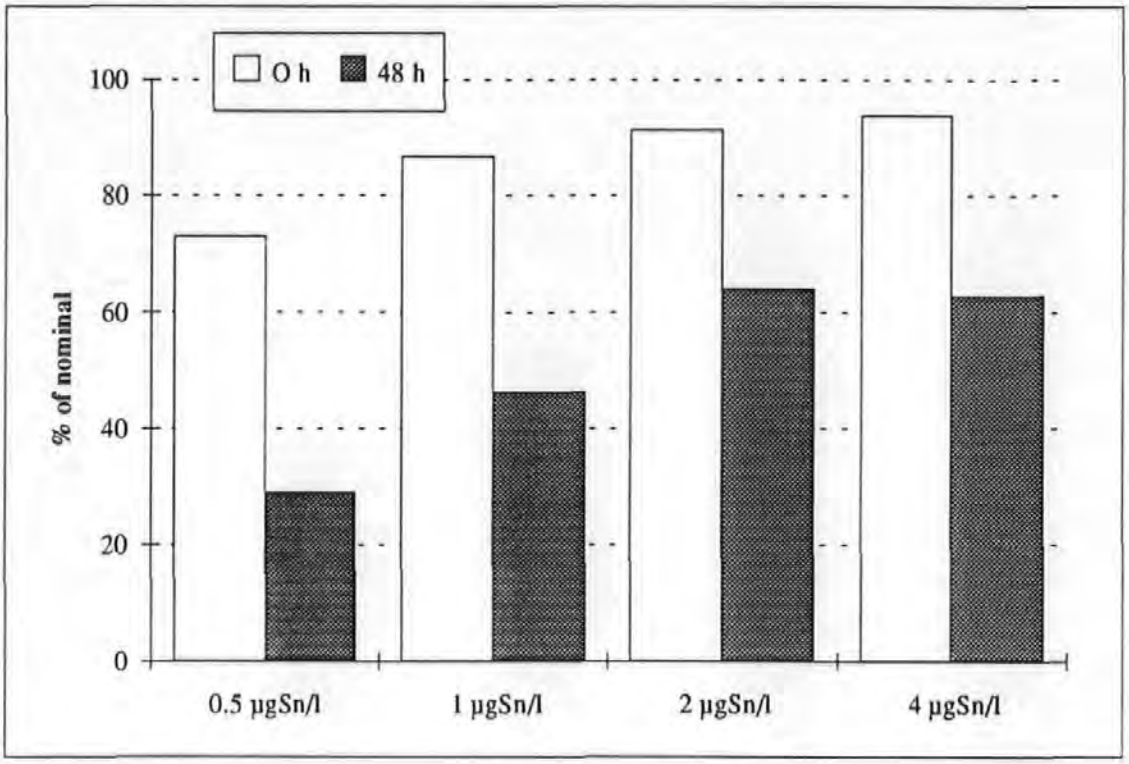


Figure 6.9: Initial (0 h) and final (48 h) tin concentrations analysed in treatments 1991 (48 h solutions after used on day 20 of experiment) expressed as % of nominals. TBT in controls was not detectable.

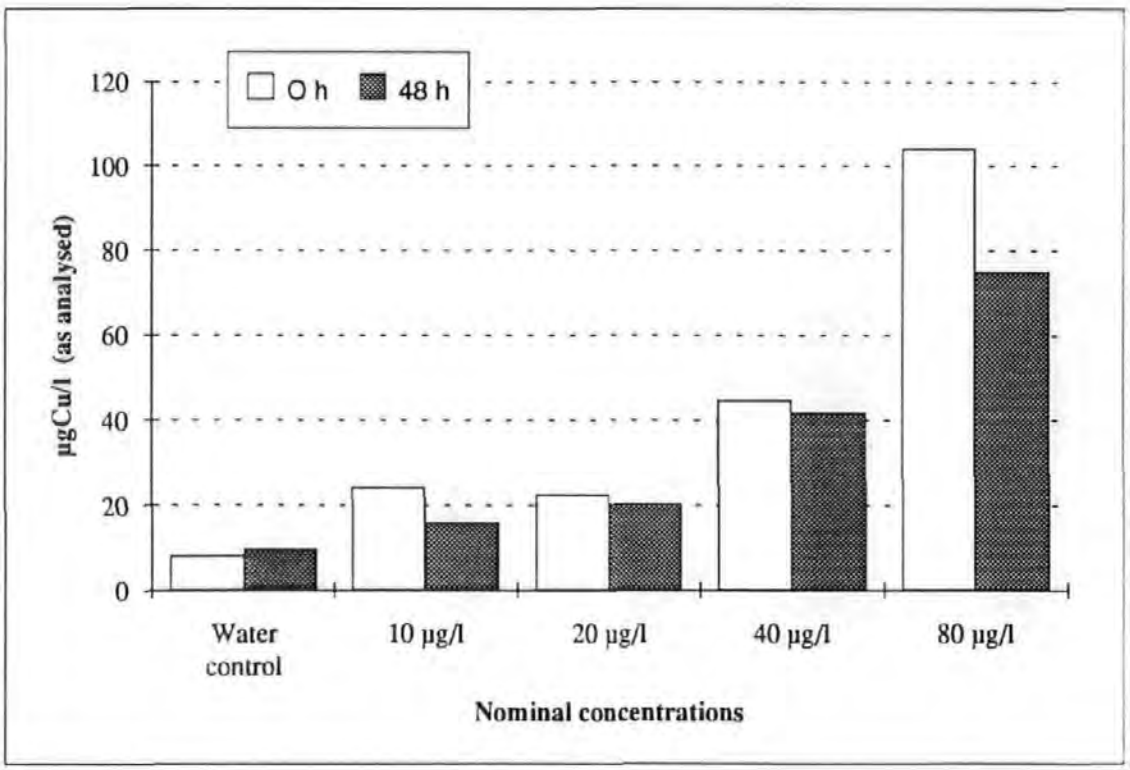


Figure 6.10: Initial (0 h) and final (48 h) copper concentrations as analysed in treatments (48 h solutions after used on day 20 of experiment).

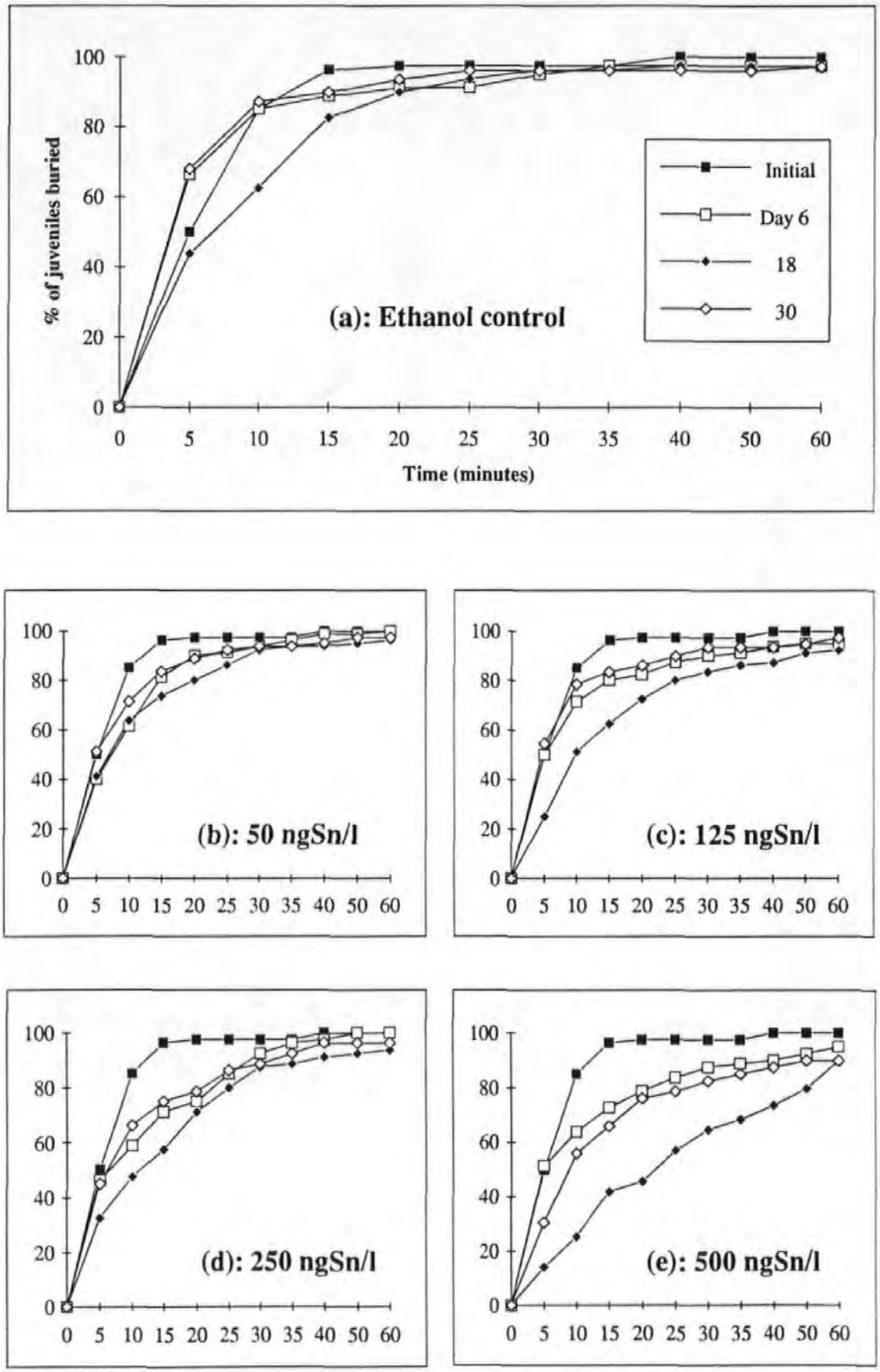


Figure 6.11: *Scrobicularia plana*. Reburying activity (mean of duplicates) displayed by juveniles exposed to TBT, expressed as ([no. buried/clams surviving to that time].100). Axes and symbols in (b), (c), (d) and (e) as in (a). For further explanation see text.

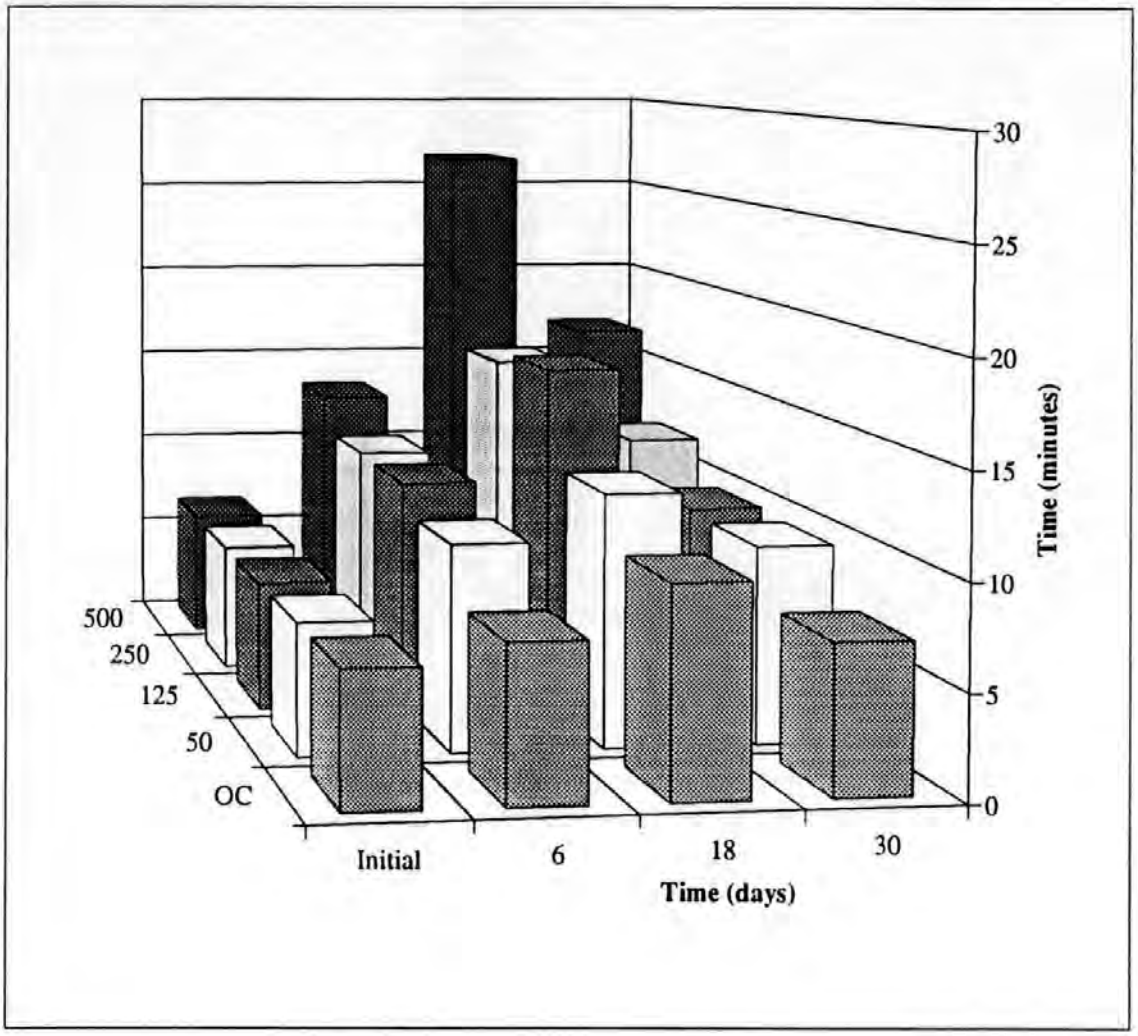


Figure 6.12: *Scrobicularia plana.* Estimated mean burying time of juveniles after exposure to TBT (50, 125, 250 and 500 ngSn/l) and ethanol (OC) control treatments in the experiment of 1992.

Table 6.5: *Scrobicularia plana.* ANOVA of burying time of juveniles after exposure to TBT doses (50, 125, 250 and 500 ngSn/l) and ethanol (OC) control treatment in the experiment of 1992 during 6, 18 and 30 days, respectively for (a), (b) and (c). SNK: treatments not underlined by the same line are significantly different at $p < 0.05$. All variances were homogeneous: $C < C.95(6,1)$.

	Source	df	MS	*F*		Pr > F							
(a): **Day 6**	Dose	5	13.3	5.3	*	0.0332	SNK:	500	250	125	50	OC	Initial
	Error	6	2.51										
(b): **Day 18**	Dose	5	99.6	32	***	0.0003	SNK:	500	125	250	50	OC	Initial
	Error	6	3.07										
(c): **Day 30**	Dose	5	28.1	5.7	*	0.0284	SNK:	500	250	125	50	OC	Initial
	Error	6	4.96										

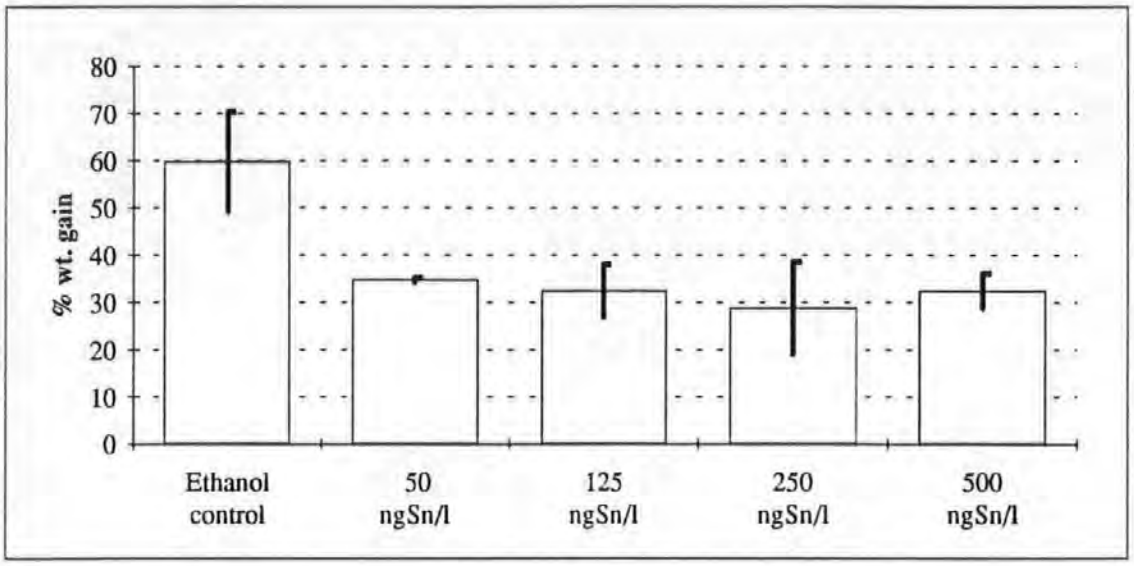


Figure 6.13: *Scrobicularia plana.* Growth of juveniles after exposure to TBT for 30 days, expressed as mean (± SD) % weight gained (corrected for mortalities) in relation to weight of initial sets.

Table 6.6: *Scrobicularia plana.* ANOVA of % weight gained by juveniles (in relation to weight of initial sets) after exposure to TBT (50, 125, 250 and 500 ngSn/l) and ethanol control (OC) treatments for 30 days in the experiment of 1992. The variances were homogeneous: C < C.95(5,1).

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

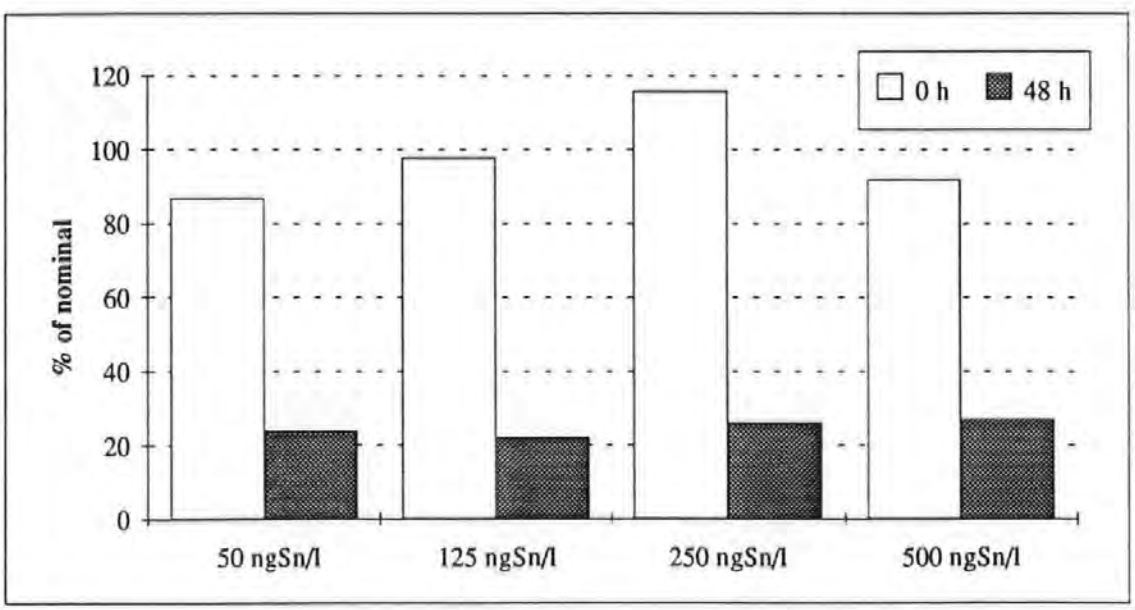


Figure 6.14: Initial (0 h) and final (48 h) tin concentrations analysed in treatments 1992 (48 h volumes after used on day 24 of experiment) expressed as % of nominals. TBT in controls was not detectable.

Chapter 7: The *Scrobicularia plana* juvenile sediment toxicity bioassay.

7.1. Introduction.

It has been shown in the previous chapter that exposure of *S. plana* spat to low levels of dissolved TBT results in some biological effects of environmental relevance to the field situation described in chapter 1. However, holding of clams in artificial sand ignored a critical factor in the juvenile stage of a deposit-feeding species: the sediment itself; this represents the primary source of food and, subsequently, heavy metals (Luoma and Bryan, 1982) and TBT (Langston and Burt, 1991). To overcome that deficiency, the solid-phase sediment toxicity bioassay described below was specifically designed to assess the suitability of field sediments for the development of *Scrobicularia plana* juveniles. Of those approaches addressing sediment quality (see for instance Power and Chapman, 1992), assessment of overall toxicity was selected because it integrates the effects of real-world substrata. Although examination of selected biological end-points (survival, growth and burying activity) was the main concern of the test, chemical analyses were also made in an effort to identify the cause of detrimental effects.

7.2. Material and methods.

7.2.1. Bioassay sediments and chemical analyses.

All 4 bioassay sediments were collected from the top 1 cm surficial layer at mid-intertidal level in the selected sites, and kept frozen within polythene bags up to 5 days before the beginning of the test (day -5). There were 2 negative (i.e. relatively uncontaminated) control sediments, collected at sites in the Torridge (July 1991) and Guernica (December 1991) estuaries; both localities support apparently healthy populations of *S. plana* (see chapters 3 and 2, respectively). Sediment from Lamiaco was also kept frozen since December 1991; it was included in the bioassay as a heavy metal contaminated sample which, despite sustaining a clam population (with juvenile recruits, see chapter 2), might not be totally innocuous to non-native animals. Finally, Cracknore

sediments were collected at a site (O.S. Grid reference SU404110, Test estuary, Southampton Water) which surficial layers had been found to contain considerable levels of TBT (mean 0.5 µgSn/g dry weight) but no *S. plana* spat since 1986 (at the latest); however, this mudflat by a dry dock facility used to host a consistent clam population up to 1978 which had died out by 1986 when its only representatives (a few old individuals) contained around 3 µgSn/g dry wt. (G.W. Bryan and P.E. Gibbs, personal communication). Since highest organotin concentrations usually occur around the summer months (see for instance Langston *et al.*, 1987) and this is the season of most active deposit-feeding in *S. plana* (Hughes, 1969; Zwarts and Wanink, 1989), Cracknore TBT-polluted sediments for bioassay were collected in July 1991.

Chemical extraction and analyses for 10 heavy metals were performed on independently frozen aliquots of the fine portion of sediments (< 100 µm) as detailed in section 2.2; the method used for estimating their organic content can also be found in that section. Content of organotin species (TBT and DBT) in separate aliquots of fine sediments was determined following the standard procedure summarised in section 3.2.6.

Although no attempt was made to determine metallic content of interstitial water, it was deemed desirable to have an estimation of the concentration of selected heavy metals (Cd, Cu, Pb and Zn) and TBT desorbed from sediments and leached into overlying water. To this end, volumes renewed in day 18 were pooled per treatment, and the resulting ≈ 500 ml (see section 7.2.3 below) were divided and analysed as follows: 400 ml per treatment were acidified and stored in the dark for TBT determination carried out as detailed in Bryan *et al.* (1986) and summarised in section 4.2.2.; heavy metal levels were directly analysed on small volumes of acidified sample in a Varian SpectrAA-20 flame (Pb, Zn) or a Varian SpectrAA-300 Zeeman graphite furnace (Cd, Cu).

7.2.2. Bioassay specimens and experimental set up.

Bioassay animals were collected as usual from the Torridge estuary in winter (February 1992), the season when in U.K. waters small clams occur in abundance and permit collection of a sufficient number of specimens. Juveniles of *Scrobicularia plana* were distributed into 8 sets (40 clams each, 2 sets per sediment treatment) of identical size classes composition (mean length 2.14 mm, see

figure 6.3) as detailed in section 6.2.2.1; weight of initial sets and of size classes individuals was also that estimated for the low-TBT experiment of the previous chapter (see Appendix 12.A), but initial burying time in sand is not pertinent for the present case.

On day -5, frozen sediments were taken to the constant temperature room (15 ± 1 ºC, 12 h illumination per day) where the bioassay was to be conducted for 36 effective days. On day -4, thawed sediments were sieved through a 500 µm pore mesh to retain large flocks and allowed to deposit overnight in plastic beakers with 24 ppt sea water. On day -3 (field trip to collect juveniles) the overlying water was decanted off and filtered (0.45 µm) 24 ppt sea water (FSW) added to settled sediments in beakers to make up a stock slurry ≈ 2:1 (sediment to FSW, by volume); aliquots (5 ml) of these stock slurries were taken to estimate dry weight of sediment samples used in bioassay. Experimental chambers were Pyrex cylindrical bowls (9 cm Ø) which had been previously cleaned with detergent, acid and twice rinsed in distilled water as usual; there were 4 bowls per sediment treatment. Stock slurry (60 ml) was poured in bowls and volume brought to 240 ml by adding 180 ml FSW to each container. Bowls were capped with a lid, and a plastic pipette tip connected to an airline was inserted through a hole in the cap so that a continuous but gentle flow of bubbles came out of the tip suspended ≈ 1 cm below the water surface; the outflow of the air pump used was passed through two wash bottles (one empty, the second with distilled water) before it reached the tips to ensure air of good quality. Bowls were then left undisturbed until day 0 when water in bowls was clear and sediments had deposited and formed a layer ≈ 1 cm thick.

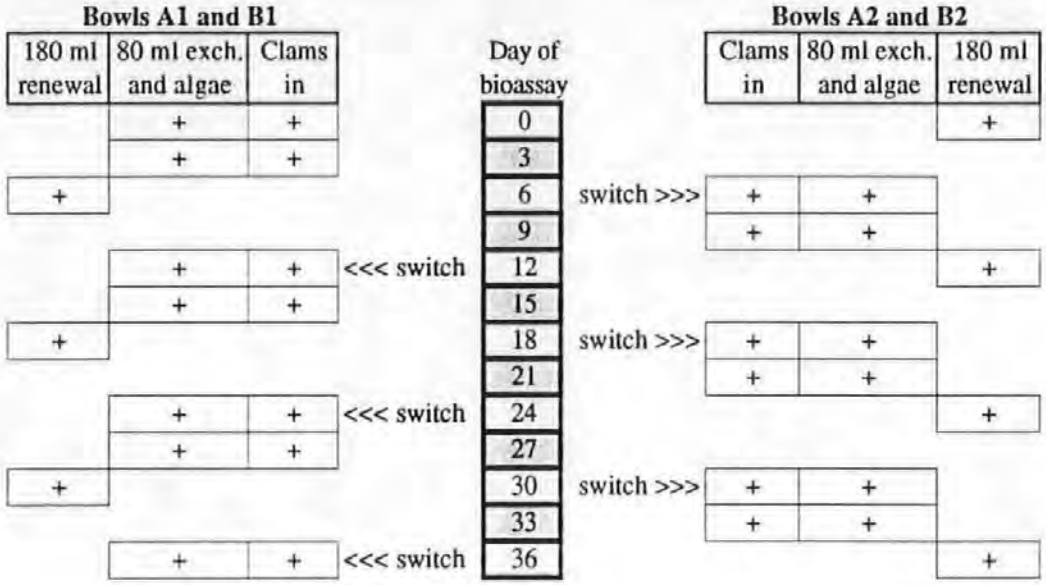
7.2.3. Initiation, procedure and termination of bioassay.

There were duplicated clam sets (A and B) to test each sediment, but 4 replicated bowls with sediment (A1, A2, B1 and B2) per treatment; this was designed to allow observation of juvenile burying activity by switching clams between bowls labelled with the same letter every 6th day as follows: on day 0 one set of animals was introduced in each of 2 bowls per treatment (A1 and B1), and the number of juveniles failing to be totally buried recorded at lapses of 2.5 min for the first 10 min, then every 5 min up to 40 min, and then at 10 min intervals to complete 1 h observation; clams were then left

undisturbed until day 6. On this day, all animals within a bowl (labelled A1 or B1) were sieved out of the sediment with a tea strainer, carefully observed under a microscope and dead animals removed for their later identification and measurement; finally, live A1 and B1 juveniles were introduced in bowls labelled A2 and B2, respectively, and burying time monitored for 1 h. On day 12, animals were dug out from sediment in bowls A2 and B2 and, after checking and removing dead ones, similarly switched to bowls A1 and B1, respectively. Alternation of bowls containing juveniles and burying assay was repeated on days 18, 24, 30 and 36, so that this last day of test animals buried into sediment in bowls A1 and B1 as they did on day 0 (see Table 7.1 in this section for summary of procedure). After 1 h burying trial on day 36, clam sets were dug out again, sized to the nearest 166 µm, blotted dry for 2½ h, weighed and frozen.

Partial exchange or total renewal of water was applied to bowls every 3 or 6 days as follows (see also Table 7.1): on days when a certain bowl was going to receive animals (including bowls 1 on day 0), one third of the total volume (i.e. 80 ml) was siphoned out beforehand and fresh FSW (80 ml) introduced carefully to produce minimal disturbance on settled sediment. On days when clams were going to be sieved out of a certain bowl and also in bowls 2 on day 0, as much overlying water as possible was decanted beforehand to a graduated cylinder trying not to discard any sediment nor clam; then, this sediment containing clams was added the same amount of FSW as collected in cylinder, plus distilled water to reach a total of 180 ml. The resulting slurry plus animals was sieved through the tea strainer to a transient bowl; then, the slurry (animals were retained in sieve) was poured back to the original bowl and left undisturbed till the next 6th day when it received clams again. On day 3 of experiment and every 6th day thereafter, 80 ml of overlying water were exchanged with equal volume of FSW as above only in bowls containing animals; pH and salinity was measured in the 160 ml resulting from pooling per treatment the volumes siphoned out of bowls. When 80 ml FSW were exchanged in bowls with or to receive clams. ≈ 2700 thousand cells of the flagellate *Isochrysis galbana* were also added as supplementary food to render a minimum of ≈ 15 cells/µl final concentration in bowls.

Table 7.1. Summary of procedure applied to sediment bioassay bowls; shadowed cells indicate days when pH and salinity were measured in the 160 ml water exchanged per treatment.



7.3. Results.

7.3.1. Sediment chemistry.

The concentrations of heavy metals detected in bioassay sediments, their estimated dry weight and their organic content are given in Table 7.2.A. Total levels (i.e. extracted with nitric acid) are generally highest in Lamiaco sediments, although some metals reach maxima in deposits from Torridge (Co, Mn), Guernica (Ag, Ni) and Cracknore (Cu, Pb); separate aliquots were extracted with 1 N hydrochloric acid to characterize the fraction of metals potentially bioavailable in sediments (Luoma and Bryan, 1981). Dry weight of samples ranged from 31 to 43 g, and their organic content from 3.5 to 5%. Butyltin levels in sediments (Table 7.2.B) were undetectable in samples from both control sites (Torridge and Guernica), low in substratum from Lamiaco and high in Cracknore deposits (i.e. TBT + DBT = 0.447 µgSn/g dry weight).

7.3.2. Water chemistry and quality.

Concentrations of Cd, Cu, Pb and Zn in bioassay overlying waters renewed on day 18 and pooled per treatment are given in Table 7.3; while Pb was not detected in any sample, maximum levels were detected in water from Lamiaco bowls for every other metal, notably Zn (447 µg/l). TBT in overlying waters (Table 7.3) ranged from below

detection limits in control samples to 36 ngSn/l in that of Cracknore; Cd, Cu, Zn and TBT K_p (sediment-water partition coefficient) values are also given for the total content of sediments in each treatment.

Values of pH and salinity in overlying waters as monitored throughout the bioassay are compiled in Appendix 14; in every case they remained consistent at ≈ 8 and ≈ 24 ppt, respectively.

7.3.3. Toxicity of Lamiaco sediments.

When juvenile clams were first deposited on Lamiaco sediments and observed for 1 h (see Appendix 16), some clear avoidance behaviour was displayed. There were some animals which probed the sediment with the foot but did not attempt to dig into it; rather, they started crawling on the sediment surface, and some of them reached and climbed up walls of the vessel to drift away on the water surface by means of fine translucent byssus threads. However, after extensive probing, most of the juveniles had buried into the sediment by 10 minutes. This burial was not permanent since a number of clams were observed to dig themselves out shortly afterwards (see Appendix16); once unearthed, they crawled all over the sediment for long periods, stopping intermittently to probe the substratum.

When bowls were checked on day 3, only few juveniles were observed to be unburied (see Appendix 14). The inhalant siphon of some buried individuals was fully extended, probably filtering the overlying waters; no siphon was seen to be cropping the sediment around the burrow entrance and, after close observation, no mark was found indicating that cropping (and therefore ingestion) of sediment had occurred at all. The sediment surface was smooth and untouched, except for a number of tracks left by the clams when crawling throughout. No juvenile was found to be dead on day 6, and when they were switched to bowls A2 and B2 reactions to toxic sediments were less intensive than in day 0. Many of the clams which managed to bury entered the substratum obliquely, and some did not stayed beneath for long; however, periods of crawling were brief and for shorter distances than on day 0. On day 9 ≈ 50% of clams were found unburied, and when checked on day 12 heavy mortalities (mean 82.5%) had occurred.

Mortality increased slightly after that day, and only 6 clams survived in one of the 2 replicated Lamiaco bowls until the end of the bioassay (see Appendix 14); no trace of cropping and ingestion of sediment was found in containers for the duration of the test.

7.3.4. Toxicity of Cracknore sediments.

Torridge, Guernica and Cracknore sediments were readily accepted as burying substrata by juvenile *S. plana*, and most clams had dug in by 2.5 minutes with apparent ease. Burial was permanent and only very few individuals were observed to be unearthed throughout the test (see Appendix 14).

Many clams displayed siphon activity shortly after burying, sampling and cropping the sediment around the burrow entrance; dark particles of sediments could be seen being ingested through the white-translucent siphons. As a result of this feeding activity, all the sediment surface in the six bowls considered had been processed in a few days, presenting an uneven and finely granulated appearance; it also showed abundant distinct formations of sediment ejected through the exhalant siphon (i.e. pseudofaeces) and fecal pellets. All sediment bowls with clams showed this same aspect for the duration of the test. Growth of juvenile sets after 36 days exposure to bioassay sediments was calculated as detailed in section 6.3.2.2 for the parallel low-TBT toxicity test; blotted dry weight of clam sets at conclusion of bioassay (Appendix 15) was expressed as a percentage of the mean weight of initial sets (Appendix 12.A) after correcting for the individual weight (see also Appendix 12.A) of the very rare mortalities occurred (Appendix 14). When transformed data were analysed by ANOVA and SNK test (Table 7.4.A), animals in Cracknore sediments were shown to have gained significantly less weight (mean 36%) than those in either Torridge or Guernica treatments (mean gain 196% and 140%, respectively). Growth also resulted in the presence of new rings in the shells of most individuals (see Appendix 15 for final length of sets). Assuming that all clams in a given set grew at a constant and common rate throughout the experiment, the individual growth rate could be calculated to have been ≈ 12 µm/day in control treatments while only ≈ 4 µm/day in Cracknore sediments (see Appendix 15).

Appendix 16 displays the burying behaviour of each lot of clams during the every-6th-day 1 h assay. Using the formula described in section 6.3.1.2, original data were transformed and the average burying time calculated for each individual observation; results are given in Appendix 16 and plotted in figure 7.1. While mean burying time in both control sediments was always kept below 4 minutes, burying time of Cracknore-treated clams exceeded that time from day 24 onwards. ANOVA displayed no significant difference between mean burying time of clams in day 0 but it showed that juveniles exposed to Cracknore sediments buried significantly slower on day 36 than those treated with either control sediment (Table 7.4.B and C, respectively).

7.4. Discussion.

Heavy metal polluted sediments from Lamiaco were acutely toxic to juveniles from the Torridge *S. plana* population, killing more than 80% of animals in 12 days. Sediments first induced some remarkable instances of clam avoidance behaviour (emergence, crawling, drifting), suggesting that clams would have moved into a sediment of a lesser toxicity should it have been available (see McGreer, 1979); later reduction in the refusal response is considered to be a result of diminished condition and fatigue of individuals rather than of amelioration of toxic conditions in bowls. TBT-polluted sediments from Cracknore did not elicit any avoidance response nor any mortality in *S. plana* juveniles, but they were chronicly toxic in that Cracknore-exposed clams grew significantly less than juveniles in control treatments; in addition, after 36 days exposure, animals buried more slowly into Cracknore sediments than into control substrata.

These results may be subject to criticism because the bioassay suffers 2 of the 4 limitations most sediment toxicity tests have: i) disruption of sediment geochemistry and the kinetic activity of bedded contaminants through sampling, storage and handling, and ii) toxicological uncertainties (Lamberson *et al.*, 1992). As for the first point, collection of intertidal surficial sediments at low tide is possibly the least disturbing sampling method that can be applied, and handling as described above mimics to some extent resuspension of the most unstable and oxic sediment layers. However, storage procedure is likely to have affected the toxicity of some contaminants (see for instance Schuytema

et al., 1989), but it was selected because freezing is the best possible way to store sediments for several months as required by bioassay characteristics; in addition, no storage technique totally respects integrity of field samples, and frozen sediments have been shown to be reasonably stable for their heavy metal and organotin content (Thomson *et al.*, 1980, and Quevauviller and Donard, 1990, respectively). It is therefore recognised that observations need to be confirmed by repeated trials using fresh sediments. In investigating the cause of toxicity of coastal sediments, we first encounter the problem of determining the actual exposure levels of, perhaps, thousands of chemicals and, secondly, quantifying the route of exposure to select those phases of concern for a given biological species. Since some sediments from the banks of Bilbao (the heavily industrialised estuary where Lamiaco is located, see chapter 2) have been characterised as severely polluted by metallic and organic compounds (Swindlehurst and Johnston, 1991), contamination of Lamiaco sediments with a myriad of potentially toxic substances is strongly suspected. On the contrary, there is neither evidence nor suspicion of Cracknore sediments being seriously polluted by chemical contamination other than TBT.

Due to analytical constraints, only the concentrations of 10 heavy metals and butyltins were determined in sediment samples (Table 7.2), and of some metals and TBT in overlying waters of bioassay bowls (Table 7.3). Interstitial water was not considered because non-migrating deposit-feeding bivalves do not interact with pore water as much as other groups (non-tubicolous amphipods, worms). *S. plana* is primarily a deposit-feeder in which tissue concentrations of heavy metals and TBT are largely controlled by the levels of these compounds in surface sediments and their partitioning between different sediment constituents such as humics, organic carbon and hydrous oxides of both Fe and Mn (Luoma and Bryan, 1982; Langston and Burt, 1991). Further, the feeding mode of *S. plana* accounts for the rarity of horizontal migrations (Hughes, 1969), and this allows the walls of its relatively permanent burrows being well ventilated and therefore oxidized; this may act as a partial barrier to remobilized metals (cf. Bryan, 1985) which may not have sufficient time to equilibrate with pore water within burrows.

Since juveniles in Lamiaco treatments did not ingest any substantial amount of sediment, dissolved toxicants must have resulted in observed lethal effects. However, whether concentrations detected in overlying waters (Table 7.3) may totally explain heavy clam mortalities is dubious. The 12 day LC50 for juvenile (2-3 mm) *S. plana* held in water was reported to be 325 µg/l for Cu and 8000 µg/l for Zn (Bryan and Gibbs, 1983); toxicity of Cu and Zn to the related clam *Abra tenuis* was observed to be additive. When similar-sized *S. plana* spat from Mylor Creek (typical concentrations in sediment of 1117 µgCu/g and 980 µgZn/g) were exposed for 18 days to Mylor Cr. and Restronguet Cr. sediments (≈ 490 µgCu/g and ≈ 494 µgZn/g), substantial mortalities (80%) only occurred in Restronguet Cr treatments; levels in the common overlying water were 40 µgCu/l and 300 µgZn/l (Bryan and Gibbs, 1983). Results of Mylor Cr., Restronguet Cr. and Lamiaco sediment tests constitute yet another example of how chemical analyses provide indications of the relative contamination among sites, but not a measure of their potential for deleterious effects (Long, 1992). On the other hand, Akberali *et al.* (1981) showed that 500 µg/l was the threshold level of dissolved Zn inducing siphonal withdrawal and valve closure in adult *S. plana* ≈ 4 cm long, but exposure did not result in significant mortalities during 14 days; similarly, concentrations of Cu in the range 20-80 µg/l have proved sublethally toxic in 30 days to juvenile clams (see chapter 6). It can then be concluded that levels of dissolved Zn and Cu in bioassay bowls stressed the juveniles, but synergism with other metals, organometals (including TBT present at detectable levels) and other toxicants should be considered to account for the acute toxicity of Lamiaco sediments. Interestingly, small *S. plana* spat were collected in Lamiaco concurrently with samples for bioassay (chapter 2); this fact, together with the existence of an enduring clam population in the mudflat (see also chapter 2) constitute preliminary evidence arguing for the native population of *S. plana* in Lamiaco to be tolerant of local toxic conditions. Increased tolerance to Cu was reported for the *S. plana* population of Restronguet Cr. (Bryan and Gibbs, 1983), where the indigenous *Nereis diversicolor* has been recently shown to have inherited tolerance to Cu and Zn (Hateley *et al.*, 1989) as suggested long before by Bryan and Hummerstone (1971). Further research on this peculiar *S. plana* population of Lamiaco is clearly

warranted, particularly considering the toxicological uncertenties raised by the use of frozen, stored sediments in the current study.

Given the *S. plana* pseudofaeces production and high organic content (Hughes, 1969), it is thought that sediment organic matter (see Table 7.2 for initial values) was not a limiting factor for juvenile growth in any bowl. In addition, since heavy metal levels in bioassay sediments and waters were not exceptionally high (Tables 7.2 and 7.3), it is suggested that performance of clams in Cracknore bowls - impaired with respect to that in both control treatments - may well be adscribed, at least in part, to TBT toxicity.

Mass balance results indicated that uptake of hexachlorobenzene (HCB) by the gut from ingested solids was the single most important phase of exposure in the clam *Macoma nasuta* (Conrad), accounting for 63 to 89% of HCB tissue residues (Boese *et al.*, 1990); these authors concluded that the importance of ingested solids to pollutant tissue residues increases for compounds with high K_{ow} (octanol-water partition coefficient). Since TBT is rather hydrophobic (K_{ow} = 5500 in seawater of 25 ppt, Laughlin *et al.*, 1986), it is likely that active ingestion of TBT-polluted Cracknore sediments enhanced organotin body burdens in juveniles and, consequently, constituted the main factor resulting in the toxic effects suffered by clams; in addition, levels of TBT in overlying waters (exceeding those 28 ngSn/l reducing *S. plana* juvenile growth, see chapter 6) surely contributed to observed results.

With respect to the other two possible shortcomings of sediment bioassays listed by Lamberson *et al.* (1992) - i.e. i) sensitivity to natural sediment features and laboratory conditions and ii) ecological relevance of test -, they are not thought to apply to the present technique. Water quality was maintained between safe limits (see Appendix 14), and all samples of fine surficial deposits were collected at mudflats containing recruiting populations of *S. plana*, except at Cracknore; behaviour of juveniles in this latter treatment clearly show that deleterious effects cannot be due to natural unsuitability of sediments. The ecological relevance of the selected end-points has already been discussed (chapter 6), and their relation to bivalve population dynamics is explicit; further validation was conferred *a priori* by field data (see chapter 1) and, thus, bioassay

results confirm the suspicion of sediment ecotoxicity to *S. plana* at TBT concentrations of ≈ 0.3 µgSn/g (Langston *et al.*, 1990).

7.5. Conclusions.

Lamiaco sediments proved fatal to *S. plana* juveniles unable to avoid them; it seems likely that the native clam population at Lamiaco has developed a tolerance of local toxic conditions originated from heavy metal and, allegedly, other pollution.

Sublethal effects of Cracknore samples strongly suggest that field sediments polluted by similar levels of TBT may act as a deadly trap to *S. plana* recruits: sediments would allow prompt settlement of drifting juveniles (and, presumably, competent pediveliger larvae) which, by means of active deposit-feeding, may experience some growth; nevertheless, this would only be ≈ 20-25% of the potential growth they would have in TBT-unaffected sediments. In a better case situation, incorporation to the reproductive stock of the population - which in U.K. waters occurs by the second summer after settlement, when clams are ≈ 2 cm long (Hughes, 1971) - will be delayed about 9 years. In the most probable scenario, bivalves forced by their size to stay in shallow sediment layers will suffer the highest mortality rate because of predation, loss and exposure to extreme temperatures (Zwarts and Wanink, 1989). If dug out by whatever factor (for instance wave action and current scour), debilitated spat would be prevented of a quick reburial, thus prolonging exposure and increasing risk of death. This is further supported by the finding of inert (but alive) *S. plana* spat lying on the sediment surface during low tide in TBT-affected areas within Poole Harbour (N. Pope, personal communication). Effects of sediment TBT will be worsened by concurrent exposure to increasing levels of dissolved TBT during the non-winter yachting season; joint action of other chemicals having similar gradients of contamination cannot be discarded.

Results of bioassay constitute sound evidence of deleterious effects of TBT-polluted field sediments on local infauna, confirming concern on the environmental relevance of TBT accumulated in deposits (Langston *et al.*, 1990; Waite *et al.*, 1991; Dowson *et al.*, 1992). Surficial sediments containing levels of TBT similar to those herein proved to be toxic to juvenile *S. plana* have recently been reported for coastal

areas throughout Atlantic Europe - Sado and Tejo estuaries in Portugal, Oléron Island and Arcachon Bay in France, Rhine and Scheldt estuaries in The Netherlands (Quevauviller and Donard, 1990), East Coast estuaries in U.K. (Waite *et al.*, 1991; Dowson *et al.*, 1992) - and, more specifically, in sites within Poole Harbour and Southampton Water where *S. plana* populations have been decimated (Langston *et al.*, 1987, 1990; Langston and Burt, 1991). It is therefore concluded that TBT in surficial sediments is likely to have prevented the successful settlement of *S. plana* spat in a number of European areas; the continued presence of moderate levels of sediment-bound TBT may render mudflats unsuitable for the development of juveniles of this and, possibly, other deposit-feeding bivalves.

Table 7.2: (A): concentration (µg/g dry weight except Fe, %) of metals in bioassay sediments (the fraction < 100 µm) as extracted with hydrochloric (Bioavailable) or with nitric (Total) acid, estimated dry wt. of sediment samples used and their organic content. (B): concentration (µgSn/g dry wt.) of organotins (Total: TBT + DBT); n.d.: not detectable.

(A)	Ag	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn	dry wt.
Bioavailable											
Torridge	0.6	0.2	6.8	2	14	0.4	475	3	32	72	43.02
Guernica	1.6	0.2	9.5	45	37	0.8	231	15	33	83	31.06
Cracknore	0.4	0.4	4.2	12	114	0.8	131	4	164	394	34.95
Lamiaco	0.8	3.7	2.7	53	71	0.7	189	7	110	479	41.14
Total											**% org.**
Torridge	0.6	0.1	9.0	40	25	2.6	650	22	33	136	4.39
Guernica	3.6	0.5	7.4	91	41	2.5	279	26	30	146	5.05
Cracknore	0.7	1.1	7.0	50	189	2.6	173	16	186	494	3.50
Lamiaco	1.7	3.9	6.7	106	118	2.7	299	22	126	646	3.69

(B)	TBT	DBT	Total	%TBT
Torridge	n.d.	n.d.		
Guernica	n.d.	n.d.		
Cracknore	0.269	0.177	0.447	60.3
Lamiaco	0.016	0.010	0.026	61.8

Table 7.3: Concentration (µg/l) of metals and TBT (ngSn/l) in supply and bioassay water (volumes renewed on day 18 pooled per treatment) as analysed with furnace (Cd, Cu, Sn) and flame Atomic Absorption (Pb, Zn); n.d.: not detectable.
Total Kp (sediment-water partition coefficient) in thousands except for Cd.

	Cd	Kp	Cu	Kp	Zn	Kp	TBT	Kp	Pb
Supply	1.7		7.8		n.d.		n.d.		n.d.
Torridge	9.6	8	6.1	4.1	11	12.4	n.d.		n.d.
Guernica	19.2	26	14.2	2.9	16	9.1	n.d.		n.d.
Cracknore	6	182	16.1	11.7	70	7.1	36	7.5	n.d.
Lamiaco	22.4	176	29.9	4.0	447	1.4	14	1.1	n.d.

Table 7.4: *Scrobicularia plana*. ANOVA of % weight gain (a) and burying time [(b) and (c)] of juveniles after exposure to sediments during bioassay. All variances [(a), (b), and (c)] were homogeneous: C < C.95(3,1).

Source	df	MS	*F*		Pr > F
(a): weight gain day 36					
Sediment	2	13146	24.25	*	0.014
Error	3	542.2			
SNK: Torridge = Guernica > Cracknore					
(b): burying time day 0					
Sediment	2	1.2	3.02	NS	0.19
Error	3	0.4			
(c): burying time day 36					
Sediment	2	3.49	34	**	0.008
Error	3	0.102			
SNK: Cracknore > Torridge = Guernica					

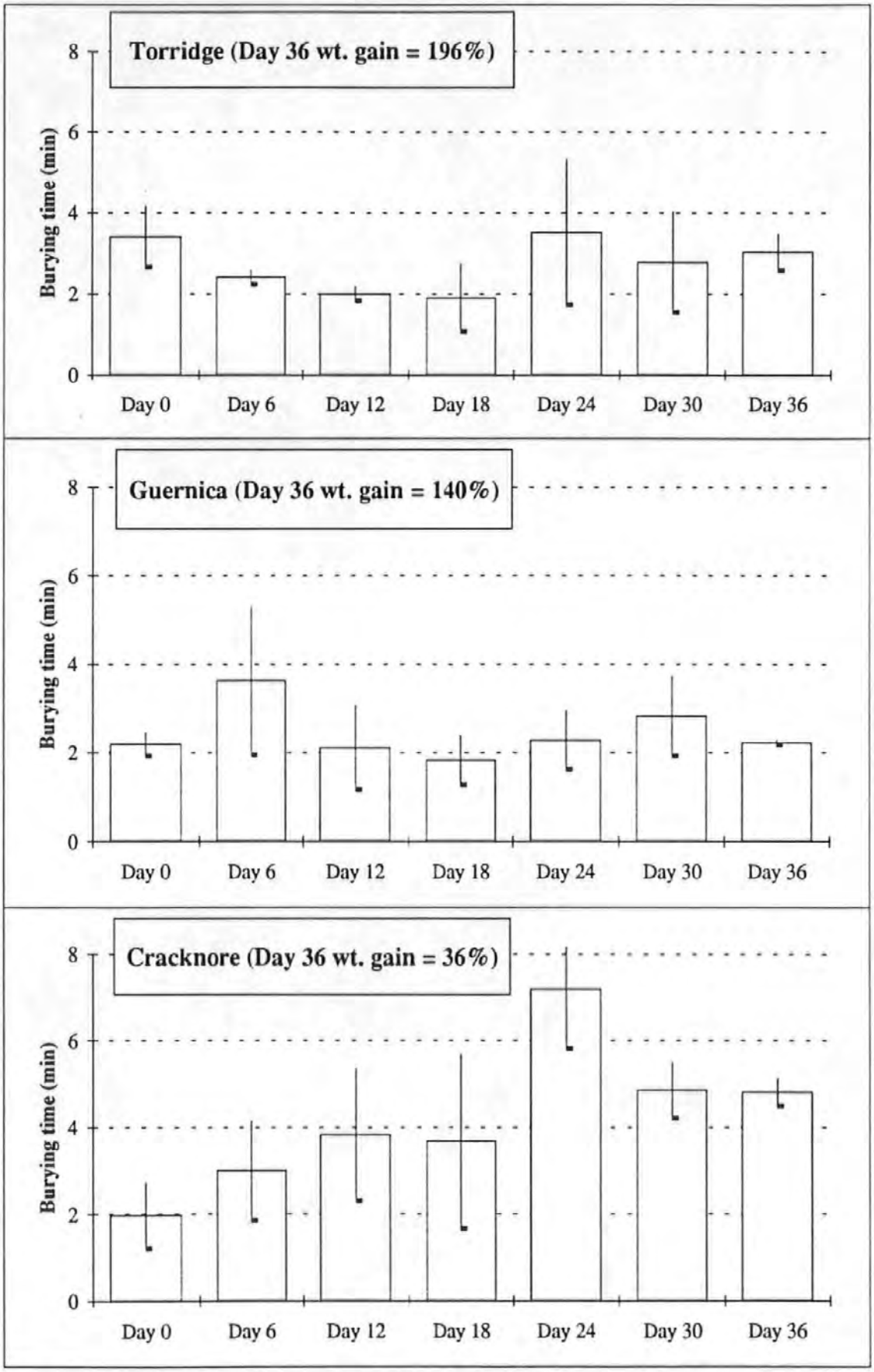


Figure 7.1: *Scrobicularia plana*. Estimated burying time (minutes, mean ± SD) of juveniles after exposure to control (Torridge and Guernica) and TBT-contaminated sediments (Cracknore).

Chapter 8: General discussion and conclusions.

8.1. Executive summary of experimental results.

Results of laboratory tributyltin toxicity tests and bioassays on the successive stages of the life cycle of *Scrobicularia plana* are summarised as follows (see also figure 8.1):

a) Langston and Burt (1991) showed that TBT is highly bioavailable relative to inorganic tin and that sediment TBT concentrations of 10 µgSn/g are invariably lethal to adult (3.5 cm long) clams in two weeks; however, they pointed out that organotin levels in that range are only likely to occur in restricted areas such as large marinas and dockyards. Similarly, concentrations of dissolved TBT had to be set at > 1 µgSn/l to kill significant proportions of *S. plana* specimens (presumably adults) maintained for ten weeks in uncontaminated sand (Beaumont *et al.*, 1989).

b) It has been found that, at least in some moderately-affected populations, an overwhelming majority of the butyltin accumulated in tissues during the reproduction season is stored as the most toxic species (i.e. TBT). However, grounds were not found indicating either impaired fecundity of spawning TBT-affected stock or increased frequency of offspring shell abnormalities at time of hatching; whether the quality of D-larvae after exposure to TBT during organogenesis may be poor or results would vary if severely-affected broodstock were involved in fertilizations was not investigated. The embryonic development of *S. plana* (48 h of duration at 20.5 ± 1 °C) has been shown to be significantly disrupted by toxicants since less than 50% of the ova (relative to controls) cultured at 188 ngSn/l (as analysed) produce fully-shelled D-larvae, and only ≈ 10% of the embryos exposed to 364 ngSn/l or 20 µgCu/l complete their development successfully; in addition, shell length of larvae hatched in toxic cultures seemed to be less than the mean 90 µm attained by control D-larvae. No effect was apparent in embryos reared at lower toxicant concentrations (i.e. 82 ngSn/l and 10 µgCu/l).

c) While the planktonic *S. plana* veliger larvae (mean length 100 µm) metamorphosed to pediveligers (230 µm long) in 10 days at 22.5 ± 1 °C under control culture conditions, vigorous veligers exposed concurrently to nominal TBT doses of 50 ngSn/l and above grew at rates which, at maximum, were only one third of that displayed by controls; probable lethal effects of levels ≥ nominal 250 ngSn/l could not be demonstrated because of reduced larval survival in ethanol controls, and no veliger was observed to have formed a misshapen shell. Modest size differences between the veligers used in larval toxicity tests and those obtained from rearing embryos on other occasions are likely to be due to holding of recently hatched larvae in culture conditions for ≈ 20 h before the experiment was initiated (see section 5.2.2).

d) Settling pediveliger control larvae of *S. plana* reared in uncontaminated fine sand had largely exceeded twice their initial size after 30 days at 20.5 ± 1 °C and, further, some were developing their siphons (i.e. were about to complete metamorphosis and become juveniles). On the contrary, pediveligers exposed to levels of dissolved TBT ≥ 70 ngSn/l (as analysed) suffered significant mortalities; more importantly, larvae reared at 23 ngSn/l showed some growth which was both substantially reduced and grossly abnormal. The fact of postlarval shell malformation indicates that TBT prevented normal calcium carbonate metabolism and deposition in exposed larvae.

e) More than 50% of field-collected *S. plana* spat (mean length ≈ 2.2 mm) kept in uncontaminated sand while exposed to TBT doses ≥ 1500 ngSn/l (as analysed) died in 30 days; also, small juveniles in treatments of ≥ 300 ngSn/l or ≥ 20 µgCu/l displayed reduced burying activity in sand. In a second experiment run similarly, small juveniles exposed to TBT at ≥ 28 ngSn/l grew significantly less than those in the control treatment; the burying activity of exposed individuals was occasionally restrained as well. In a specifically-devised sediment toxicity bioassay, similarly-sized *S. plana* spat held in heavy metal polluted sediment (Lamiaco) suffered massive mortalities in 12 days. Juveniles exposed for 36 days to butyltin-contaminated sediment (Cracknore, TBT + DBT = 0.4 µgSn/g) did not display any avoidance behaviour nor limited survival, but

both their growth and burying activity were significantly reduced relative to those of juveniles kept in control sediments (Torridge and Guernica).

8.2. Extrapolation to field populations: conclusions.

Extrapolation of laboratory results to the field is always a delicate matter, particularly when, as in the present case, single toxicants are tested on single species. However, this set of experiments was not intended to predict environmental risk but, rather, it was prompted by a considerable body of literature suspicious of TBT harmful effect on U.K. populations of *S. plana* through prevention of successful recruitment; the present toxicological laboratory studies focusing on environmentally relevant end-points are thought to provide the best nowdays-possible comprehensive evidence confirming the implications raised by time-extense ecological surveys. Thus, it is concluded that in U.K. coastal areas where TBT in water during the summer-autumn months over the 1980s ranged from ≈ 20 ngSn/l to ≈ 200 ngSn/l (i.e. ≈ 50-500 ngTBT/l) and *S. plana* populations disappeared or declined markedly, a cause-effect relationship is most likely to exist between the former and the latter through the deleterious effects of the chemical on the early life stages of the clam:

Firstly, a large proportion of the embryos developing in estuarine waters (either surface or subsurface) contaminated with TBT at ≈ 188 ngSn/l would not complete successfully their organogenesis. The veligers produced and, indeed, any others imported from clean areas could grow during August in North Atlantic Europe water bodies containing TBT at nominal ≥ 50 ngSn/l at a curtailed rate which would not allow them to reach the pediveliger stage competent for settlement any sooner than November. If, however, initially settling pediveligers were transported to colonise by the end of August tidal flats exposed to average TBT levels ≥ 70 ngSn/l, they would suffer significant mortalities and, at lower concentrations (≥ 23 ngSn/l), their restrained growth rate would result in postlarvae not having completed metamorphosis by December-January; these estimations are conservative in that no likely toxicity originating from sediment-bound TBT is considered. Finally, should small spat be transported by byssus-drifting to settle in polluted mudflats, both TBT levels in water (≥ 28 ngSn/l) and organotin

concentrations deposited in sediments (0.4 μgSn/g) would promote juvenile mortality through increased exposure to surface predators, and slow-growing surviving individuals would be delayed from incorporation to the population reproductive stock for several years. As in most environmental studies, possible contribution of other pollutants to observed field effects, synergism and antagonism of TBT with other natural or anthropogenic factors, and noxious effects of lower TBT concentrations on these and/or other ecological parameters cannot be fully discarded.

The above hypothesis is not so directly applicable to explain the disappearance and/or lack of recruitment of *S. plana* populations in other European estuaries (see figure 1.1) due to lack of concurrent monitoring of both dissolved and sediment-bound TBT. However, there are three characteristics inherent to TBT from antifouling paints which argue for this toxicant to be considered a quite possible cause, or largely contributing factor, of declining clam populations: **i)** its unprecedented toxicity, **ii)** its, in the early 1980s, unexpected occurrence in otherwise non-polluted areas and **iii)** its seasonal dynamics, reaching maximum levels in the summer months; this is of chief importance to the present case since natural spawning of *S. plana* throughout North Atlantic waters concentrates in August and, therefore, earlier planktonic stages will suffer the brunt of TBT toxic action. Later, when levels of dissolved TBT start declining in Autumn after the yachting season is over, natural conditions will have become less favourable so that spat not having completed their metamorphosis and grown large enough (≈ 2 mm) to reach a minimum depth refuge to overwinter will not stand a chance of resisting the combined pressure of predation and low temperatures characteristic of an average winter at these latitudes. If, however, some small spat (either native or originating from not-avoided secondary settlement of postlarvae and juveniles developed elsewhere) do survive until the next spring, ingestion of TBT-polluted sediment (alone or, more probably, in combination with increasing levels of dissolved TBT leached from newly painted boats) will impair normal growth rates and result in debilitated individuals which will be in clear disadvantage to overcome the dynamic and risky existence typical of the unstable top layers of the intertidal sediment flats.

This is thought to be the most plausible available theory to account for the unexplained non-winter mortality of *S. plana* recruits reported by Essink *et al.* (1991) since, in addition, chemical monitoring of European coastal environments has detected over the mid and late 1980s levels of TBT within the range now proved to be noxious to the early stages of the clam; literature data reveal toxic TBT concentrations in waters and/or sediments from the Atlantic (Denmark (Zoulian and Jensen, 1989; L.K. Kure and M.H. Depledge, personal communication), The Netherlands (Ritsema *et al.*, 1991), France (Alzieu *et al.*, 1989), Portugal (Quevauviller *et al.*, 1988)) to the Mediterranean (from Spain to Italy (Alzieu *et al.*, 1991)) and all the way through (Quevauviller and Donard, 1990). Interestingly enough, in some of these areas (i.e. the Dutch Eastern Scheldt) the native population of the specific TBT-biomonitor *Nucella lapillus* (see Gibbs *et al.*, 1987) has been exterminated by organotin pollution (Ritsema *et al.*, 1991) and populations of *S. plana* are reported to be declining (Essink *et al.*, 1991).

8.3. Extrapolation to the community level.

8.3.1. Why is *S. plana* disappearing while other bivalves seem to survive?.

In view of the likely ecotoxicity of TBT, an immediate question raised by this work is why other invertebrate species, usually cohabitant of TBT-affected estuarine habitats, are not suffering a decline similar to that shown by *S. plana*. A response is equally immediate: it is impossible at the present level of environmental compromise to monitor the variety of species found in European Coasts, and lack of denunciatory reports does not necessarily mean absence of impact; the general conclusion of Bryan and Gibbs (1991), arguing that untold consequences of releasing TBT are likely to have occurred through subtle toxicity to the reproduction and early life stages of marine organisms, must be fully accepted. It is also obvious that the conditions for the much-needed multidisciplinary pollution monitoring and control (to include laboratory toxicity bioassays) are, unfortunately, many times not gathered and, if accomplished, it is often after severe environmental and/or commercial damage has been extensively noticed. For practical purposes, let us focus on molluscan species inhabiting mostly soft substrata to look into other more detailed answers.

Thus, Minchin *et al.* (1987) found strong correlation between declining populations of scallops and flame-shells and increased use of organotin net-dips on salmonid farms in the Irish Coast, but suspicion of TBT detrimental effects on the reproduction and larval development of the bivalves could not be proved. Similarly, circumstantial evidence linking poor recruitment of *Littorina littorea* (L.) in estuaries of the English East Coast with substantial levels of dissolved TBT was shown (Matthiesen *et al.*, 1991), but only very recently impaired periwinkle reproductive and larval hatching success have been found to be induced in laboratory experiments by 100-330 ngTBT/l (P. Matthiesen, personal communication). The fact that there are some characteristics of the life cycle of this gastropod which are shared by *S. plana* (i.e. longevity and planktonic veliger development) leads to the suggestion that, if *L. littorea* populations have not been harmed as much as those of the clam, it is probably because of their multiple periods of laying annually. Since clams in Southern Europe recruit two or even three times a year (Paes-da-Franca, 1956; Bachelet, 1981), this may also account for the absence of reports on affected *S. plana* populations in meridional areas well-known to have been polluted by TBT (e.g. Arcachon Bay); successive larval and juvenile cohorts distributed from April to October and average Autumn-Winter conditions less adverse than in Northern waters probably ameliorate effects of TBT and aid in maintenance of populations.

No consistent decline of populations as that reported for *S. plana* by Essink *et al.* (1991) has been noticed in concurrent studies on other common sediment-dwelling bivalves (see Desprez *et al.* (1991) and Ducrotoy *et al.* (1991) for data about *Macoma balthica* and *Cerastoderma edule*, respectively). On the contrary, an inverse correlation between environmental levels of TBT and bivalve recruitment has been observed for years in U.K. embayments (Langston *et al.*, 1987, 1990). Thus, a conspicuous impoverishment of bivalve spat was found at sites in Southampton Water by monitoring from the mid 1980s, and only some of the less polluted sites have shown a few signs of reestablished recruitment in 1991-92 (G.W. Bryan and P.E. Gibbs, personal communication); of a range of species surveyed (*S. plana*, *M. balthica*, *Mya arenaria* (L.), *Abra tenuis* (Montagu)), only *A. tenuis* seem to have bred successfully in the

presence of TBT. Since this probably monotelic clam lays its eggs (July-August in U.K. waters) in a mass within the sediment and, following direct development, miniature adults are hatched (Gibbs, 1984), it is likely that its peculiar life cycle (lasting only 1-2 years) has determined the better survival of the species. The remainder have planktonic larvae which are exposed to considerable risks (both natural and anthropogenic) during their development and, particularly, in near bottom waters at time of settlement (see chapter 5). Nevertheless, for species reproducing only once a year in North Atlantic European waters, larval settling before water TBT is raised to seasonal maxima (for *M. balthica* and *M. arenaria* in the Wadden Sea settlement occurs in spring, see Günther 1991 and 1992, respectively) usually allows spat to grow to 3-5 mm by the end of August, thereby guaranteeing to some extent the acquirement of the subsequent depth refuge to overwinter.

In addition, a multitude of factors, both physiological (feeding mode in relation to sediment ingestion, specific TBT metabolism and sensitivity, ...) and ecological (abiotic such as sediment granulometry, biotic such as competition between settlers and accessibility to epibenthic predators) may interact to result in the observed rarity of successful bivalve recruits, notably *S. plana*. For instance, it is generally accepted that burial depth in bivalves is largely determined by length of siphons (Zwarts and Wanink, 1989) and shape of shell (Trueman, 1983); thus, Stanley (1970) showed that most rapid burrowers have developed shells that are streamlined (e.g. Tellinacea) while strongly ornamented and thick valves are employed primarily by shallow burrowers for stability near the sediment surface (e.g. Cardiacea). Additionally, although anti-predator shell features are subtle, shallow-burying bivalves may counterbalance increased availability to surface predators by reduced vulnerability conferred by inflated, thick and adorned shells (Kauffman, 1969). It could be speculated that in tidal flats where several bivalve species coexist sharing a common predator pressure, TBT (both dissolved and sediment-bound) will deprive *S. plana* spat of their natural strategies to avoid predation (i.e. ability to grow fast by secreting a thin shell and attainment of deep reburial rapidly); this would shift off crab attention from equally shallow-buried but thicker-shelled *C. edule* of a similar size (since they will be probably rejected in favour of a prey with less-resisting

shell and, therefore, shorter handling times, see Boulding, 1984) and also from similarly-shelled spat of species such as *A. tenuis*, *M. balthica*, *M. arenaria* (which, for reasons described above, will have reached a safer size and depth refuge) to concentrate predator foraging on increasingly vulnerable *S. plana* recruits. This indirect effect of TBT may be of high environmental relevance because decapod crustaceans appear to be relatively resistant to TBT (see review by Bryan and Gibbs, 1991) due to their fairly efficient ability to degrade it (Rice *et al.*, 1989) and, as a result, crab activity is possibly undiminished by organotin pollution.

8.3.2. Why is *S. plana* able to withstand severe heavy metal contamination in Bilbao but not TBT pollution elsewhere ?.

A further question is posed by the work described above: why is *S. plana* able to survive and breed amidst extreme ambient pollution in Bilbao while some Northern clam populations have been decimated by TBT ?. Tentatively, an answer can be put forward: clams in Bilbao may have evolved to become a tolerant strain of the species while adaptation to organotins has not been possible. Certainly, precedents exist showing increased tolerance to some heavy metals and methylmercury of several aquatic invertebrates (see review by Mulvey and Diamond, 1991) including one *S. plana* population in Restronguet Creek (Bryan and Gibbs, 1983), whereas no such an instance is known to have been reported with respect to TBT; further, results of both field and laboratory work (chapters 2 and 7, respectively) constitute preliminary evidence supporting the first assumption. In addition, if it has to be born in mind that acquisition of metal tolerance by Bilbao *S. plana* would be exceptional, there are other indications compelling to consider the possibility, for instance: **i)** progressivity of pollution which, although not documented, it can be assumed to have been the case since iron mining and related industrial development is known to have flourished in the area for centuries, notably from the 1800s onwards. **ii)** exclusion of other bivalves and invertebrates common in neighbour estuaries from Lamiaco and Sestao (particularly *C. edule* which, in Bilbao, are only found in the more downstream site (Arriluce) but are unable to colonize permanently those other two localities where *S. plana* is settled) and exclusion of *S.*

plana itself from increasingly polluted reaches further upstream such as Asua where the fossil record shows it used to be established some 2000 years ago (A. Cearreta, personal communication). Thus, the present *S. plana* in Bilbao are a relict sample of former widespread populations which would have died off as industrialization and associated pollution progressed.

Among the factors concluded by Mulvey and Diamond (1991, see Table 1 and figure 1) as capable of influencing the evolution of tolerance in specific populations exposed to metals, it is worth noting some which may have had a converse effect on the population of *S. plana* in Bilbao and those disappearing in Northern Europe: **i)** genetic factors such as fitness: when juveniles clams from a naive population (Torridge) were exposed to Lamiaco sediment (see chapter 7), > 90% of individuals were killed in 36 days (82% in 12 days); the small proportion of survivors constitute the extreme of the phenotypic distribution and may be tolerant genotypes, showing *S. plana* potentiality for rapid development of adaptative response. **ii)** reproductive factors such as generation times, shorter in Bilbao (*S. plana* is most likely to reproduce twice a year at this latitude) and longer in Northern populations (see above). **iii)** ecological factors such as **(a)** homogeneity of pollution in Bilbao and isolation of populations within the estuary (see chapter 2); on the contrary, TBT pollution usually displays a clear spatial gradient resulting in diversely affected areas which are likely to trade off larvae and spat, thus diminishing the rate of evolutive reaction, and **(b)** differential sensitivity of life stages which will influence the effectiveness of selection; in this respect, it has been shown that developing embryos of *S. plana* resist about 16 times more Cu than TBT (LC90 ≈ 20 µgCu/l or 1.25 µgTBT/l, see chapter 4) and burying activity of small juveniles in sand was not affected by exposure to 80 µgCu/l for 6 days while it was significantly decreased by 1.25 µgTBT/l in the same period (i.e. TBT > 64 times more toxic than Cu, see chapter 6, experiment of 1991).

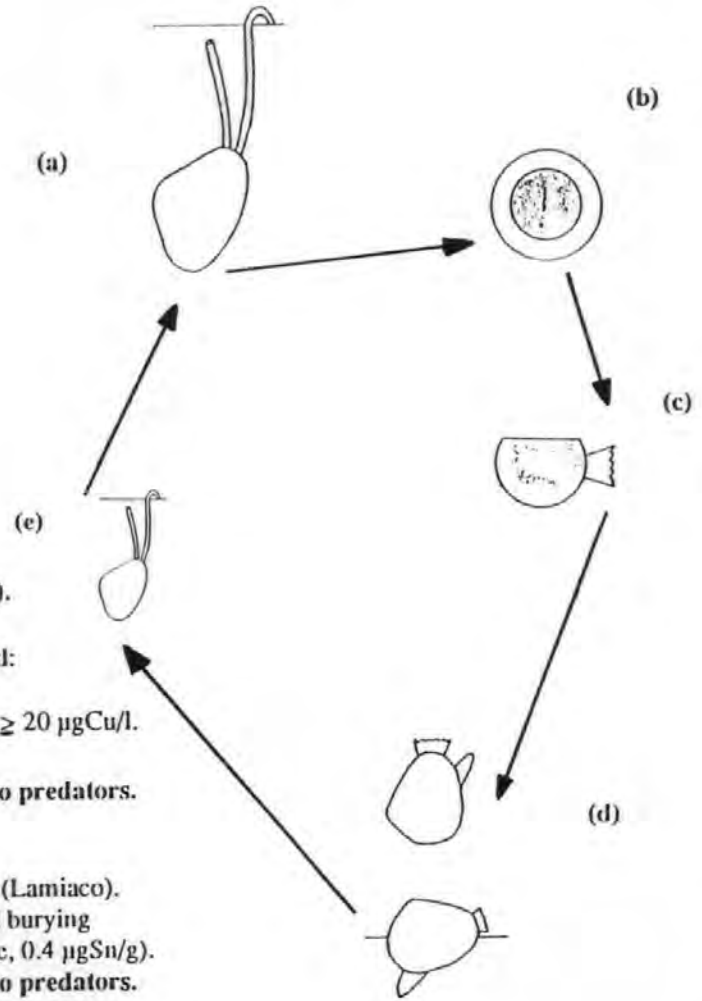
However, if further evidence may be gained by ecotoxicological experiments, only genetic laboratory studies can undoubtedly establish causative relationships between metallic pollution and evolution of tolerance by determinate biological populations,

particularly in places such as Bilbao where a myriad of pollutants concur (see for instance Azkona *et al.*, 1984; Swindlehurst and Johnston, 1991).

8.4 Corollary.

The present work confirms once again that TBT from antifouling paints constitutes a milestone in marine pollution research (see for example Mee and Fowler, 1991); although it has been produced after ecological damage occurred, it may still be useful for the safeguarding of coastal species since it supports with biological effects evidence the concern expressed on sediments acting as reservoirs of TBT at toxic levels (Langston *et al.*, 1990; Waite *et al.*, 1991; Dowson *et al.*, 1992) and on the continued use of organotin-based formulations on large ships (Bryan and Gibbs, 1991). Clearly, combined chemical and biological monitoring of *S. plana* and other populations must be promoted, giving itemised consideration to possible subtle effects of ecological relevance induced by low toxicant ambient concentrations on the early stages of the life cycle of our biota. As for pollution in Bilbao estuary, if only opportunities to work were expanded ...

(a) Adult (2-7 cm, up to 18 years),
data from Langston and Burt (1991):
- field CF tissue/sediment (0.4 µgSn/g) ≈ 10.
- field CF tissue/water (6-80 ngSn/l) ≈ 1 x 10^5.
- sediments 10 µgSn/g are lethal to clams (3.5 cm) in 2 weeks.



(b) 48 h (20 °C) embryonic development,
from zygote (80 µm Ø) to D-larva (90 µm long):
- NOEL = 82 ngSn/l or 10 µgCu/l.
- **EC50 < 188 ngSn/l** or < 20 µgCu/l.

(c) 10 days (22 °C) veliger development,
from D-larva to pediveliger (230 µm):
- growth reduced at nominal ≥ 50 ngSn/l.
- acute effects possible at nominal ≥ 250 ngSn/l.

Unlikely to reach pediveliger stage.

(d) 30 days (20 °C) postlarval development
(in uncontaminated sediment), from settling pediveliger to siphon-bearing juvenile (800 µm):
- significant mortalities at ≥ 70 ngSn/l.
- reduced and abnormal shell growth at ≥ 23 ngSn/l.

Unable to reach juvenile stage.

(e) Juvenile stage (1-20 mm, 1-2 months to 2 years).
Exposure of spat (mean length ≈ 2.2 mm) to
(i) dissolved TBT for 30 days in uncontaminated sand:
- LC50 < 1500 ngSn/l.
- reduced burying activity in sand at ≈ 300 ngSn/l or ≥ 20 µgCu/l.
- reduced growth at ≥ 28 ngSn/l.

Prolonged juvenile existence, increased exposure to predators.

(ii) natural sediments for 36 days,
- heavy mortalities in metal-contaminated sediments (Lamiaco).
- no avoidance response elicited, reduced growth and burying activity in TBT-contaminated sediments (Cracknore, 0.4 µgSn/g).

Prolonged juvenile existence, increased exposure to predators.

Figure 8.1: Summary of effects of TBT (and Cu) on the life stages of *Scrobicularia plana*.

(A)

	Santoña	Guernica			Bilbao			
	S	G-2	G-3	G-4	Arriluce	Sestao	Lamiaco	Asua-1
Sediments	Jan.'91	Jan.'91	Jan.'91	Jan.'91			Jan.'91	Jan.'91
	May'91	May'91					May'91	May'91
	Dec.'91	Dec.'91	Dec.'91	Dec.'91	Feb.'92	Feb.'92	Dec.'91	
Scrobicularia plana	Jan.'91	Jan.'91	Jan.'91				Jan.'91	
	May'91	May'91					May'91	
	Dec.'91	Dec.'91	Dec.'91		Feb.'92	Feb.'92	Dec.'91	
Nereis diversicolor	Jan.'91	Jan.'91	Jan.'91				Jan.'91	Jan.'91
Ligia oceanica								May'91
Orchestia sp.								May'91
Lycastopsis sp.								May'91
Cerastoderma edule					Feb.'92			

(B)

Date and transplant	no. of clams (unrestricted)	Date and no. recovered
Jan. 1991, G-2 ——> S	36	May 1991, 12 clams
Jan. 1991, G-2 ——> G-2	40	May 1991, 12 clams Dec. 1991, 5 clams
May 1991, G-2 ——> Lamiaco	25	Dec. 1991, 16 clams

Appendix 1: (A): List of samples collected in Cantabrian estuaries for assessing metallic pollution impact. (B) *S. plana*. Scheme of transplants between sites of estuaries thought to follow a decreasing gradient of pollution: Lamiaco (Bilbao) > G-2 (Guernica) > S (Santoña).

	Santoña				Guernica-2			Guernica-3				Lamiaco			
	a	b	c	TBT	a	b	c	a	b	c	TBT	a	b	c	TBT
Jan. of 1991															
n =	6	6	6	8	6	6	6	6	6	6	4	12	12	12	22
Avg.	41	42	41	35	40	40	40	41	42	41	38	26	27	27	22
SD	4	4	4	1	2	2	2	3	2	1	1	1	2	2	2

	Santoña			Guernica-2					Lamiaco			
	Natives	Transpl.		Natives		Transplants			a	b	AA	EM
		a	b	a	b	a	b	box				
May of 1991												
n =	7	6	6	6	6	6	6	4	12	12	4	5
Avg.	41	40	39	41	40	39	38	37	28	27	27	27
SD	3	1	1	1	1	3	3	1	2	2	1	1

	Santoña	Guern.-2		Guern.-3		Arriluce		Sestao		Lamiaco			
		Natives		Tra.		Feb. 1992		Feb. 1992		Natives		Transpl.	
		a	b		a	b	a	b	a	b	a	b	a	b
Dec. of 1991														
n =	5	6	6	5	9	9	11	11	11	11	9	9	7	7
Avg.	45	41	41	38	34	33	32	31	32	32	32	31	36	37
SD	4	2	2	3	2	2	2	2	3	1	1	1	1	2

Appendix 2: *Scrobicularia plana*. Number of individuals which soft-parts were pooled together for replicate (a, b, c) heavy metals analyses (except TBT: organotin analyses), and their average length and standard deviation (mm). AA, EM: data of clams which tissues were disected and pooled for examination with Atomic Absorption or X-ray Electron Microscopy, respectively.

		Day 2		Day 4		Day 6		Day 8		Day 10	
		Total	Live	Total	Live	Total	Live	Total	Live	Total	Live
WC	A	157	113	84	65	62	41	63	48	27	14
	B	119	90	123	89	113	74	73	57	114	100
OC	A	120	57	38	18	71	24	33	6	27	11
	B	79	43	47	12	98	34	37	8	31	9
50	A	79	27	68	26	76	27	52	10	35	5
	B	84	35	91	38	74	18	53	17	37	12
125	A	112	47	34	16	76	28	49	24	52	15
	B	99	47	38	13	41	22	71	25	37	5
250	A	95	51	32	16	70	30	59	11	66	5
	B	102	57	29	10	34	9	53	7	54	3
500	A	101	41	38	15	55	23	27	3	19	0
	B	57	27	46	27	65	26	26	0	54	0

Appendix 3: *Scrobicularia plana.* Number of total and live veliger larvae found in duplicated (A, B) vessels during toxicity test after exposure to TBT (50, 125, 250 and 500 ngSn/l) and water (WC) and ethanol (OC) control treatments for 2, 4, 6, 8 and 10 days.

																										Mean	SD
Initial		102	100	101	106	106	107	104	102	105	102	103	102	98	102	98	89	98	93	94	98	96	99	100	97	95	99.88	4.4
Day 2	OC	113	115	111	121	119	118	117	115	117	116	112	109	115	117	108	113	112	114	119	121	110	117	109	112	114	114.6	3.68
	50	115	109	112	114	109	116	121	110	114	111	112	112	108	115	112	105	109	119	118	111	113	109	110	106	114	112.2	3.87
	125	113	109	113	108	110	114	107	115	106	110	108	119	116	113	110	113	111	108	107	114	110	111	113	107	105	110.8	3.45
	250	108	104	102	102	103	110	109	105	104	111	106	107	105	108	103	105	107	110	102	108	104	111	109	102	101	105.8	3.13
	500	98	107	106	106	102	103	103	101	99	105	108	110	105	106	104	102	100	99	98	104	106	108	104	105	97	103.4	3.5
Day 4	OC	148	130	136	143	143	149	150	151	168	153	146	139	126	157	158	141	138	153	150	135	159	145	140	150	149	146.3	9.53
	50	111	117	131	124	123	127	126	127	119	110	107	106	103	123	108	108	128	121	105	118	124	121	118	117	123	117.8	8.25
	125	107	125	117	106	115	105	112	109	125	120	119	117	121	136	128	125	136	114	119	115	111	118	125	120	115	118.4	8.18
	250	114	124	115	125	127	120	114	118	109	131	119	115	124	116	123	114	132	119	121	117	107	110	119	121	116	118.8	6.26
	500	106	113	105	108	102	108	111	109	119	105	108	110	116	102	108	114	107	105	107	102	112	115	109	108	105	108.6	4.42
Day 6	OC	166	169	184	187	161	205	166	172	179	171	164	172	175	185	165	169	179	189	163	165	180	174	177	166	181	174.6	10.3
	50	134	129	131	133	132	119	127	128	124	134	121	129	130	119	136	125	127	119	137	131	125	128	121	126	130	127.8	5.27
	125	127	131	120	116	130	115	117	115	123	129	118	121	125	114	126	118	132	128	119	125	124	127	120	119	123	122.5	5.39
	250	121	122	108	117	113	113	122	119	111	116	121	117	119	109	125	120	118	122	117	116	120	114	121	112	116	117.2	4.39
	500	112	109	105	117	115	118	107	114	112	108	110	117	106	103	117	115	116	109	110	116	114	107	108	111	117	111.7	4.41
WC	Day 2	119	114	114	106	113	117	114	113	110	108	110	105	104	115	117	119	120	113	119	120	120	120	113	112	110	113.8	4.89
	Day 4	140	142	125	154	135	134	136	142	154	151	133	137	129	141	151	172	133	139	144	147	137	136	137	135	162	141.8	10.6
	Day 6	188	192	164	167	180	188	182	179	185	151	163	169	155	165	165	170	173	149	167	190	177	169	168	174	186	172.6	11.9
	Day 8	203	170	215	223	180	210	203	192	204	185	194	205	178	200	208	214	218	196	205	201	188	196	193	205	207	199.7	12.8
	Day 10	208	241	247	245	220	240	231	230	228	217	216	220	210	233	229	237	242	233	225	228	234	227	221	230	242	229.4	10.5

Appendix 4: *Scrobicularia plana*. Valve length (µm) of veliger larvae at initiation of toxicity test and after exposure to TBT (50, 125, 250 and 500 ngSn/l) and ethanol control (OC) treatments for 2, 4 and 6 days, and to water control (WC) for up to 10 days.

	Day	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
WC a	live	65	65	65	65	65	65	65	65	65	65	65	65	65	65	65	65
	dead		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
WC b	l	69	67	67	67	67	67	67	67	67	67	67	67	67	67	67	67
	d		2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OC a	l	66	65	54	48	46	45	45	45	45	45	45	45	45	45	45	45
	d		1	11	6	2	1	0	0	0	0	0	0	0	0	0	0
OC b	l	63	63	56	53	53	51	50	50	50	50	50	50	50	50	50	50
	d		0	7	3	0	2	1	0	0	0	0	0	0	0	0	0
50 a	l	68	68	64	61	59	59	57	57	57	57	56	56	55	44	34	32
	d		0	4	3	2	0	2	0	0	0	1	0	1	11	10	2
50 b	l	70	69	65	61	61	61	58	57	56	56	55	55	54	54	52	45
	d		1	4	4	0	0	3	1	1	0	1	0	1	0	2	7
125 a	l	69	69	59	58	56	56	56	56	56	54	50	50	49	48	46	37
	d		0	10	1	2	0	0	0	0	2	4	0	1	1	2	9
125 b	l	66	65	49	41	35	34	34	33	33	33	33	31	28	22	21	20
	d		1	16	8	6	1	0	1	0	0	0	2	3	6	1	1
250 a	l	66	66	61	61	61	59	59	58	57	54	51	47	47	45	35	16
	d		0	5	0	0	2	0	1	1	3	3	4	0	2	10	19
250 b	l	65	64	49	48	47	47	47	42	42	42	42	36	29	27	24	22
	d		1	15	1	1	0	0	5	0	0	0	6	7	2	3	2
500 a	l	70	69	60	54	52	47	47	33	32	32	32	29	29	25	18	5
	d		1	9	6	2	5	0	14	1	0	0	3	0	4	7	13
500 b	l	69	69	53	51	45	45	45	45	45	41	35	33	33	28	14	0
	d		0	16	2	6	0	0	0	0	4	6	2	0	5	14	14

Appendix 5: *Scrobicularia plana*. Number of live and dead post-larvae found in duplicated (a, b) bowls every 2nd day during exposure to TBT (50, 125, 250 and 500 ngSn/l) and water (WC) and ethanol (OC) control treatments for 30 days.

	Initial	WC		OC		50		125		250	Model
	233	567	495	712	679	387	336	251	225	247	280
	237	454	500	683	577	419	319	255	247	255	459
	247	475	553	704	621	398	294	223	259	271	420
	258	529	565	511	548	355	334	256	263	256	422
	240	584	522	671	816	343	318	286	278	251	481
	220	517	568	536	636	310	347	302	293	257	504
	239	578	600	674	680	337	351	274	287	275	390
	238	523	514	490	723	302	328	256	263	262	424
	242	416	537	562	567	321	332	245	298	258	412
	235	506	573	651	628	293	337	283	233	273	493
		592	566	613	667	311	305	238	247	265	458
		553	448	581	622	309	313	257	252	237	439
		478	527	708	649	328	341	309	264	250	
		576	575	608	611	315	299	260	273	256	
		515	565	637	685	287	334	255	281	257	
		546	449	625		367	329	274	269	259	
		532	452	543		261	345	263	237	265	
		470	482	633		311	321	286	275	256	
		586	524	584		279	328	230		263	
		488	530	666		347	276	250		241	
		518	504	770		349	315	267		232	
		648	628	643		256	329	260		269	
		551	545	593		296	331	254		258	
		444	528	670		327	311	251		256	
		521	516	651		257	317	261		264	
		515	571	730		288	326	225		271	
		562	611	516		322	306	269		242	
		489	549	622		289	322	285		237	
		566	497	709		324	316	273		248	
		653	513	520		320	322	281		253	
		503	694	726		288	335	236		261	
		721	742	562		301	327	248		247	
		486		774		311	297	254		277	
		579		568		277	306	259			
		467		790		345	293	241			
n =		10		67		50		70		53	33	12
Mean		239		540		639		320		262	257	432
SD		10		63		75		28		20	11	36

Appendix 6: *Scrobicularia plana*. Valve length (µm) of post-larvae at initiation of toxicity test and after exposure to TBT (50, 125 and 250 ngSn/l) and water (WC) and ethanol (OC) control treatments for 30 days. Model = larvae reared in parallel culture which were kept as a reference of normal shell shape.

	Day	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
Water control a	surviving	40	40	38	38	38	38	38	38	38	38	38	38	38	38	38	38
	on surface	-	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0
Water control b	surviving	40	40	39	39	39	39	39	39	39	39	39	39	39	39	39	37
	on surface	-	0	1	0	1	0	1	0	1	2	2	2	2	2	0	1
Ethanol control a	surviving	40	38	38	37	37	37	37	37	37	37	37	37	37	37	37	34
	on surface	-	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0
Ethanol control b	surviving	40	39	39	39	39	39	39	39	39	39	39	39	39	39	39	38
	on surface	-	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0
0.5 µgSn/l a	surviving	40	40	40	40	40	40	40	40	40	40	40	40	39	39	39	39
	on surface	-	0	2	0	0	1	0	0	2	5	4	3	11	14	10	8
0.5 µgSn/l b	surviving	40	39	39	39	39	39	39	39	39	39	39	39	39	38	38	38
	on surface	-	0	3	1	1	1	2	4	7	10	7	8	7	6	9	4
1 µgSn/l a	surviving	40	40	40	40	40	40	40	40	40	39	39	39	39	39	39	39
	on surface	-	0	2	5	10	11	11	13	12	17	25	20	24	25	30	25
1 µgSn/l b	surviving	40	40	40	40	40	40	40	40	40	40	40	40	39	39	39	39
	on surface	-	0	1	6	9	10	15	21	19	20	31	27	30	30	30	27
2 µgSn/l a	surviving	40	40	40	40	40	40	39	38	38	37	36	35	34	32	29	19
	on surface	-	12	17	20	19	26	22	29	31	33	34	30	33	31	28	19
2 µgSn/l b	surviving	40	40	40	40	40	40	40	39	39	39	39	39	29	23	11	11
	on surface	-	11	16	19	23	25	31	34	35	35	39	37	29	23	11	11
4 µgSn/l a	surviving	40	38	38	37	36	36	29	28	25	23	18	11	10	6	5	5
	on surface	-	22	29	31	32	32	26	27	23	23	18	11	10	6	5	5
4 µgSn/l b	surviving	40	40	40	40	39	35	32	31	24	22	16	11	10	8	5	2
	on surface	-	26	33	34	37	34	32	31	24	22	16	11	10	8	5	2

	Day	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
10 µgCu/l a	surviving	40	38	37	37	37	37	37	37	37	37	37	37	37	37	36	36
	on surface	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10 µgCu/l b	surviving	40	36	36	36	36	36	36	36	36	36	36	36	36	36	35	35
	on surface	-	0	1	0	0	1	1	1	2	3	3	3	1	2	1	0
20 µgCu/l a	surviving	40	40	40	38	38	38	38	38	38	38	38	38	38	38	38	37
	on surface	-	0	0	0	0	0	1	1	1	0	0	4	4	10	14	3
20 µgCu/l b	surviving	40	39	39	39	39	39	39	39	39	39	39	39	39	39	39	39
	on surface	-	0	1	0	0	0	1	0	2	2	1	2	2	3	9	4
40 µgCu/l a	surviving	40	39	38	38	37	37	37	37	37	37	37	37	37	37	37	37
	on surface	-	0	2	1	1	3	2	4	5	6	8	13	15	17	24	21
40 µgCu/l b	surviving	40	38	38	38	38	37	37	37	37	37	37	37	37	37	37	37
	on surface	-	0	2	1	1	0	1	2	0	5	6	11	17	19	22	23
80 µgCu/l a	surviving	40	38	38	38	38	38	38	38	38	38	38	38	38	38	38	38
	on surface	-	12	9	4	6	14	17	21	27	31	31	34	33	35	37	38
80 µgCu/l b	surviving	40	40	40	40	40	40	40	40	40	40	39	39	39	39	39	39
	on surface	-	13	13	7	8	15	20	27	31	33	35	35	37	38	38	39

Appendix 7: *Scrobicularia plana*. Total number of juveniles surviving and those live specimens which were found on surface in duplicated bowls (a and b) at time of renewal every other day of the 30 day long 1991 experiment.

(A)

Water Control

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	38	39	38	39	38	39	38	39	38	37
5	11	13	12	14	12	18	12	19	6	18
10	6	7	7	7	3	8	3	8	2	10
15	2	4	5	5	1	6	1	4	1	3
20	0	1	0	4	0	4	0	2	0	0
25	0	1	0	1	0	3	0	2	0	0
30	0	0	0	1	0	1	0	1	0	0
35	0	0	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0

Ethanol Control

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	37	39	37	39	37	39	37	39	34	38
5	15	14	11	13	14	17	14	19	8	11
10	3	6	3	5	9	5	3	7	5	5
15	2	3	2	2	6	3	1	1	2	2
20	1	1	2	0	4	1	1	1	2	1
25	1	0	2	0	1	1	1	1	1	1
30	0	0	1	0	0	1	0	1	0	1
35	0	0	0	0	0	0	0	1	0	1
40	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0

0.5 µg Sn/l

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	40	39	40	39	40	39	39	39	39	38
5	21	21	22	28	27	20	23	21	32	28
10	16	15	18	12	20	14	22	19	27	24
15	11	9	14	8	16	11	20	15	24	21
20	6	9	10	5	14	9	18	14	22	18
25	5	1	6	3	13	9	16	13	22	18
30	4	1	6	1	10	8	13	13	22	17
35	3	0	4	1	7	8	12	13	21	17
40	3	0	4	1	7	8	12	11	21	16
50	1	0	3	0	5	8	10	10	20	15
60	1	0	3	0	5	8	9	10	20	14

1 µg Sn/l

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	40	40	40	40	39	40	39	39	39	39
5	23	22	24	23	32	33	33	31	33	36
10	15	14	16	18	26	28	29	28	32	34
15	12	12	13	15	24	27	29	28	32	33
20	9	9	12	13	18	27	26	26	32	33
25	8	8	11	12	17	27	25	25	32	32
30	8	8	10	12	17	25	25	25	32	31
35	7	7	9	11	17	23	24	25	32	31
40	7	7	8	11	16	23	24	25	31	31
50	7	6	7	9	14	20	24	24	31	31
60	7	6	7	9	14	19	24	24	30	31

2 µg Sn/l

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	40	40	39	40	37	39	34	29	19	11
5	26	28	31	32	35	36	34	28	19	11
10	22	26	25	27	31	36	34	28	19	11
15	19	19	23	25	31	36	32	28	19	11
20	19	19	22	23	31	34	31	28	19	11
25	18	19	21	22	31	34	29	28	19	11
30	18	19	19	21	31	33	29	28	19	11
35	18	18	16	20	30	33	29	28	18	11
40	17	18	16	19	30	33	29	28	18	11
50	17	18	16	19	29	33	29	28	18	11
60	17	18	16	19	29	33	29	28	18	11

4 µg Sn/l

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	37	40	29	32	23	22	10	10	5	2
5	36	38	26	28	23	22	10	10	5	2
10	35	36	23	27	23	22	10	10	5	2
15	31	35	22	26	23	22	10	10	5	2
20	30	34	20	25	22	22	10	10	5	2
25	30	34	20	25	22	22	10	10	5	2
30	28	33	20	24	22	22	10	10	5	2
35	27	31	20	23	21	22	10	10	5	2
40	27	30	19	23	21	22	10	10	5	2
50	25	30	19	21	21	22	10	10	5	2
60	24	30	19	19	21	22	10	10	5	2

(B)

	Day 6		Day 12		Day 18		Day 24		Day 30	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Water control	5.4	0.6	6.1	0.7	6.1	2.1	5.9	1.8	5.2	2.1
Ethanol control	5.5	0.1	5.2	0.2	6.6	0.7	5.8	0.9	5.3	0.2
0.5 µgSn/l	10.7	1.5	12.4	3.3	19.0	1.0	24.6	1.4	35.3	4.6
1 µgSn/l	17.2	0.5	20.7	1.9	36.5	5.4	43.8	0.1	53.5	0.8
2 µgSn/l	32.5	1.2	35.4	2.1	55.4	2.0	60.6	3.7	64.1	1.2
4 µgSn/l	51.8	1.7	47.4	1.2	63.3	2.5	65.0	0.0	65.0	0.0

Appendix 8: *Scrobicularia plana*. Control and TBT treatments. (A): Number of juveniles not buried as observed in duplicated bowls (a and b) at time (minutes) intervals every 6th day of the 30 day long 1991 experiment. (B): estimated burying time of juveniles in minutes. See also Appendix 9.

(A)

10 µg Cu/l

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	37	36	37	36	37	36	37	36	36	35
5	3	6	10	9	8	12	11	9	4	6
10	1	3	3	3	3	3	1	4	3	4
15	0	2	1	2	3	2	0	2	2	4
20	0	1	0	1	1	2	0	1	1	3
25	0	1	0	1	0	2	0	1	1	2
30	0	1	0	1	0	2	0	1	1	2
35	0	1	0	1	0	2	0	1	1	2
40	0	1	0	1	0	2	0	1	1	2
50	0	1	0	1	0	1	0	1	1	2
60	0	1	0	1	0	1	0	1	1	1

20 µg Cu/l

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	38	39	38	39	38	39	38	39	37	39
5	11	6	18	19	9	11	15	9	18	18
10	3	5	10	6	2	3	9	4	14	14
15	1	1	6	4	2	3	8	3	12	9
20	1	1	1	1	1	2	5	2	10	9
25	0	1	0	1	1	2	5	1	10	8
30	0	1	0	1	1	2	4	1	10	5
35	0	1	0	1	1	2	4	1	9	5
40	0	1	0	1	1	1	4	1	9	5
50	0	1	0	1	0	1	4	1	8	4
60	0	1	0	1	0	1	4	1	8	4

40 µg Cu/l

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	38	38	37	37	37	37	37	37	37	37
5	5	3	9	7	13	10	23	22	35	34
10	2	1	5	3	6	6	17	14	33	32
15	1	1	5	1	5	4	13	11	30	31
20	1	1	5	0	3	3	12	11	30	29
25	1	0	3	0	2	2	10	9	28	28
30	1	0	3	0	1	2	10	9	28	26
35	1	0	2	0	1	1	8	8	23	25
40	1	0	1	0	1	0	8	7	21	25
50	1	0	1	0	0	0	8	7	20	24
60	1	0	0	0	0	0	8	7	20	24

80 µg Cu/l

	Day 6		D 12		D 18		D 24		D 30	
Time	a	b	a	b	a	b	a	b	a	b
0	38	40	38	40	38	40	38	39	38	39
5	5	9	24	31	33	35	35	37	38	39
10	3	4	13	27	31	32	31	35	38	39
15	1	1	9	22	28	31	29	33	38	38
20	1	1	7	20	27	28	28	33	38	36
25	0	1	7	17	24	26	28	33	38	36
30	0	0	7	14	24	25	25	32	37	35
35	0	0	7	14	23	25	25	32	35	35
40	0	0	7	13	21	25	25	32	34	36
50	0	0	7	11	20	23	25	32	33	36
60	0	0	7	9	19	21	25	32	32	36

(B)

	Day 6		Day 12		Day 18		Day 24		Day 30	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
WC	5.4	0.6	6.1	0.7	6.1	2.1	5.9	1.8	5.2	2.1
10 µgCu/l	4.2	1.6	5.1	1.0	5.7	1.7	5.0	1.3	6.1	1.4
20 µgCu/l	4.9	0.5	7.3	0.2	5.7	1.0	8.9	4.3	17.0	4.0
40 µgCu/l	4.0	1.1	5.6	2.3	6.6	0.4	20.0	1.4	46.9	1.9
80 µgCu/l	4.2	0.5	22.7	7.6	42.7	1.1	51.1	5.8	60.9	0.1

Appendix 9: *Scrobicularia plana*. Copper treatments. (A): Number of juveniles not buried as observed in duplicated bowls (a and b) at time (minutes) intervals every 6th day of the 30 day long 1991 experiment. (B): estimated burying time of juveniles in minutes. See also Appendix 8.

	Controls		TBT (µgSn/l)		Copper (µg/l)			
treatment	Water	EtOH	0.5	1	10	20	40	80
n =	74	72	76	77	71	76	73	76
mg/juvenile	1.11	0.97	0.93	0.68	1.14	0.81	0.95	0.54

Appendix 10: *Scrobicularia plana*. Weight of surviving juveniles (pooled per treatment) after 30 day exposure to control, TBT and copper treatments, experiment of 1991.

	Day	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
Ethanol a control	surviving	40	40	40	40	40	40	40	40	40	40	40	40	39	38	38	38
	on surface	-	0	0	0	0	0	0	2	1	1	2	1	1	1	1	0
	size of dead: 2.33, 2.0.																
Ethanol b control	surviving	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
	on surface	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50 ngSn/l a	surviving	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
	on surface	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50 ngSn/l b	surviving	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
	on surface	-	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
125 ngSn/l a	surviving	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
	on surface	-	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
125 ngSn/l b	surviving	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	39
	on surface	-	0	0	0	0	0	0	0	0	0	1	1	2	1	2	1
	size of dead: 2.33.																
250 ngSn/l a	surviving	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
	on surface	-	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
250 ngSn/l b	surviving	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
	on surface	-	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
500 ngSn/l a	surviving	40	40	40	40	40	40	40	40	40	39	39	39	39	39	39	39
	on surface	-	1	2	1	2	3	3	3	3	2	1	0	0	0	2	1
	size of dead: 2.5.																
500 ngSn/l b	surviving	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
	on surface	-	0	0	1	1	2	2	2	2	1	2	2	2	0	1	1

Appendix 11: *Scrobicularia plana*. Total number of juveniles surviving, those live specimens which were found on surface in duplicated bowls (a and b) at time of renewal every other day and length of clams (mm) dead on course of the 30 day long 1992 experiment.

(A)

	Initial	
	Set 1	Set 2
n =	40	40
mg total	15.30	15.20
mg/juvenile		

Size class				
1.83	2.00	2.17	2.33	2.50
11	23	10	3	6
3.2	7.5	4.1	1.5	4.9
0.291	0.326	0.410	0.500	0.817

(B)

treatment	OH control		50 ngSn/l		125 ngSn/l		250 ngSn/l		500 ngSn/l	
replicate	a	b	a	b	a	b	a	b	a	b
n =	38	40	40	40	40	39	40	40	39	40
mg total	22.4	25.5	20.6	20.5	20.8	19.1	18.6	20.7	19.0	20.6

Appendix 12: *Scrobicularia plana*. (A): Blotted dry weight of juveniles in initial sets and weight of individuals per size class as estimated from spare specimens. (B): Dry weight of surviving juveniles in treatments sets after conclusion of the 30 day long experiment of 1992.

(A)

Initial sets

Time	1	2
0	40	40
5	18	22
10	4	8
15	1	2
20	0	2
25	0	2
30	0	2
35	0	2
40	0	0
50	0	0
60	0	0

Ethanol Control

Time	Day 6		D 18		D 30	
	a	b	a	b	a	b
0	40	40	40	40	38	40
5	15	12	19	26	13	12
10	6	6	13	17	5	5
15	4	5	5	9	4	4
20	4	3	4	4	3	2
25	4	3	4	1	2	1
30	2	2	2	1	2	1
35	0	2	2	1	2	1
40	0	2	2	0	2	1
50	0	2	2		2	1
60	0	2	2		2	0

50 ngSn/l

Time	Day 6		D 18		D 30	
	a	b	a	b	a	b
0	40	40	40	40	40	40
5	27	21	30	17	24	15
10	20	11	16	13	15	8
15	6	9	9	12	8	5
20	2	6	9	7	6	3
25	2	5	6	5	4	2
30	1	4	3	3	3	2
35	1	2	3	2	3	2
40	0	1	3	2	2	2
50	0	1	2	2	1	1
60	0	0	1	2	1	1

125 ngSn/l

Time	Day 6		D 18		D 30	
	a	b	a	b	a	b
0	40	40	40	40	40	39
5	18	22	31	29	15	21
10	9	14	17	22	8	9
15	6	10	14	16	5	8
20	6	8	11	11	5	6
25	3	7	7	9	3	5
30	3	5	6	7	1	4
35	2	5	6	5	1	4
40	1	4	6	4	1	4
50	1	3	3	4	1	3
60	1	3	2	4	0	2

250 ng Sn/l

Time	Day 6		D 18		D 30	
	a	b	a	b	a	b
0	40	40	40	40	40	40
5	17	26	23	31	21	23
10	14	19	18	24	13	14
15	11	12	15	19	10	10
20	10	10	10	13	8	9
25	6	6	7	9	4	7
30	4	2	3	7	4	5
35	1	2	3	6	3	3
40	1	1	2	5	1	2
50	0	0	2	4	1	2
60	0	0	2	3	1	2

500 ng Sn/l

Time	Day 6		D 18		D 30	
	a	b	a	b	a	b
0	40	40	39	40	39	40
5	18	21	31	37	26	29
10	15	14	29	30	16	19
15	11	11	23	23	12	15
20	6	11	21	22	7	12
25	5	8	17	17	7	10
30	4	6	14	14	5	9
35	4	5	13	12	4	8
40	4	4	11	10	3	7
50	4	2	10	6	3	5
60	3	1	4	4	3	5

(B)

	Initial		Day 6		Day 18		Day 30	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Initial	6.4	1.5						
Ethanol control			7.4	0.8	9.9	0.1	7.1	1.3
50 ngSn/l			10.0	0.2	12.3	1.5	9.6	2.3
125 ngSn/l			11.3	3.2	17.0	0.9	9.8	2.9
250 ngSn/l			11.4	1.2	16.3	3.6	11.9	1.2
500 ngSn/l			13.2	0.4	26.7	0.7	16.8	3.2

Appendix 13: *Scrobicularia plana*. Initial, control and TBT treatments. (A): Number of juveniles not buried as observed in duplicated bowls (a and b) at time (minutes) intervals during the 30 day long 1992 experiment. (B): estimated burying time of juveniles in minutes.

Day	0	3	6	9	12	15	18	21	24	27	30	33	36	Avg	SD
Bowl	1	1	2	2	1	1	2	2	1	1	2	2	1		
Torridge															
A: surviving	40	-	40	-	40	-	40	-	40	-	40	-	40		
on surface	-	0	0	0	0	0	0	0	0	0	0	0	0		
B: surviving	40	-	40	-	40	-	40	-	40	-	40	-	38		
on surface	-	0	0	0	0	0	0	0	0	0	0	0	0		
Size of dead: 2.0, 2.33															
pH		7.9		8.3		8.3		8.2		8.2		8.1		8.2	0.1
S (ppt)		26		24		25		25		23		24		24.5	1.0
Guernica															
A: surviving	40	-	40	-	40	-	40	-	40	-	40	-	40		
on surface	-	0	0	0	0	0	0	0	0	0	0	0	0		
B: surviving	40	-	40	-	40	-	40	-	40	-	40	-	40		
on surface	-	0	0	0	0	0	0	0	1	0	0	0	2		
pH		7.9		8		8		8		8.2		8.1		8.0	0.1
S (ppt)		24		25		23		26		23		24		24.2	1.2
Cracknore															
A: surviving	40	-	40	-	40	-	40	-	40	-	40	-	40		
on surface	-	0	0	0	0	0	0	0	0	0	0	0	0		
B: surviving	40	-	40	-	40	-	39	-	39	-	39	-	39		
on surface	-	0	0	1	1	1	1	0	0	2	2	1	1		
Size of dead: 1.83															
pH		7.8		8.1		7.6		7.8		7.8		8.1		7.9	0.2
S (ppt)		25		24		26		24		23		25		24.5	1.0
Lamiaco															
A: surviving	40	-	40	-	5	-	1	-	1	-	0	-	-		
on surface	-	1	4	17	28	5	4	1	1	1	1	-	-		
B: surviving	40	-	40	-	9	-	8	-	6	-	6	-	6		
on surface	-	2	4	20	30	8	6	4	3	2	0	2	1		
pH		7.9		8.3		8.3		8.2		8.1		8.1		8.2	0.1
S (ppt)		24		24		24		25		23		24		24.0	0.6

Appendix 14: *Scrobicularia plana*. Number of juveniles surviving as assessed every 6th day and clams which were found on surface every 3rd day in duplicated treatments (A and B) in alternating bowls (1 and 2); length of specimens (mm) dead during the bioassay is included for sediments other than Lamiaco. Also given pH and salinity (parts per thousand) as determined in volumes renewed. For further explanation see text.

sediment	Torridge		Guernica		Cracknore	
replicate	a	b	a	b	a	b
n =	40	38	40	40	40	39
mm total	105	98	105	102	91	90
µm/day	523	454	537	449	139	162
mg total	45.9	43.5	40.7	32.4	19.7	21.5

Appendix 15: *Scrobicularia plana*. Length, daily growth rate and blotted dry weight of juvenile sets at conclusion of sediment bioassay; initial length of sets and weight of initial sets and size classes individuals are given in figure 6.3 and Appendix 6, respectively.

	Time (minutes)		0	2.5	5	7.5	10	15	20	25	30	35	40	50	60	bt
Torridge	Day 0	A	40	15	5	3	2	0	0	0	0	0	0	0	0	2.88
		B	40	25	14	4	0	0	0	0	0	0	0	0	0	3.94
	Day 6	A	40	6	4	1	1	1	1	0	0	0	0	0	0	2.28
		B	40	11	4	2	1	1	0	0	0	0	0	0	0	2.53
	Day 12	A	40	10	3	1	0	0	0	0	0	0	0	0	0	2.13
		B	40	7	3	0	0	0	0	0	0	0	0	0	0	1.88
	Day 18	A	40	9	7	4	0	0	0	0	0	0	0	0	0	2.50
		B	40	1	0	0	0	0	0	0	0	0	0	0	0	1.31
	Day 24	A	40	15	9	5	3	2	1	1	1	1	1	1	1	4.78
		B	40	6	3	2	2	1	0	0	0	0	0	0	0	2.25
	Day 30	A	40	7	1	1	1	0	0	0	0	0	0	0	0	1.91
		B	40	9	4	3	1	1	1	1	1	1	1	1	1	3.66
	Day 36	A	40	18	3	1	1	0	0	0	0	0	0	0	0	2.72
		B	38	18	7	2	2	1	0	0	0	0	0	0	0	3.36
Guernica	Day 0	A	40	12	5	1	0	0	0	0	0	0	0	0	0	2.38
		B	40	11	1	0	0	0	0	0	0	0	0	0	0	2.00
	Day 6	A	40	19	10	7	2	2	2	2	2	1	0	0	0	4.81
		B	40	10	6	3	0	0	0	0	0	0	0	0	0	2.44
	Day 12	A	40	13	6	4	1	0	0	0	0	0	0	0	0	2.78
		B	40	3	0	0	0	0	0	0	0	0	0	0	0	1.44
	Day 18	A	40	9	3	2	1	0	0	0	0	0	0	0	0	2.22
		B	40	3	0	0	0	0	0	0	0	0	0	0	0	1.44
	Day 24	A	40	8	4	3	2	2	1	0	0	0	0	0	0	2.75
		B	40	8	1	0	0	0	0	0	0	0	0	0	0	1.81
	Day 30	A	40	14	1	0	0	0	0	0	0	0	0	0	0	2.19
		B	40	12	7	4	3	2	2	0	0	0	0	0	0	3.47
	Day 36	A	40	12	4	0	0	0	0	0	0	0	0	0	0	2.25
		B	40	11	3	1	0	0	0	0	0	0	0	0	0	2.19
Cracknore	Day 0	A	40	14	6	0	0	0	0	0	0	0	0	0	0	2.50
		B	40	3	0	0	0	0	0	0	0	0	0	0	0	1.44
	Day 6	A	40	5	4	3	2	0	0	0	0	0	0	0	0	2.19
		B	40	4	4	4	4	2	1	1	1	1	1	1	1	3.81
	Day 12	A	40	15	8	1	0	0	0	0	0	0	0	0	0	2.75
		B	40	22	11	3	1	1	1	1	1	1	1	1	1	4.91
	Day 18	A	40	9	5	2	0	0	0	0	0	0	0	0	0	2.25
		B	39	19	9	6	6	2	2	1	1	1	1	0	0	5.10
	Day 24	A	40	19	10	10	9	5	3	2	1	1	1	0	0	6.22
		B	39	15	10	7	6	5	3	3	3	3	3	3	3	8.17
	Day 30	A	40	26	17	9	6	2	0	0	0	0	0	0	0	5.31
		B	39	12	7	2	2	2	2	1	1	1	1	1	1	4.39
	Day 36	A	40	16	9	7	5	1	1	1	1	1	1	1	1	5.03
		B	39	14	9	5	2	1	1	1	1	1	1	1	1	4.58
Lamiaco	Day 0	A	40	24	17	14	13	17	21	22	14	18	12	18	15	
		B	40	22	19	14	14	12	13	6	17	18	20	14	13	
	Day 6	A	40	16	12	7	6	4	3	4	2	3	4	5	3	
		B	40	15	15	13	10	9	8	7	7	6	6	6	6	

Appendix 16: *Scrobicularia plana*. Numbers of juveniles not totally buried as observed in bowls (duplicates A and B) at time intervals every 6th day of the sediment bioassay, and estimated average burying time (bt); raw data for Lamiaco sets are not given for days of reduced survival (see text).

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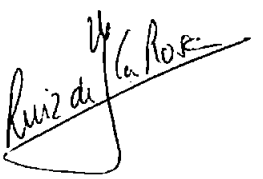

Signed: **Jose M. Ruiz.**