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BIOACCUMULATION AND EFFECTS OF TBT ON MOLLUSCS FROM SOUTHERN PORTUGAL

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ABSTRACT

The present work intended to study the impact of the highly toxic compound tributyltin (TBT) in one of the most important mollusc species in southern Portugal, the clam *Ruditapes decussatus.*

The first Chapter provides a general overview of the TBT problem in the marine environment.

A summary of the analytical methods employed to analyse organotins in water, sediments and biological tissues is presented in Chapter 2.

The effects of sublethal tributyltin concentrations on the growth and development of *R. decussatus* larvae were studied (Chapter 3). Veliger larvae of *Ruditapes decussatus* were exposed to TBT nominal concentrations of 25, 50, 75 and 100 ng L^{-1} Sn in the water for a period up to 13 d. Larval growth and development were chosen as endpoints for evaluation of TBT toxicity. Growth of *R. decussatus* larvae was severely affected by TBT concentrations (25-100 ng L^{-1} Sn). A 3- to 6-fold reduction in growth was observed in these early larval stages. Furthermore, *R. decussatus* larvae exposed to TBT did not develop further than D-larvae while larvae from control cultures reached the umbonated stage.

The effects of sublethal concentrations of tributyltin (TBT) on growth of clam juveniles, *R. decussatus*, were determined in clams exposed to nominal TBT concentrations of 50, 100 and 250 ng L^{-1}Sn in sea water, for a period up to 2 years (Chapter4). *R. decussatus* juveniles increased regularly in length and weight over the whole experimental period. Final length and weight were affected by TBT exposure. However, growth rates (in length and weight) were not significantly different amongst treatments, after 2 years of TBT exposure. Thus, under the described experimental conditions, although a decrease in growth (length and weight) of *R. decussatus* juveniles was observed with increasing TBT concentrations, it was not significantly affected by TBT exposure.

The relative importance of water, sediments and food as vectors of TBT uptake was assessed for the infaunal, suspension-feeding bivalve, *Ruditapes decussatus* (Chapter 5). Accumulation of TBT was determined in *R. decussatus* exposed for 60 days to, moderately high, but environmentally realistic, levels of TBT dissolved in water (100 ng L^{-1} Sn) and sediments (0.8 µg g^{-1} Sn dw), separately or in combination, using constant-flow systems. The results indicate that this species accumulates TBT predominantly from water. Although some accumulation from sediments does occur, the processing of large amounts of water needed to sustain the filter-feeding habits of this species is a prime determinant of TBT uptake. The route of exposure is reflected in tissue distributions of TBT in *R. decussatus.* However, gills are the most important sites for accumulation of TBT from water, irrespective of whether contaminated sediments are present or not. In addition, the relative importance of phytoplankton as a vector of TBT uptake was assessed in the same species. Accumulation of TBT via the algal diet was determined by experimental exposure of *R. decussatus* to ^{14}C-TBT labelled phytoplankton *Isochrisis galbana*, for a period up to 60 days. *Ruditapes decussatus* exhibited increasing TBT burdens during a period up to 40 days, after which an apparent steady state was achieved. Within the body, the digestive tract of these clams initially accumulated TBT. After a few weeks of exposure, internal remobilization resulted in a more widespread partitioning of TBT amongst tissues.

From the results of these experiments (Chapter 5), in which *R. decussatus* were exposed to TBT in water, sediments and food partitioned realistically, it seems clear that this species accumulates TBT predominantly from water. Although some accumulation from sediments and food does occur, the contribution from these phases was negligible when compared to water, at least under the conditions and concentrations used in this experiment.

In a filed survey (Chapter 6), organotin concentrations were measured in water, sediments and clams (*Ruditapes decussatus*) from eleven sites in the Ria Formosa lagoon, Portugal, in 1992-1993. Results showed a marked spatial pattern of tributyltin (TBT) and dibutyltin (DBT) concentrations. Highest organotin concentrations were observed at Olhão (site 5), where the most important fishing harbour of the Southern coast of Portugal is located. Results indicated that fishing vessels, moored in the harbour at Olhão (site 5), were the major source of organotin contamination to the lagoon. No significant seasonal trend was observed, suggesting a continuous input of organotin compounds throughout the year.

In order to assess the incidence of imposex in neogastropods (Chapter 7), a field survey was carried out in Algarve between Vila Real de Santo António, at the eastern border, and Zavial, near Sagres, at the western end. The degree of imposex was determined in six neogastropod species: *Ocenebra erinacea, Hexaplex trunculus, Murex brandaris, Conus ventricosus, Nassarius reticulatus* and *Nucella lapillus,* using the two indices RPLI and VDSI. Imposex was observed in females from all the sampled sites in Algarve, except in Zavial (13), where all females *Nucella lapillus* were unaffected by imposex.

The final discussion is presented in the last Chapter (8). It emphasizes that results from this work constitute a further step to confirm that TBT is among the most toxic chemicals introduced in the marine environment. Its potential for negative impact on the benthic community is evident. In the particular case of *R. decussatus* cultured in Ria Formosa and other important mariculture sites, a continued input of organotins will probably cause serious damage to shellfish species, particularly to its early life-stages. According to the World Health Organisation (WHO, 1990), recommendations for protecting human and environmental health against the damaging effects of TBT include (1) the establishment of restrictions on the use of TBT compounds; (2) evaluation of organotins inputs to the environment from sources other than antifouling paints; (3) improvement of methods for the safe application removal and disposal of organotins. Furthermore, future research needs comprise improvement of detection and analysis of organotins and better knowledge of toxicity, namely immunological effects, mechanisms of toxicity, endocrine effects and mammalian toxicity including studies on potential carcinogenic effects. Human intake of TBT should also be carefully studied; specific consumption recommendations should eventually be published and maximum residue levels (MRL) for TBT, and other organotin compounds, in seafood products should be set to minimize human risks. Attention should also be given to the search for other sensitive bioindicator species, including freshwater organisms and finally more information is required on butyltin residues in fish and shellfish for human consumption.

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Chapter 1

GENERAL INTRODUCTION

The present work intends to study the impact of the highly toxic compound tributyltin (TBT) in one of the most important mollusc species in southern Portugal, the clam *Ruditapes decussatus*, together with an assessment of the situation of TBT contamination in this region.

1.1. Fouling and antifouling paints

From the multitude of species that inhabit the oceans some marine organisms live in the water column or in the sea-bed, others such as sea weeds and barnacles, have to find a hard surface on which to settle and metamorphose in order to fulfil their life-cycle. Settlement can take place on natural substrates such as rocks or shells of other organisms and also on artificial structures, such as pipes, boat hulls and buoys. This settlement of plant spores and animal larvae in man-made structures in the sea is called "fouling" (Champ & Pugh, 1987). It has been estimated that the number of species involved in fouling is in the order of 4000 - 5000. Thus, in contrast with many other forms of pest control which deal with specific species, antifouling protections must function effectively against a whole diversity of species.

The problem of unwanted growth of marine organisms on vessel's hulls has existed ever since boats were immersed in the sea. For more than 2000 years, wax and tar have been used on ships to protect wooden hulls. The ancient Phoenicians and Chartaginians were said to have used pitch and probably copper strips in the hulls of their ships to inhibit the growth of fouling organisms (Champ & Pugh, 1987). Arsenic and sulphur mixed with oil are also said to have been used in 412 BC (Callow, 1990). The Greeks and the Romans are believed to have used lead sheets to prevent the action of boring and fouling in wooden ships. In the 1500s lead sheeting was widely used by Spanish, English and French against fouling and has been the most frequently material used prior to the 1700s (Callow, 1990).

In the 18th century, the use of lead decreased due to its corrosive effect on iron ships and by the turn of the 20th century, with the arrival of steel ships lead and copper sheeting were abandoned due to the severe galvanic corrosion. As an alternative, other coatings using copper sulphate appeared.

From these early formulations cuprous oxide antifouling paints were developed and widely used.

Antifouling paints efficacy is based on the use of biocidal components which are incorporated in the paint matrix. These components will slowly leach into the surrounding water, inhibiting settlement or even killing most fouling organisms (Champ & Pugh, 1987). The main biocide used in antifouling paints has been cuprous oxide. Although this compound is effective against a wide range of animal fouling, many plants are resistant to it. Furthermore, in contact with sea water cuprous oxide forms insoluble salts in the paint surface, interfering with biocide release and thus leading to a loss of the paint efficiency (Anderson & Dalley, 1986).

In order to increase the biocidal characteristics of copper, several other chemical compounds were added to antifouling paints, including organomercury, arsenic and organolead compounds. These chemical compounds are no longer in use, due to the recognition of its toxicity and environmental impact. It was in the search for other biocides to "boost" the performance of cuprous oxide in antifouling paints that, in the early 1960's, another chemical compound, tributyltin (TBT), was first used in antifouling paints. Initially, TBT paints seemed favourable due to their toxicity to fouling animals and plants at low concentrations, low mammalian toxicity and also to its rapid degradation into less toxic products. These characteristics made TBT an obvious replacement for the far more toxic compounds previously used. In addition to TBT, triphenyltin (TPT) was also used as a biocide in antifouling preparations (UNEP, 1996).

An important advantage of organotin compounds was the fact that they were colourless, enabling them to be used in brightly coloured antifoulings for pleasure boats. Furthermore, in conditions of "heavy" fouling such as in tropical oceans, while antifouling copper paints were found to be effective for less than one year, organotin-based paints would prevent fouling for 5-7 years (Champ & Pugh, 1987). Because of their longer useful lifespan, the popularity of organotin paints rose in the decades of 1960s and 1970s, as their cost-effectiveness became widely recognised by paint users (Goldberg, 1986). As a result, TBT has increasingly been used in antifouling preparations over the

80s and 90s decades, both as "booster" in copper-based paints, and also as the sole biocidal agent (at concentrations as high as 3% Sn (dw)) (Batley, 1996). The reduction in fuel costs, together with less frequent dry-docking maintenance and re-painting of ship's hulls were estimated to be worth about 5.7 billion USD (or euros) to the shipping industry in the mid 1990s. As a result, TBT-based antifouling paints dominated the market and, in 1996, were still applied to approximately 70% of the world's commercial shipping fleet (Abbott, 2000; Evans *et al.*, 2000).

1.2. The 'TBT problem'

Taking into account its extremely effective biocide action against a wide variety of fouling organisms, it is hardly surprising that TBT should also affect non-target organisms, especially in areas with high boating activity such as ports, marinas and shipyards (Bryan & Gibbs, 1991).

The deleterious effects of TBT leached from antifouling paints on non-target organisms became apparent in the late 1970s when it was pointed out as the responsible for the decline in oyster (*Crassostrea gigas*) production along the Atlantic Coats of France and in the UK. Abnormal shell formation and poor recruitment of larval oysters were shown to occur in the presence of only a few ng litre $^{-1}$ of TBT in the water (Alzieu *et al.*, 1989; Chagot *et al.*, 1990), illustrating the high toxicity of TBT. In Arcachon Bay, one of the most TBT affected sites in France, economic losses in oyster production were estimated at 150 million dollars, between 1977 and 1983.

More evidence of the deleterious effects of TBT was provided by the responses induced in gastropods. In the group of neogastropods TBT leached from antifouling paints was linked to a reproductive anomaly whereby male sex characteristics are superimposed on female sex organs ("imposex"), at a concentration of 1 ng litre $^{-1}$ (Smith, 1981a-c; Gibbs *et al.,* 1987). Above 5 ng l^{-1} TBT *Nucella lapillus* females became progressively sterilised leading to a population decline (Gibbs & Bryan, 1986; Gibbs *et al.*, 1987).

As a result of its ecological damage to non-target organisms, TBT was considered the most toxic substance ever deliberately introduced into the marine environment (Goldberg, 1986; Maguire, 1987; Mee & Fowler, 1991).

Because of the extreme toxicity of TBT many countries have conducted extensive surveys to assess the impacts of TBT in the marine environment, particularly in coastal areas. Consequently, severe restrictions were imposed on the use of TBT based antifouling paints and the European Union adopted, on the 5th May 2003, a Regulation on the prohibition of organotin compounds on ships (Regulation (EC) 782/2003 of the 14th April 2003). At the moment in Portugal, legislation follows the EU Directives and thus, the use of TBT based paints is banned; however no Environmental Quality Standard was implemented for coastal waters. Although some countries have extensively monitored TBT levels in coastal environments, in southern Portugal data on TBT contamination is still limited.

1.3. Tributyltin compounds

1.3.1. Physical and chemical properties

Tin (Sn) is a metal of the IV group of the periodic table, with an atomic number 50 that can be found in the environment in two oxidation states (Sn^{2+} or Sn^{4+}). It can be present in a wide variety of both inorganic and organic compounds. Essentially, in all organotin compounds, tin is in the oxidation state (+4). Organotin compounds differ from inorganic tin compounds both in environmental behaviour and effects.

Organotins are characterised by the presence of at least one covalent carbon-tin bond. Depending on the number of organic ligands, organotin compounds are classified as mono-, di-, tri-, and tetraorganotins with the general structure $RSnX_3$, R_2SnX_2, R_3SnX, R_4Sn. In compounds of industrial importance, R is usually a butyl, octyl, or phenyl group, while X is usually a chloride, fluoride, oxide, hydroxide, carboxylate or thiolate (Snoeij *et al.*, 1987; UNEP, 1996). The discovery of triorganotin compounds biocidal properties, in the 1950s, led to its use in antifouling paints. It was recognised that different

triorganotin compounds presented different toxicities to distinct living organisms. Tributyltin (TBT) compounds were found to be the most toxic of the triorganotins to bacteria, fungi and a wide range of aquatic organisms (see reviews by Hall & Pinkney, 1984; WHO, 1990; Bryan & Gibbs, 1991). Toxicity of triorganotin compounds led to the use of TBT and to a lesser extent TPT (triphenyltin), as biocides in antifouling paints preparations (Fent & Hunn, 1991; UNEP, 1996). Antifouling paints represent the greatest source of TBT in the marine environment and thus, the present review will focus on these compounds.

Tributyltin compounds are characterised by the existence of three covalent bonds between three carbon atoms and the tin atom. They conform to the general chemical formula $(n\text{-}C_4H_9)_3Sn\text{-}X$, where X is an anion or a group linked covalently to the tin atom. The nature of X influences its physico-chemical properties, especially the relative solubility in water and non-polar solvents as well as the vapour pressure (WHO,1990).

There is little consensus in the literature regarding tributyltin speciation in sea water. Nuclear Magnetic Ressonance (NMR) analysis performed with typical sea water extracts showed the presence of tributyltin chloride (TBTCl), tributyltin oxide (TBTO) and tributyltin carbonate ($TBTCO_3$) (Laughlin *et al.*, 1986a). TBT speciation in sea water is influenced by the pH, according to the scheme:

$Bu_3\text{-}Sn\text{-}OH_2^+$ and $Bu_3\text{-}Sn\text{-}Cl$ for $pH < 7$

$Bu_3\text{-}Sn\text{-}Cl$, $Bu_3\text{-}Sn\text{-}OH$ and $Bu_3\text{-}Sn\text{-}CO_3^-$ for $pH \approx 8$

$Bu_3\text{-}Sn\text{-}OH$ and $Bu_3\text{-}Sn\text{-}CO_3^-$ for $pH > 10$

Thus, under typical sea water conditions (at $pH \approx 8$), it is considered that the three butyltin species (chloride, hydroxide and carbonate), remain in equilibrium (Laughlin *et al.*, 1986a).

TBT by itself is unstable and will break down in the environment unless it is combined with an element such as oxygen. One of the most common TBT compounds is bis(tributyltin) oxide (TBTO) (CAS No. 56-35-9). TBTO is a

mild oxidising agent; it is flammable, but does not form explosive mixtures with air. TBTO vapour pressure is low, with values in the order of 10^{-3} Pa (20°C), indicating a low affinity of the compound to the air (WHO,1990). A study carried out with a TBTO solution of 1 mg L^{-1} showed a negligible volatility of this compound from solution to the air, after a period of two months (Maguire *et al.,* 1983), and also negligible release from fresh water-sediment mixture to the air over a period of eleven months (Maguire & Tkacz, 1985). TBTO solubility in sea water is also relatively low (1 to 2 mg L^{-1} Sn) (Meinema *et al.,* 1978; Inaba *et al.*, 1995). In contrast, this compound dissolves readily in organic solvents such as ethanol, ether and hexane (UNEP, 1996).

1.3.2. Partitioning coefficients

The chemical properties of TBT, particularly its poor water solubility, negligible volatility and lipophilic nature are such that when TBT is introduced into water, a partition process occurs and TBT will preferentially adsorb onto particles (Dooley & Homer,1983; Langston & Pope, 1995). Adsorption of TBT to suspended particles is an important process which will facilitate its transport to benthic sediments.

The partitioning of contaminants between water and sediments is traditionally described by the sediment-water partition coefficient (Kd), the ratio between the concentration of a compound measured in the sediments and that in the overlying water, at equilibrium. Kd is highly dependent on a number of variables including the levels of TBT contamination in overlying water, particle size, salinity, pH and the characteristics of the sediment (Langston and Pope, 1995). Due to the many variables that influence this coefficient, measurements of Kd values in field samples, shown in Table 1.1, presented considerable discrepancy, with values ranging from 227 to 55 500.

In field samples, TBT concentrations in benthic sediments are always several orders of magnitude higher than in the overlying water, confirming the tendency of TBT to adsorb to particles. Once bound to sediments this compound will remain strongly adsorbed, even after long periods of time. An *in-situ* study at the sediment-water interface in Pearl Harbour (Hawaii)

suggested that TBT does not significantly desorbs from sediments after a period of 2.5 months (Stang & Seligman 1987). Moreover, a time series (1985-1992) of TBT levels from Poole harbour (UK) sediments and water, indicated 'half times' in sediments in the order of 50 months (Langston & Pope, 1995). Experiments carried out in the present work assessed the importance of sediments as vectors of TBT uptake to a sediment- dwelling clam, *Ruditapes decussatus* (Chapter 5).

Table 1.1- TBT partition coefficient between benthic sediments and overlying water (Kd) in different environmental samples.

Site	TBT in water (ng L^{-1} Sn)	Kd	Reference
Poole harbour, UK	44.4	5 170	Langston & Pope, 1995
	16.5	10 992	Langston & Pope, 1995
	6.9	32 969	Langston & Pope, 1995
Tamar River, UK	1.7	20 798	Langston & Pope, 1995
Algarve coast, Portugal	4	31 400	Langston *et al.*, 1997
Sado estuary, Portugal	48.5	7 270	Langston *et al.*, 1997
Tejo estuary, Portugal	316	1 930	Langston *et al.*, 1997
San Diego bay, USA	57	227	Valkirs *et al.*, 1986a,b
	44	1 590	Valkirs *et al.*, 1986a,b
	193	4 610	Valkirs *et al.*, 1987
	213	38 900	Valkirs *et al.*, 1987
Sarah creek, USA	7	2 500	Unger *et al.*, 1988
	4	2 600	Unger *et al.*, 1988
	9	5 500	Unger *et al.*, 1988
Pearl Harbour, Hawaii	7	6 250	Stang & Seligman, 1987
	6	55 500	Stang & Seligman, 1987

It is also important to stress that slow desorption of TBT from sediments, over extended periods of time, can result in significant TBT levels in the overlying water and may be of long-term ecotoxicological significance (Dowson *et al.*, 1993b; Langston & Pope, 1995).

In addition to a tendency to adsorb to the particulate phase, chemicals with poor water solubility and negligible volatility, present a high affinity for the

lipid phase in biota and thus can be stored and concentrated in tissues with high lipid content (Mackay, 1982; Rand *et al.*, 1995). This affinity is described by the octanol:water partition coefficient, K_{ow} (the ratio between concentration in *n*-octanol and in water, for the two phases at equilibrium). Typically, logarithmic values of K_{ow} higher than 3 classify a compound as lipophilic (Connell, 1988).

For sea water, TBTO exhibits a moderately high K_{ow}, presenting values between $5x10^3$ and $6x10^3$ (Laughlin *et al.*, 1986a).

1.3.4. TBT degradation

Degradation of organotins in the environment proceeds by successive debutylation steps. This degradation is considered completed with the liberation into water of an inorganic tin compound, as follows (Blunden & Chapman, 1986):

$$R_4Sn \Rightarrow R_3SnX \Rightarrow R_2SnX_2 \Rightarrow RSnX_3 \Rightarrow SnX_4$$

R - butyl, octyl, or phenyl group;

X - chloride, fluoride, oxide, hydroxide, carboxylate or thiolate

For TBT, degradation pathways involve sequential debutylation steps, via dibutyltin (DBT) and monobutyltin (MBT) to inorganic tin (SnX_4). This stepwise loss of butyl groups is followed by a progressive decrease in toxicity. Thus, in the environment TBT compounds were supposed to exert their biocidal function on target organisms and degrade to less toxic compounds in a short period of time. In fact, early studies of TBT emphasised the "disappearance" of the compound in the water.

Degradation of TBT in marine waters can proceed by both biotic and abiotic routes, occurring simultaneously in the environment. Biological processes are probably the most important mechanisms of TBT degradation in the marine environment and several studies suggest a rapid degradation of TBT by microorganisms (fungi, bacteria and phytoplankton) in natural waters

(Lee *et al.,* 1987; Maguire *et al.*, 1984; Maguire & Tkacz, 1985; Olson & Brinckman, 1986; Seligman *et al.,* 1986a; 1986b; Stang & Seligman,1986;).

Like other processes, biodegradation is dependent on a number of factors such as light, temperature, pH, dissolved oxygen, level of mineral elements, presence of biodegradable organic substances, and the nature and density of the microorganisms, including phytoplankton (Lee *et al.,* 1987, 1989; WHO, 1990). A number of laboratory microcosm experiments have been carried out on the degradation kinetics of TBT in spiked sea water samples from harbours and estuaries. Results indicate half-life values of 3-13 days for TBT in sea water (Lee *et al.,* 1987; 1989; Seligman *et al.*, 1986b;)

In the case that phytoplankton does not degrade TBT, algal cells may accumulate this compound and act as a vector of TBT uptake for organisms higher in the food chain, such as molluscs. Laboratory studies are required to evaluate the role of phytoplankton as vector of TBT uptake for marine organism, particularly filter feeding bivalves. This was one of the objectives of the present work (see Chapter 5).

TBT degradation by microorganisms also depends on the concentration of this compound in the water. When a high level of TBT (744 $\mu g\ L^{-1}$), higher than the lethal or inhibitory threshold for microorganisms, was added to natural sea water, under light and ambient temperature, no degradation was observed over a period of 144 days. At such high concentrations, biodegradation was probably inhibited by direct toxicity of TBT (Seligman *et al.,* 1986a).

Besides biological degradation two other processes can be defined as promoting TBT degradation in marine waters; these are photodegradation by ultraviolet radiation (Maguire *et al.,* 1983; Maguire *et al.,* 1986) and hydrolysis (Maguire *et al.,* 1983; Maguire & Tkacz, 1985). Two further processes, with no environmental significance, are capable of inducing TBT degradation; these are thermal cleavage (since temperatures above 200 °C are needed to break the Sn-C bond) and gamma radiation (owing to its negligible intensity at the earth's surface) (Blunden & Evans, 1990).

Although degradation can occur in a short period in marine waters, several studies indicated that degradation in other environmental

compartments, such as sediments, are slower processes that may pose long-term ecological threats (Dowson *et al.*, 1993b; Kilby & Batley, 1993; Langston & Pope, 1995; de Mora *et al.*, 1989). TBT degradation in sediments is also dominated by microorganisms, leading to the same final chemical products as in water but presenting a slower rate, with half-lives ranging from months to 4 years (Dowson *et al.*, 1993b; Langston & Pope, 1995; Stang & Seligman,1986).

Long half-life of TBT in sediments coupled with a slow desorption of this compound from contaminated sediments into the water column is of major ecological importance and confirms the importance of sediments as a TBT reservoir in the marine environment. Even if restrictions are set on the use of TBT, levels of this compound in some environmental compartments, particularly sediments, may not decrease for several years, as confirmed by field studies in the UK (Langston *et al.*, 1997; Waite *et al.*, 1991). Thus, repeated field sampling of sediments, as performed in the present study (Chapter 6), is crucial to determine the extent of TBT contamination in areas of ecological importance.

1.4. TBT in the environment

1.4.1. Environmental sources

It is important to emphasise that butyltin compounds are not produced naturally and therefore their environmental presence is due entirely to anthropogenic inputs (Maguire, 1996).

In the marine environment, antifouling paints represent by far the greatest source of TBT and can be used in several structures such as boats, ships, quays, buoys, fish nets and cages. In addition to antifouling paints, the stripping of old paints from ships hulls, by surface blasting with water, sand or other abrasive agents can also represent a source of TBT to the marine environment (Langston *et al.*, 1995b). Other less important sources of TBT into marine waters include their use as wood preservatives, as slimicides on masonry, as biocides for cooling systems - particularly in power station

cooling towers - and effluents of domestic waste plants, pulp and paper mills, leather processing and textile mills (see reviews by Blunden & Chapman, 1986; WHO, 1990).

1.4.2. Environmental concentrations

1.4.2.1. Water

Since its first use in marine antifouling paints, TBT concentrations have been extensively monitored in marine and estuarine waters world-wide. Ranges of TBT concentrations in sub-surface waters from different areas of the world, are shown in Table 1.2.

In estuarine waters, measured TBT concentrations ranged typically from non detected levels up to 50 ng L^{-1} TBT (Table 1.2).

Mariculture areas generally exhibited TBT levels in water from less than 1 ng L^{-1} up to 300 ng L^{-1}, although prior to the TBT ban exceptionally high concentrations were registered in the UK (500-700 ng L^{-1} TBT) (Table 1.2). Maricultures can be considered highly sensitive areas to TBT contamination since this compound is likely to pose a threat to molluscs, traditionally cultured at these sites. Most TBT data obtained in European maricultures concerns France and the UK. Data on TBT levels in Portuguese waters is scarce. Thus, further surveillance is required to assess the impacts of TBT, in coastal waters, particularly in areas where molluscs are cultured, such as Ria Formosa. This is the aim of the field survey carried out in the present study (Chapter 6).

At locations with intensive boating activity, such as marinas and shipyards, high TBT concentrations in water (up to 4000 ng L^{-1} TBT) were detected (Table 1.2). In Portugal, measurements of TBT in waters in the vicinity of shipyards such as Solisnor (Sado estuary) and Lisnave (Tejo estuary), revealed high levels of this compound in the water (up to 1000 ng L^{-1} TBT) at these sites (Table 1.2). Taking into account that estuaries, where major shipyards are located, are preferential reproduction and nursery areas for many marine organisms and include mariculture areas (e.g. Sado

estuary), continuous monitoring should be performed to assess impacts of TBT in these areas.

Table 1.2- Tributyltin concentrations (expressed as ng L^{-1} TBT) in estuarine and marine subsurface waters.

Sampling area	Location	TBT (ng L^{-1}) min	max	Reference
Estuaries / bays	Portugal	✧ 2.5	20	Langston *et al.*, 1997
	UK	✧ < 3	44	Langston & Burt, 1991
	Chesap.Bay, USA	nd	24	Hall *et al.*, 1987
		5	48	Hall *et al.*, 1988
	San Diego, USA	1	13	Valkirs *et al.*, 1991
	Thailand	<1	3	Larsen, 1997
Maricultures	Portugal	✧ 2	28	Langston *et al.*, 1997
	France	< 2	17	Gabrielides *et al.*, 1990
	UK	530	730	Waldock & Miller, 1983
	Australia	< 1	40	Batley & Scammell, 1991
	Hong-Kong	< 90	285	Lau, 1991
Harbours	UK	✧ 19	157	Langston & Burt, 1991
	Turkey	< 8	935	Kubilay *et al.*, 1996
	San Diego, USA	< 5	350	Seligman *et al.*, 1989
	Thailand	7	11	Larsen, 1997
Marinas	Portugal	10	1000	Langston *et al.*, 1997
	UK	< 80	2250	Waldock & Miller, 1983
		✱ < 8	289	Cleary & Stebbing, 1987
		< 1	3260	Waite *et al.*, 1989
		✧ 19	575	Langston & Burt, 1991
		< 3	100	Dowson *et al.*, 1993a
	Italy	10	3930	Gabrielides *et al.*, 1990
	Turkey	< 8	353	Kubilay *et al.*, 1996
	Holland	120	4000	Ritsema *et al.*, 1991
	Denmark	✧ 60	950	Kure & Depledge,1994
	Chesap.Bay, USA	nd	998	Hall *et al.*, 1987
		77	1801	Hall *et al.*,1988
	San Diego, USA.	17	120	Valkirs *et al.*, 1991
	Hong-Kong	< 90	1000	Lau, 1991
	Australia	100	249	Batley & Scammell, 1991
Naval base	Portugal	✧ 8	120	Langston *et al.*, 1997
	San Diego, USA	3	14	Valkirs *et al.*, 1991
Shipyard	Portugal - Sado	✧ 125	1030	Langston *et al.*, 1997
	Portugal - Tejo	✧ 100	790	Langston *et al.*, 1997

✧ Converted from concentrations originally expressed as ngL^{-1} Sn (Sn x 2.5 = TBT)
✱ Expressed as concentration of total organotins
nd - not detected

In addition to field surveys, studies should be carried out on the impact of dissolved TBT on bivalves. Although bivalves are benthic organisms, their life-cycle includes planktonic larval stages which live in the water column. These early life-stages can be adversely affected by the presence of TBT in

the aqueous phase (Beaumont & Budd, 1984; Laughlin *et al.*, 1988; Lapota *et al.*, 1993; Ruiz *et al.*, 1995a). Studies on the deleterious effects of TBT on the early life-stages of *R. decussatus* are addressed in Chapters 3 and 4 of this work.

Considerable evidence has shown that TBT concentrations in water are notably enriched in the so-called surface microlayer (1000 µm in thickness) (Gucinski, 1986; Cleary & Stebbing, 1987; Hall *et al.*, 1987; Battley & Scammell, 1990; Hardy & Cleary, 1992; GESAMP, 1995). Probably, enhanced levels of TBT present in the surface microlayer are ecologically significant, since they may cause adverse biological effects. Organisms that inhabit the microlayer permanently such as bacteria, ciliates and algae, termed neuston, together with temporary inhabitants including eggs and larvae of a great number of fish and invertebrate species, may be severely affected by exposure to high TBT levels (Hardy *et al.*, 1987; Cleary *et al.*, 1993; GESAMP, 1995). Still, *in situ* studies are required to assess the magnitude of damaging effects caused by TBT enhanced contamination in the surface microlayer

1.4.2.2. Sediments

As for waters, there have been a significant number of studies which report TBT concentrations in estuarine and marine sediments. Examples, from different locations world-wide, are shown in Table 1.3.

Table 1.3 - Tributyltin concentrations (expressed as ng g^{-1} TBT *) in surface sediments from different locations world-wide

Sampling area	Location	TBT (ng g^{-1}) * min	max	Reference
"Clean" sites	Portugal	✧ nd	23	Langston *et al.*, 1997
	Puget Sound, USA		< 2	Krone *et al.*, 1989
	Egypt		35	Gabrielides *et al.*, 1990
Coastal zone	Chesap. Bay, USA	< 1	6	Espourteille *et al.*, 1993
	USA	< 5	187	Wade *et al.*, 1990
Estuaries / bays	Portugal	✧ < 8	53	Quevauviller *et al.*, 1988
		✧ nd	70	Langston *et al.*, 1997
	UK	✧	25	Langston & Burt, 1991

Sampling area	Location	TBT (ng g^{-1}) * min	max	Reference
Estuaries / bays	San Diego, USA	2	78	Valkirs *et al.*, 1991
	Chesap. Bay, USA	1	93	Espourteille *et al.*, 1993
	Korea	✧ 55 000	307000	Hwang *et al.*, 1999
Fjord	Denmark	< 10	84	Kure & Depledge, 1994
Mariculture	Portugal	✧ nd	90	Langston *et al.*, 1997
	Italy	★ 13	19	Cardellicchio *et al.*, 1992
		★ 14	29	Cardellicchio *et al.*, 1992
	Thailand	4	81	Kan-Atireklap, 1997
	Hong-Kong	52	1100	Lau, 1991
Harbour	UK	✧ 40	2875	Langston & Burt, 1991
	Thailand	36	4500	Kan-Atireklap, 1997
Marina	Portugal	✧ nd	60 000	Langston *et al.*, 1997
	UK	✧ 250	550	Langston & Burt, 1991
		< 3	4207	Dowson *et al.*, 1993a
	Denmark	45	74	Kure & Depledge, 1994
	Egypt	260	975	Gabrielides *et al.*, 1990
	Puget Sound, USA	✧ < 7	8283	Krone *et al.*, 1989
	San Diego, USA	37	280	Valkirs *et al.*, 1991
	Thailand	9	880	Kan-Atireklap, 1997
	Hong-Kong	60	1160	Lau, 1991
	Fiji Islands	✧ 40	95380	Stewart & de Mora, 1992
Naval base	Portugal	✧ 120	160	Langston *et al.*, 1997
	Italy	★ 45	51	Cardellicchio *et al.*, 1992
	San Diego, USA	15	1100	Valkirs *et al.*, 1991
Shipyard	Portugal	✧ < 8	1305	Quevauviller *et al.*, 1988
		✧ 580	1910	Langston *et al.*, 1997
		✧ 1520	1820	Langston *et al.*, 1997
	Chesap. Bay, USA	24	4000	Espourteille *et al.*, 1993

✧ Converted from concentrations originally expressed as Sn (Sn x 2.5 = TBT)
* TBT concentrations in dry weight (dw), except where indicated
★ Concentrations expressed in wet weight
nd - not detected

In sediment samples from "clean" sites, levels of TBT were generally <50 ng g^{-1} TBT (dw) (Table 1.3). In estuaries, fjords and mariculture sites TBT concentrations ranged typically from non detected levels up to 100 ng g-1 (dw). However, unusually high TBT contaminations were detected in sediments from a mariculture in Hong-Kong (up to 1 µg g^{-1} dw) and from Chinhae Bay in Korea (up to 307 µg g^{-1} dw) (Table 1.3).

In the vicinity of marinas and shipyards TBT levels were generally high and concentrations up to 8 µg g^{-1} (dw) were observed (Table 1.3).

Table 1.4 - Tributyltin concentrations (expressed as ng g^{-1} TBT *) in the whole soft tissues of several species of clams, oysters and mussels, collected in different locations world-wide.

Species	Location/ type		TBT (ng g^{-1}) min.	max.	Reference
Clams					
Ruditapes decussatus	Spain / harbour	✧	420	710	Morcillo *et al.*, 1997
	Spain / mariculture			nd	Morcillo *et al.*, 1997
Scrobicularia plana	Portugal / estuary	✧	nd	730	Langston *et al.*, 1997
	Portugal / shipyard	✧	4 450	14 600	Langston *et al.*, 1997
	UK / estuary	✧	88	131	Langston & Burt, 1991
	UK / commercial harbour	✧	1 255	5 120	Langston & Burt, 1991
	UK / marina	✧	3 790	12 775	Langston & Burt, 1991
Mya arenaria	Denmark/coastal zone	✧	628	1 330	Kure & Deplege, 1994
	Denmark / fjord	✧	9 638	36 900	Kure & Deplege, 1994
Anadara scapha	Fiji / marina	✧		226	Stewart & de Mora, 1992
Oysters					
Crassostrea gigas	Japan / coastal zone		50	300	Mizuishi *et al.*, 1989
	Korea / bay	✧	250	4 500	Hwang *et al.*, 1999
	Australia / estuary	✧		439	Batley & Scammell, 1991
Crassostrea virginica	USA / coastal zone		7	180	Espourteille *et al.*, 1993
	USA / coastal zone		10	4 030	Uhler *et al.*, 1993
	USA / bay	✧	< 13	4 209	Wade *et al.*, 1991
			43	2 000	Espourteille *et al.*, 1993
	USA / shipyard		1000	5 600	Espourteille *et al.*, 1993
Ostrea angasi	Australia /estuary	✧		< 3	Batley & Scammell, 1991
Ostrea sandvencis	USA / marina		970	5 280	Uhler *et al.*, 1993
Saccostrea commercialis	Australia / estuary	✧	< 5	879	Batley & Scammell, 1991
Mussels					
Mytilus edulis	Holland / estuary		< 1	530	Ritsema *et al.*, 1991
	Holland / marina		350	2 300	Ritsema *et al.*, 1991
	Norway / fjord		870	1 580	Page & Widdows, 1991
	UK / naval base		540	1 880	Page & Widdows, 1991
	UK/ oil terminal		330	1 560	Page & Widdows, 1991
	USA / bay		10	1 380	Uhler *et al.*, 1993
	USA /bay -estuary	★	27	390	Valkirs *et al.*, 1991
Mytilus edulis	USA/ naval base	★	11	1 700	Page & Widdows, 1991
	USA/ marina	★	110	2 100	Page & Widdows, 1991
	Australia / bay	★	18	166	Batley & Scammell, 1991

Species	Location/ type	TBT (ng g^{-1}) min.	max.	Reference
Mytilus galloprovincialis	Portugal / coastal zone	✧ 40	100	Quevauviller *et al.*, 1988
	Portugal / shipyard	✧ 60	286	Quevauviller *et al.*, 1988
		✧4 500	8 100	Langston *et al.*, 1997
	Spain / harbour	✧ 308	2 900	Morcillo *et al.*, 1997
	Italy / mariculture	★ 10	24	Cardellicchio *et al.*, 1992
		★ 10	13	Cardellicchio *et al.*, 1992
	Italy / naval base	★ 23	34	Cardellicchio *et al.*, 1992
	Korea / bay	✧ 300	3000	Hwang *et al.*, 1999
Mytilus californianus	USA / bay	10	470	Uhler *et al.*, 1993

* TBT concentrations in dry weight , except where indicated
★ concentrations expressed in wet weight
✧ Converted from concentrations originally expressed as Sn (Sn x 2.5 = TBT)
nd - not detected

A wide range of TBT levels were detected in marine bivalves. TBT concentrations in clam tissues ranged from non detected levels in *S. plana* up to 37 μg g^{-1} in *Mya arenaria* (Table 1.4). Oysters exhibited TBT burdens from less than 3 ng g^{-1} in *Ostrea angasi* up to 5.6 μg g^{-1} in *C. virginica* (Table 1.4). Mussels exhibited TBT concentrations from less than 1 ng g^{-1} in *M. edulis* up to 8.1 μg g^{-1} in *M. galloprovincialis* (Table 1.4). The wide rage of TBT levels detected in bivalves collected in the field is probably a reflection of their proximity to organotin sources, as well as distinct accumulation, feeding habits and ability to degrade TBT compounds in different species.

Extensive assessments of TBT contamination in bivalves were performed in several countries such as the USA, UK and France among others. As for TBT contamination in waters and sediments, data on TBT burdens in bivalves is scarce in Portugal. A survey on TBT burdens in bivalves from the Portuguese coast seems crucial in order to evaluate the accumulation of organotins in these species, hence their inclusion in the field survey described in Chapter 6. Furthermore, it is important to stress that several bivalve species, as *R. decussatus*, are important edible resources in Portugal. Thus, continued surveillance to monitor TBT burdens in bivalve's

tissues should be performed also in order to assess potential human consumption of this compound.

1.5. Routes of TBT uptake in marine bivalves

It is clear from numerous studies that marine organisms can obtain and accumulate pollutants directly from sea water and also from sediments and from contaminated food (see, for example, Bryan, 1979; Fowler, 1982). In bivalves, routes of uptake include the water, benthic sediments and food (including phytoplankton and particles in suspension). Extensive reviews on TBT bioaccumulation from water and sediments were published during the last decade (Alzieu, 1996; Bryan & Gibbs, 1991), but very limited information exists for *R. decussatus* and related species (Gomez-Ariza *et al.*, 1999). Thus in order to understand, more fully, the implications and risks of TBT impact in this species, a study of uptake rates and routes is required (see Chapter 5).

1.5.1. Uptake of TBT from water

In respect to the major organic pollutants, uptake by marine organisms is dominated by a passive diffusion from solution, although there is evidence that carrier-mediated uptake may also occur (Van den Berg *et al.*, 1995). In marine organisms, the bioconcentration mechanism involves transfer from water to gills, or body surface, then to the circulatory fluid followed by either metabolism and excretion of the products or storage in body lipids. Molecules having a high degree of liposolubility are usually absorbed more rapidly and certainly the lipophilic properties of TBT as well as its moderately high octanol-water partitioning coefficient (K_{ow}) contribute to its bioaccumulation by marine organisms (Fowler, 1982; Connell, 1988).

The ratio between the steady-state concentration of a compound in the organism and its levels in water (expressed in the same unit) is usually referred to as the Bioconcentration Factor (BCF). Bioconcentration increases with increasing lipophilicity as measured by the K_{ow} and theoretically, the K_{ow}

coefficient provides the possibility of predicting the BCF (Connell, 1988). For organic compounds the relationship between BCF and K_{ow}, in aquatic organisms, was examined by several authors and direct relationships were obtained between BCF and K_{ow} (see review by Connell, 1988). In the case of TBT (K_{ow} $5x10^3$- $6x10^3$) predicted BCFs for molluscs, including clams, varied between 78 and 91 (Hawker & Connell, 1986). These predicted BCF values, appear to be very low in comparison with "true" values determined in field and laboratory experiments (Table 1.5), suggesting that lipid partitioning is not the only factor involved in the bioconcentration of pollutants in molluscs.

Bioconcentration from water is influenced by a number of abiotic and biotic factors including exposure time, the physico-chemical form of the pollutant, salinity, temperature, competitive effects with other substances as well as stage of the life-cycle, physiology and feeding strategy of the organism. Considering the multitude of factors that impinge on the bioaccumulation process it is not surprising that, for TBT, BCFs appear to vary across a wide range. Examples of BCFs determined for a variety of bivalve organisms, exposed to TBT in the water, are given in Table 1.5.

Table 1.5 - Bioconcentration factors (BCF) determined for several species of marine bivalves exposed to TBT in the water.

Species	Lab. / field study	TBT conc. in water (µg L^{-1})	BCF	Reference
Scrobicularia plana	laboratory	* 0.25	13 000	Langston & Burt, 1991
	field	* 0.17	49 487	Bryan & Gibbs, 1991
Macoma balthica	field	* 0.17	67 258	Bryan & Gibbs, 1991
Mercenaria mercenaria	field	* 0.17	126 818	Bryan & Gibbs, 1991
Mya arenaria	field	* 0.17	539 690	Bryan & Gibbs, 1991
Crassostrea gigas (juveniles)	laboratory	0.15 1.6	11 400 2 300	Waldock & Thain, 1983
Crassostrea gigas (adults)	laboratory	0.15 1.25	6 000 2 000	Waldock *et al.*, 1983
	field	* < 20 - 88	25 000	Shim *et al.*, 1998
Ostrea edulis	laboratory	0.15 1.25	1 500 1 000	Waldock *et al.*, 1983
Mytilus edulis	field	* <0.10 0.50	50 000 5 000	Zuolian & Jensen, 1989
	laboratory	0.023 0.045	1300	Laughlin & French, 1988

Species	Lab. / field study	TBT conc. in water (µg L^{-1})	BCF	Reference
Perna viridis	laboratory	2.8 10	32 174 7 503	Karande *et al.*, 1993
Cerastodeme edule	field	* 0.17	60 528	Bryan & Gibbs, 1991

* Converted from concentrations originally expressed as Sn (Sn x 2.5 = TBT)

Results demonstrate considerable interspecific variability with respect to bioconcentration of TBT, suggesting that mussels and oysters accumulate TBT to a lower extent (BCF from 1000 to 50000) than cockles and clams (BCF from 13000 to 540000) (Table 1.5). Large interspecific differences in BCF values are probably related with variation in feeding habits, uptake rates and distinct abilities to degrade TBT *in vivo* (Kaag *et al.*, 1997; Meador, 1997).

Information on TBT bioconcentration in bivalves found in the Portuguese coast is scarce. Long-term experiments, using environmentally realistic concentrations, along with field surveys are required to assess accumulation of TBT from water, in commercially important bivalve species such as *R. decussatus*. This is one of the main objectives of the current research (see Chapters 5 and 6).

1.5.2. Uptake of TBT from sediments

It is generally accepted that sediments act mainly as a medium for sequestration and degradation of organic pollutants and comparatively little attention has been given to its role as a potential pathway for pollutant uptake. Taking into account the adsorption of TBT to particles and consequent accumulation in surface sediments, this should be regarded as a potential route of uptake for benthic organisms. In fact, it has been suggested that TBT bound to sediments may be a threat to benthic species namely deposit feeding bivalves (Langston & Burt, 1991). For the deposit feeder *S. plana*, laboratory studies demonstrated that sediments constitute a preferential route of TBT uptake when compared to water (Langston & Burt, 1991). Similarly, it was shown that sediment was the major uptake route of other organic pollutants (PCBs and PAHs) for the clam *Macoma balthica*

(Kaag *et al.*, 1997). Sediment-dwelling clams, *Mya arenaria*, from a contaminated harbour were also shown to concentrate TBT from sediments (Langston *et al.*, 1987). Moreover, freshwater mussels *Elliptio complanata* exposed to TBT contaminated sediments exhibited concentration factors, between mussels and sediments, from 0.1 to 18.1 (Chau *et al.*, 1989).

Studies on TBT bioaccumulation from sediments is very limited, especially for suspension feeding bivalves and further studies are necessary to evaluate the importance of sediments as vectors of TBT uptake. As with waters, environmentally realistic concentrations should be used in order to ensure ecological relevance. The study of sediment as potential vector of TBT uptake in clams *R. decussatus* is one of the aims of this work (Chapter 5).

1.5.3. Uptake of TBT from food

In marine benthic organisms uptake of pollutants from food and water exhibits different mechanisms. According to Fowler (1982) when uptake from food occurs, a contaminant is probably taken up by cells in the digestive tract and then distributed to other tissues. Once assimilated from the digestive tract, many of the same physiological factors affecting pollutants absorbed from water will determine the fate of ingested contaminants. Thus, tissue accumulation of pollutants taken up from food depends on the assimilation efficiency, subsequent metabolism and excretion or storage of the contaminant (Fisher & Reinfelder, 1995).

Several laboratory experiments have been carried out using different prey and predator species in order to determine transfer of organic compounds from particles of food. In a study carried out for 60 days, mussels *M. edulis*, showed an ability to accumulate TBT directly from the algae *Dunaliela* sp. previously exposed to low levels of this compound (20-500 ng L^{-1}) (Guolan & Yong, 1995). In addition, differential tissue accumulation was observed in *M. edulis* exposed to TBT contaminated phytoplankton (*Isochrysis galbana*). Higher levels of this compound were detected in the viscera, while the gills, mantle and the adductor muscle exhibited lower TBT concentrations (Laughlin *et al.*, 1986 b).

Rouleau *et al.* (1995), studied the distribution kinetics of tributyltin from food in a marine invertebrate, the starfish *Leptasterias polaris*, fed with TBT-

contaminated mussels. This study, using whole-body autoradiography, indicated that TBT was absorbed in the stomach, transferred to pyrolic *caeca* (where absorption of food takes place) and then from this tissue to the rest of the body.

Relative tissue distributions of contaminants are insufficient to identify accumulation pathways. Understanding the bioaccumulation of TBT in bivalves requires a quantitative appreciation of the relative importance of each source (water, sediments and food), as well as the kinetics of TBT uptake in these organisms. As mentioned earlier, environmentally realistic concentrations have to be used in experiments to ensure ecotoxicological relevance. Assessment of TBT uptake from contaminated food, using these sorts of concentrations, is the objective of the work described in Chapter 5.

Studies on bioaccumulation of TBT are of particular importance when attempting to set environmental quality criteria for the protection of aquatic life. In countries, like Portugal, where Environmental Quality Standards should be defined despite existing legislation, in order to protect marine organisms, standards should focus on the media which constitutes the main route of TBT uptake.

1.6. Toxic effects of TBT

Since TBT eventually degrades to harmless inorganic tin (SnX_4) in the environment it initially seemed to have acceptable advantages over the previously used organometallic biocides. Thus, TBT rose in popularity until it was recognised as one of the most damaging compounds ever introduced in the marine environment.

Toxic effects induced by low concentrations of TBT have been catalogued in a large spectrum of marine organisms, including bacteria (Dooley & Kenis, 1987), phyto and zooplankton (Laughlin & French, 1980), macroalgae (Burridge *et al*, 1995), worms (Moore, 1991; Walsh *et al.*, 1986a), echinoderms (Mercier *et al.*, 1994; Walsh *et al.*, 1986b), crustaceans (Burridge *et al.*, 1995; Laughlin *et al.*, 1983; Lignot *et al.*, 1998; Weis & Perlmutter, 1987) and fish (Pinkney *et al.*, 1990; Ward *et al.*, 1981).

Nevertheless, substantial evidence indicates that molluscs are the most sensitive *taxa* to TBT contamination (Bryan & Gibbs, 1991). A remarkable example of sensitivity to TBT comes from neogastropods, such as *Nucella lapillus*, which display 'imposex' - a peculiar reproductive anomaly caused by TBT, which has been used as biological indicator of TBT contamination.

1.6.1. Imposex

In females of different species of neogastropods, TBT induces 'imposex' - the imposition of male characters on females (Smith 1971), with the formation of a penis and vas deferens - following an exposure to TBT concentrations of only a few parts per trillion (ng L^{-1}) (Bryan *et al.* 1986, 1987; Gibbs *et al.*, 1987; Smith, 1981a-c). Effects on populations vary considerably and for some gastropod species, such as *Nucella lapillus* and *Ocenebra erinacea*, TBT can cause an impairment in the breeding activity, sterility of females and, consequently, a reduction in the population's abundance which can eventually lead to extinction (Bryan *et al.*, 1986). In others, such as *Ilyanassa obsoleta* and *Nassarius reticulatus*, imposex appears to cause little interference with the reproductive activity of the affected female, hence reproduction and population ecology do not appear to be affected (Smith, 1981a-c). Demonstrating the global nature of the problem, imposex has been observed in over 150 gastropod species in coastal waters world-wide (Bettin *et al.*, 1996; Gibbs & Bryan, 1994; Schulte-Oehlmann *et al.*, 2000). Studies with *N. lapillus* have shown that the extent of imposex depends on the ambient TBT concentrations; concentrations as low as 1-2 parts per trillion TBT (as Sn) (1-2 ng L^{-1} Sn) can induce the development of a penis and a vas deferens in females and lead to oviduct blockage. Levels of 3-5 ng L^{-1} Sn can sterilise females, thus leading to an inhibition on reproduction (Gibbs *et al.*, 1987; 1988).

High sensitivity of neogastropods to TBT can be used as a valuable tool to assess TBT contamination, since lowest effect levels are similar to detection limits reported for this compound. Thus, studies on the degree of imposex in neogastropod species should be performed in order to further monitor the extent of TBT contamination in coastal areas. An assessment of

the degree of imposex in different species of neogastropods from the Algarve coast, was the objective of Chapter 7.

In addition to imposex, TBT causes other damaging effects on many molluscan species, in particular bivalves. Several reviews on TBT lethal and sublethal toxicity on marine organisms were published in the last decades (Bryan & Gibbs, 1991; Hall & Pinkney, 1984; Maguire, 1987; Rexrode, 1986; Waldock, 1994; Waldock *et al.*, 1987; WHO, 1990).

1.6.2. Lethal toxicity of TBT to bivalves

Assessment of pollutants toxicity is commonly carried out by measurement of short-term lethality and thus, standard ecotoxicological procedures are known to include this type of toxicity testing. For a given substance, this involves determining the median lethal concentration (LC_{50}) of the chemical compound to which test organisms are exposed. The LC_{50} is the concentration estimated to produce mortality in 50% of a test population of organisms over a specific time period (Duffus, 1980; Rand *et al.*, 1995) and constitutes a major part of the ecological risk assessment of potential pollutants (Forbes & Forbes, 1994). Besides LC_{50}, data found in the literature on lethal toxicity refers to the "concentration that causes high mortality to organisms (generally >50%)". A summary of lethal effects of TBT to different life stages of bivalves, is presented in Table 1.6.

Table 1.6- Lethal concentrations of TBT to embryos, larvae, post-larvae, juveniles and adults of different bivalve species, exposed to TBT in the water.

Stage of life-cycle	Species	Exposure time	LC_{50} (µg L^{-1})	Concentration causing high mortality (µg L^{-1})	Reference
Embryo	*M. mercenaria*	48 h	1.13		Roberts, 1987
	C. virginica	48 h	1.30		Roberts, 1987
Larvae	*M. mercenaria*	48 h	1.65		Roberts, 1987
		8 d		>0.6	Laughlin *et al.*, 1989
	S. plana	10 d		>0.312 *	Ruiz, 1993
	C. gigas	24 h		>5	His & Robert, 198(
		48 h	1.6		Thain, 1983

Stage of life-cycle	Species	Exposure time	LC_{50} (µg L^{-1})	Concentration causing high mortality (µg L^{-1})	Reference
Larvae	*C. gigas*	10 d		>0.05	His *et al.*, 1983
	C. virginica	48 h	3.96		Roberts, 1987
	M. edulis	24 h	0.635 *		Stenalt *et al.*, 1997
		48 h	2.3		Thain, 1983
		5 d		10	Beaumont & Budd 1984
		10 d		1	Beaumont & Budd 1984
		15 d	0.1		Beaumont & Budd 1984
		25 d	0.05 - 0.13		Lapota *et al.*, 1993
Post-larvae	*M. mercenaria*	25 d		10	Laughlin *et al.*, 1989
		25 d	7.5 <LC50<1(		Laughlin *et al.*, 1989
	S. plana	30 d		>0.312 *	Ruiz, 1993
	C. gigas	45 d		>0.24	Thain & Waldock, 1985
	O. edulis	45 d		>0.24	Thain & Waldock, 1985
Juvenile	*S. plana*	30 d		>5 *	Ruiz, 1993
	R. decussatus	45 d		2.6	Thain & Waldock, 1985
	M. edulis	14 d		2.6	Thain & Waldock, 1985
Adults	*C. gigas*	21 d		1.25	Waldock *et al.*, 1983
		30 d		2	Alzieu & Portmann, 1984
	O. edulis	21 d		1.25	Waldock *et al.*, 1983
	Saccostrea cucullata	96 h	25		Karande *et al.*, 1993
		28 d	10		Karande *et al.*, 1993
	M. edulis	66 d	0.97		Valkirs *et al.*, 1987
	Mytilopsis sallei	96 h	56		Karande *et al.*, 1993
		28 d	13		Karande *et al.*, 1993
	Perna viridis	96 h	4.8		Karande *et al.*, 1993
		28 d	0.28		Karande *et al.*, 1993

* Converted from concentrations originally expressed as Sn (Sn x 2.5 = TBT).
LC_{50} - Median lethal concentration;
h - hours, d - days

Generally, estimated LC_{50} values demonstrate considerable variability reflecting a range of different sensitivities to TBT in water. Data presented in Table 1.6 suggests that early life stages of different species are more sensitive to TBT contamination than other stages of the life-cycle. Embryos

and larvae of bivalves exposed to TBT for 24 or 48 hours exhibited LC_{50} in the order of 0.6-4 µg L^{-1} TBT, while adult bivalves exposed to this compound for a period of 96 hours displayed LC_{50} from 5 to 56 µg L^{-1} TBT. As expected, when the exposure time increased (≥ 15 days) bivalves presented lower LC_{50}, but again early life stages of bivalves presented higher sensitivity to TBT than adults (Table 1.6).

Concerning TBT concentrations that cause high mortality (>50%) to bivalves, in exposures for longer periods, a wide range of values has also been reported. For larvae, lethal concentrations were shown to be as low as 0.05 µg L^{-1} TBT (*C. gigas*) while for post-larvae the lowest reported value was 0.24 µg L^{-1} TBT (*C. gigas* and *O. edulis*). In the case of juveniles, high mortality was observed in *R. decussatus* and *M. edulis* above a threshold concentration of 2.6 µg L^{-1} TBT. For adult bivalves, the lowest lethal concentrations was reported for oysters *C. gigas* and *O. edulis* with a value of 1.25 µg L^{-1} TBT.

In addition to the reported studies (Table 1.6), experiments were also carried out exposing deposit feeding clams (*S. plana*) to 10 µg g^{-1} TBT (Sn) in the sediments. After 12 days all adult individuals were dead showing that TBT contaminated sediments can also be lethal to benthic organisms (Langston & Burt, 1991).

Information provided by lethal toxicity tests is limited because, in most cases, extremely high concentrations are utilised to elicit mortality. This concentration range is usually not found in the environment and thus lethal toxicity tests lack realism. In contrast, chronic toxicity tests permit an evaluation of possible adverse effects of a chemical compound under conditions of long-term exposure at low, environmental realistic, concentrations. Toxicity tests will only attain ecological relevance if carried out under such conditions.

1.6.3. Chronic toxicity of TBT to bivalves

Chronic toxicity studies with pollutants include a wide range of sublethal effects including reductions in growth rates and condition index,

development of abnormal larvae, reduction in burying activity of juveniles as well as deleterious effects on reproduction. Furthermore, data obtained in chronic toxicity tests allow an estimation of different parameters such as the highest concentration of a chemical compound at which no deleterious effect is observed (NOEC - No Observed Effect Concentration); the lowest concentration that produces an effect (LOEC) and the median effect concentration (EC_{50}) - concentration of a chemical compound estimated to produce a specific effect in 50% of a test population - which are crucial for risk assessment studies (Rand *et al.*, 1995).

Sublethal effects caused by TBT have now been described in a wide range of marine organisms. Phytoplankton growth was inhibited at levels of 100 ngL^{-1} (Beaumont & Newman, 1986); growth of coelentrates *Laomeda flexuosa* was restrained at a concentration of 500 ngL^{-1} TBT; arm regeneration of sea star *Ophioderma brevispina* was inhibited at a concentration of 100 ngL^{-1} TBT (Walsh *et al.*, 1986b); crustaceans (*Gammarus oceanicus* and *Hommarus americanus*) presented a reduction in growth at levels of 300-1000 ngL^{-1} TBT (Laughlin & French, 1980; Laughlin *et al.*, 1984); reproduction of mysids *Acanthomysid sculpta* was affected at a concentration of 140 ngL^{-1} TBT (Davison *et al.*, 1986); larvae of tunicates *Styela plicata* presented an inhibition of metamorphosis at 63 $\mu g\ L^{-1}$ TBT; a reduction in growth and viability was also observed in fish larvae (*Morone saxatilis*) exposed to 800 ngL^{-1} TBT (Pinkney *et al.*, 1990). However, the most sensitive marine biota to TBT are molluscs, especially gastropods and bivalves. Examples of different sublethal effects caused by exposure to low concentrations of TBT in several species of bivalves are presented in Table 1.7.

As with lethal toxicity, chronic toxicity studies indicate that early life stages of bivalves are the most sensitive stage to TBT exposure. Sublethal effects of pollutants on early life stages of bivalves are important to consider since they involve a potential effect on recruitment and consequently in the abundance of species (Ruiz *et al.*, 1994).

For the development of embryos to larvae *S. plana* exhibited an EC_{50} of $<0.625\ \mu g\ L^{-1}$ TBT, while a delay in development was observed at

concentrations ranging from 0.01 to 1 $\mu g L^{-1}$ TBT in embryos of *M. mercenaria* and *C. virginica* (Table 1.7). Larvae of different bivalve species presented reductions in growth at concentrations between 10 and 600 $ng L^{-1}$ (Table 1.7). Inhibition of metamorphosis and development of abnormal larvae was observed at concentrations as low as 0.1 $\mu g L^{-1}$ (Table 1.7).

A variety of sublethal effects were also reported for bivalve post-larvae and juveniles exposed to low levels of TBT for long periods. Deleterious effects on growth were observed above a threshold concentration of 0.02 $\mu g L^{-1}$ TBT. Reductions in feeding rates and oxygen consumption were detected at concentrations of 0.05 $\mu g L^{-1}$ TBT and a decrease in the condition index was observed in post-larvae of *C. gigas* exposed to 1.6 $\mu g L^{-1}$ TBT (Table 1.7).

Low exposure concentrations (0.04 - 0.2 $\mu g L^{-1}$ TBT) during a period of 56 days, did not affect growth rates of *M. edulis* juveniles. In an analogous experiment, levels of TBT in water within the same range ($\geq$0.07 $\mu g L^{-1}$), during 196 days caused significant reductions in growth only after a first phase of 60 days (Table 1.7). For *C. gigas* spat, a LOEC (4 months) of 10 ng L^{-1} TBT was indicated for shell thickening (Table 1.7)

Burying activity of juvenile *S. plana* was also shown to be affected by the presence of low concentrations of dissolved TBT (>1.25 $\mu g L^{-1}$) (Table 1.7). Studies with adult bivalves revealed that concentrations above 0.03 $\mu g L^{-1}$ TBT could cause weight reductions after a few months of exposure (Table 1.7). A decrease in the condition index of *C. virginica* was observed with increasing TBT concentrations from 0.04 -1.89 $\mu g L^{-1}$ TBT (Table 1.7).

In most studies concerning TBT deleterious effects on bivalves, toxicity is expressed as an external concentration in the exposure medium. However, toxic effects can also be expressed as internal concentrations in tissues. For *M. edulis*, a TBT tissue concentration of 5 $\mu g g^{-1}$ TBT (dw) induced a reduction in growth (EC_{50}) while a reduction in feeding rate was detected at a level of 3-4 $\mu g g^{-1}$ TBT (dw) in tissues (Table 1.7).

Table 1.7- Sublethal effects of TBT to embryos, larvae, post-larvae, juvenile and adults of bivalves, exposed to TBT in water.

Stage of life cycle	Species	Exposure Time	Concentration of TBT ($\mu g\ L^{-1}$)+	Effects	References
Embryos	*M. mercenaria*	48 h	≥ 0.77	Delay in development	Roberts, 1987
	S. plana	48 h	< 0.625 ° 0.312 °	EC50 (development) NOEC	Ruiz, 1995b
	C. gigas	24 / 48 h	> 50	No embryogenisis	His & Robert, 1980
	Pinctada fucata	1 wk	0.191	Decrease in embryo developmental success	Inoue *et al.*, 2004
	C. virginica	24 / 48 h	≈ 1	Abnormal shell development in embryos	Roberts, 1987
Larvae	*M. mercenaria*	14 d	>0.01 >0.1	Growth reduction No metamorphosis and no feeding	Laughlin *et al.*, 1988
		8 d	0.6 - 7.5	Growth reduction	Laughlin *et al.*, 1989
	S. plana	10 d	>0.312 °	Growth reduction; abnormal larvae; no foot development	Ruiz, 1993
	C. gigas	24 / 48 h	>5	No larval development	His & Robert, 1980
		10 d	>0.05 ≥ 0.2	Growth reduction Abnormal D larvae	His *et al.*, 1983
	M. edulis	25 d	>0.05 0.050 0.006	Growth reduction LOEC NOEC	Lapota *et al.*, 1993
Post- larvae	*R. decussatus*	45 d	0.24	Growth reduction	Thain & Waldock, 1985
	R. semidecussatus	45 d	2.6	Growth reduction	Thain & Waldock, 1985
	S. plana	30 d	>0.312 °	Growth reduction Abnormal shell growth.	Ruiz, 1993
	C. gigas	15 d	0.05 0.05 0.02	Reductions in : oxygen consumption feeding rate growth rate	Lawer & Aldrich, 1987
		56 d	0.15 1.6	Reductions in: growth condition index	Waldock & Thain, 1983
		45 d	≥0.24	Growth reduction	Thain & Waldock, 1985
		49 d	0.002-0.02	NOEC (for shell thickening)	Thain *et al.*, 1987

Stage of life cycle	Species	Exposure Time	Concentration of TBT ($\mu g\ L^{-1}$)+	Effects	References
Post- larvae	*C. gigas*	5 wks	2.0	Growth reduction	Thain, 1986
	O. edulis	45 d	2.6	Growth reduction	Thain & Waldock, 1985
Juvenile	*S. plana*	30 d	>0.125 * >1.25 *	Reductions in: growth rate burying activity	Ruiz, 1993 Ruiz *et al.*, 1994
	C. gigas	2- 3 m (field)	0.23- 0.45	Reduction in growth rate	Smith *et al.*, 1987
	M. edulis	12 wks (field)	> 0.070 μgL^{-1} in water >1.5 µg g-1 in tissues 0.025 μgL^{-1} in water 0.5 µg g-1in tissues	Reduction in growth NOEC	Salazar & Salazar, 1991
		12 wks (field)	≥ 0.2	Growth reduction	Salazar & Salazar, 1988
		196 d 56 d	≥0.07 0.04-0.16	Reduction in growth (after 60 days) No significant effects detected	Salazar & Salazar, 1987
		7 d	≥ 0.4	Reduction in growth	Stromgren & Bongard, 1987
		45 d	0.24	Reduction in growth	Thain & Waldock, 1985
Adults	*C. gigas*	4 - 5 m (field)	> 0.03 0.01	Shell thickening and weight reduction LOEC	Stephenson, 1991
		1 m	0.002	Shell chambering	Chagot *et al.*, 1990
	C. virginica	70 d	0.04-1.89	Decrease in condition index with increasing TBT conc.	Valkirs *et al.*, 1985
	M. edulis	4 d	5 $\mu g\ g^{-1}$ dw - tissues 3-4 $\mu g\ g^{-1}$ dw - tissues	EC 50 (growth) Reduction in feeding rate	Widdows & Page, 1993
Reproduction	*O. edulis*	75 d	0.24 2.6	No larvae produced; change in reproduction pattern. Inhibition of gametogenisis	Thain, 1986

+ Reported concentrations are for TBT dissolved in water, expressed as TBT ion, except where indicated

* Converted from concentrations originally expressed as Sn (Sn x 2.5 = TBT)

h - hours, d - days, wks - weeks, m - months ; dw- dry weight

NOEC - No observed effect concentration; LOEC - Lowest observed effect concentration; EC50 - Median effect concentration

Deleterious effects on reproduction were reported for *O. edulis* exposed to TBT concentrations of 0.24 and 2.6 µg L^{-1} TBT. Breeding oysters exposed to the lower level of toxicant (0.24 µg L^{-1}TBT) exhibited a change in the reproduction pattern and did not produce any larvae. Oysters exposed to the higher TBT concentration (2.6 µg L^{-1} TBT) showed similar effects coupled with an inhibition of gonad development (Table 1.7).

In respect to TBT concentrations at which no effect is observed (NOEC) in bivalves, studies suggest that NOEC values are in the order of a few nanograms per litre (parts per trillion) of TBT in the water (Table 1.7). A NOEC (25 days) of 6 ng L^{-1} TBT was estimated for the growth of *M. edulis* larvae, while a NOEC (12 weeks) of 25 ng L^{-1} TBT was indicated for juveniles of the same species. Following a study with oyster spat, Thain *et al.* (1987), determined that the NOEC (49 days) for the shell thickening response to TBT was between 2-20 ng L^{-1} TBT. In contrast, Chagot *et al.* (1990) reported that a concentration as low as 2 ng L^{-1} TBT could initiate shell chambering in *C. gigas*.

The reported chronic toxicity studies with bivalves focused mainly on different species of oysters, mussels and clams. However, information on *Ruditapes decussatus* is limited to one study performed with post-larvae (Thain & Waldock, 1985). Further studies should be carried out to assess impacts of low concentrations of TBT to different life stages of *R. decussatus*. Special attention should be given to effects of TBT on the early life stages of bivalves, namely larvae and juveniles, since these are considered the most sensitive stages. This was the objective of the toxicity studies reported in Chapter 3 and 4, respectively.

1.7. TBT Legislation

1.7.1. History

Based on data of persistence, bioaccumulation and toxicity in the marine environment, the group of organotin compounds was included in the original List I or "black list" of dangerous substances of the European Union,

since **1976** (Directive 76/464/EEC). Based on this Directive, in 1982, the European Commission named 129 substances, belonging to List I and Tributyltin Oxide (TBTO) was designated as number 115. Moreover, an Environmental Quality Standard (EQS) in the order of 0.001 μg L^{-1} TBTO was suggested by the Scientific Advisory Committee on Toxicity and Ecotoxicity (SCTE) of the European Commission yet, no EQS was implemented in European Directives. The EQS is defined as the maximum concentration of a pollutant allowed in water over a particular period or geographical area.

In **1982**, the French government, due to concerns over their Atlantic coast oyster culture industry, implemented the first legal restrictions on the use of TBT-based antifouling paints. The French temporary ban was set for paints containing more than 3% TBT, by weight; this measure proved to be immediately effective in improving spatfall, oyster condition and commercial production. Since 1987 similar restrictions have been imposed throughout Europe, North America, South Africa, Asia, Australia and Japan.

In the UK organotins were included in the "Initial priority red list" of contaminants after **1985**, and an EQS of 20 ng L^{-1} TBT (8 ng L^{-1} Sn) was set in 1985 for the protection of marine organisms. However, it did not offer a sufficient margin of safety to protect the most sensitive species and in 1987 the EQS was reduced to 2 ng L^{-1} TBT (0.8 ng L^{-1} Sn) (reviewed by Bryan,1992; Langston, 1995). As expected, TBT concentrations in water were higher prior to the partial bans and decreased at most sites monitored, as a result of TBT legislation. However, levels in sediments did not decline rapidly following the partial restrictions on TBT usage (Dowson *et al.,* 1993a; Langston & Burt, 1991; Waite *et al.,* 1991) and its consequences are yet to be fully assessed.

In the Netherlands a ban on the use of organotin paints in small vessels (< 25m) was imposed in 1990 (Ritsema *et al.* 1991).

A total ban on the use of TBT based paints in freshwater environments has also been established by a few nations - Germany, Austria and Switzerland (Stewart, 1996).

In the European Union, in **1989**, new legislation on the use of TBT was introduced by the Directive 89/677/EEC, of the 21st of December, stating that

organotin compounds were not admitted as constituent of antifouling preparations for use on:

1) hulls of vessels < 25m in length;
2) pens, buoys, nets or any other equipment used in aquaculture;
3) any equipment partially or totally immersed.

Moreover, marketing of organotin antifouling paints was restricted to certified operators and allowed to be sold only in 20 L containers. In addition, use of these substances was not permitted in preparations utilised to treat industrial effluents.

These regulations on the use of TBT-based antifouling paints followed the assumption that TBT contamination in coastal waters was mainly caused by small pleasure vessels rather than large commercial ships. Thus, TBT paints were prohibited only on vessels smaller than 25 m in length, with the exception of boats with aluminium hulls due to corrosion problems. In this way, vessels larger than 25 m in length were still entitled to use TBT based antifouling paints. This arises from the argument that TBT derived from commercial shipping had no impact in coastal waters, due to the enormous dilution factor in open seas. Nevertheless, more recent information refuted this argument based on the observation of TBT deleterious effects in open seas, particularly in major shipping lanes (Ten Hallers-Tjabbes *et al.*, 1994; 2002), together with evidence that in estuaries and confined areas, TBT contamination is related to sedimentary sinks of the compound and to the presence of larger vessels, entitled to use TBT antifoulings (Langston *et al.*, 1997).

During the last decade in **Portugal**, legislation on TBT usage was based on the referred EU Directive 89/677/EEC and was published on the Portuguese Official Journal in **1993** (Dec.-lei 53/93), banning the use and marketing of organotin compounds, as mentioned above. In 1992, the Portuguese Navy set a total ban on the use of TBT paints on their ships.

In **1995**, large vessels were estimated to account for 90% of the total antifouling market, by volume, and about two-thirds of coatings contained organotin (Langston, 1995). These types of vessels need to perform their

maintenance in shipyards. Inputs from ship maintenance are related with spillage and contamination during paint spraying and, most of all, wastes from removal of old coatings giving rise to locally harmful inputs of TBT. In fact, high levels of organotins - between one and two orders of magnitude above EQS values - have been detected in water, sediments and biota collected near large shipyards in several countries including Portugal (Langston *et al.*, 1997; De Bettencourt *et al.*, 1999).

In some countries as in the UK, guidelines were set on containment practices during maintenance activities. Nevertheless, in water samples collected in the vicinity of docks and maintenance slipways, extremely high levels of TBT were still observed (Alzieu, 1991; De Bettencourt *et al.*, 1999; Langston *et al.*, 1994; 1997; Waldock *et al.*, 1988). In these cases, TBT contamination is probably related to discharges of blasting wastes, due to the high cost of containment practices. Furthermore, some of the most important countries regarding building and repairing of large ships, including Greece, Portugal, Singapore and Korea do not have restrictions on elimination of the waste from blasting.

It seems clear that TBT contamination is a global problem and although restrictions on the use and elimination of TBT-based paints were imposed in some countries, continued environmental impact appears to be inevitable in most coastal areas, especially in locations near dockyards and maintenance facilities. In a number of countries restrictions on the use of TBT led to a reduction on TBT inputs to coastal environments from leisure vessels. However, the persistence of TBT in sediments and contamination in areas which are influenced by commercial shipping, leisure and fishing boats and maintenance facilities are still of great concern (De Bettencourt *et al.*, 1999; Langston *et al.*, 1997; Santos *et al.*, 2002).

Facing TBT contamination as a global problem, the UN's International Maritime Organization adopted, on the 5th October **2001**, a new "International Convention on the Control of Harmful Anti-fouling Systems on Ships" (**AFS-Convention**). Even though it was adopted, it will only enter into force 12 months after 25 States (representing 25% of the world's merchant shipping

tonnage) have ratified it. Although the Convention was opened for signatures during 2002, it has not been ratified until October 2004. This new convention will prohibit the use of harmful organotins in antifouling paints used on ships and will establish a mechanism to prevent the potential future use of other harmful substances in antifouling systems.

Based on available data on TBT toxicity, EPA set, in **2002**, an ambient water quality criterion, to protect saltwater aquatic life from chronic toxic effects, of 0.001 μg L^{-1} TBT (EPA, 2002).

1.7.2. Current Legislation

Based on the AFS Convention the European Union adopted, on the 5th May **2003,** a Regulation on the prohibition of organotin compounds on ships (Regulation (EC) 782/2003 of the 14th April 2003), as an attempt to "reduce or eliminate the adverse effects on the marine environment and human health caused by organotin compounds". A system of certification and surveillance of antifouling coatings applied on ships is established on the Regulation. It states that organotin compounds which act as biocides shall not be applied or reapplied on ships operating under the authority of a Member State, from the 1st of July 2003. For all other ships, this prohibition will be applied from the 1st January **2008**. Thus, after this date, the Regulation will apply to any ship sailing to or from ports of the EU Member States, regardless of its flag.

Although this Regulation was published in the EU, most of the world countries do not comprise severe restrictions on the use of organotins. Thus, on a global scale the proportion of the world's coastline protected by legislation is rather small. It should be taken into account that if legislation is not applied in all countries, then global contamination will continue and it seems obvious that the only measure capable of avoiding further contamination of TBT is a global ban.

In 2003, the basis towards a global ban was set by the AFS Convention and there is an expectation that this will soon be achieved. However, some authors argue that this global ban has been absent of a

debate and that there are strong arguments against it (Abel, 2000; Champ, 2000; Ritterhoff, 1998; Strandenes, 2000).

Nevertheless, it must be stressed that in the near future, even with the establishment of a TBT global ban, continued surveillance should be carried out for years, in order to asses the effectiveness of this measure. Results from this thesis intended to contribute to this debate in terms of impact assessment in southern Portugal.

1.8. Studied species - Ruditapes decussatus

Most of the laboratory and field work carried out in this thesis is focused on clams *Ruditapes decussatus* (Linnaeus, 1758); hence, a brief description of its biology and commercial importance is given below.

The grooved carpet shell clam *R. decussatus* (Linnaeus, 1758) has a wide distribution in European waters, extending from western and southern shores in Britain, south to the Iberian Peninsula, into the Mediterranean (including the Suez channel) and along the Atlantic coast of Morocco and Senegal (Tebble, 1976).

Ruditapes decussatus is a suspension feeder and has an annual reproductive cycle which includes a gametogenisis from March to the end of June being the gametes first expelled at the end of July. This is followed by a partial restoration of the gonad which can result in a smaller spawning in September. However, in the southern part of its distribution this species reproduces over large part of the year (Barnabé, 1994).

This clam species is gonochoric, i.e., has separate sexes but there is no external difference between males and females. In a spawning event gametes are released to the water where fertilisation occurs. Spawning can be synchronised throughout the population by means of chemical "signals" (hormones) (Morales, 1983). After fertilisation the first stage larva known as the trocophore comes into existence. This is a free swimming larva which is part of the plankton. Just about two days after fertilisation, larvae turn into the veliger stage with a characteristic ciliated organ, the 'velum', which is used for

swimming and food catching. In this stage larvae have a typical 'D shape' and thus are also known as D stage or straight-hinge stage larvae (Morales, 1983). A few days later the umbo (hinge) starts to develop and the larva reaches the umbonated stage. At the end of the larval phase (pediveliger stage) a contractile foot appears and larvae have all the adult characteristics becoming benthic. Reproduction as well as larval development depend upon several environmental factors such as temperature, salinity and nutrition. Thus larvae develop through the described stages, but there is no fixed length of time to achieve each larval stage.

In Portugal there are several species of bivalves which support significant coastal fisheries and *R. decussatus* is one of the most exploited species. It is extensively cultured in the southern part of the country, particularly in the Ria Formosa lagoon, where it attains a high commercial value. In this region, clam culture is a very important activity both social and commercially, since approximately 10 000 people are involved in the activity and at least 8000 tons of clams are produced per year, representing roughly 70 million euros (Cachola, 1996) and corresponding to 90% of the Portuguese production for this species (Chícharo & Chícharo, 2001). Generally, there is a scarcity of studies on the effects of pollutants on this species and thus, work on the impact of TBT on *R. decussatus* appears to be of particular relevance in this area.

1.9. Objectives of this study

This review brings together information about TBT and deleterious effects caused by this compound in a wide variety of species, notably bivalve molluscs. It emphasises the need for studies both in the laboratory and in the field to assess TBT sublethal effects on commercially important bivalve species.

Since the recognition of the deleterious effects of TBT, several countries have monitored their coastal environments and carried out extensive research on effects of this compound. As a result, restrictions have

been imposed and a reduction in TBT levels has been observed at many sites.

In Portugal, although legislation on these compounds exists since 1993, there is a lack of information on TBT levels in coastal areas. Mariculture sites, like Ria Formosa, are particular cases for concern due to the high sensitivity of molluscs to TBT. In addition to monitoring of TBT levels, experimental work should be performed to assess the effects of this compound on different life-stages of commercial bivalve species, such as *Ruditapes decussatus*.

This work constitutes an attempt to evaluate the effects of TBT, on *R. decussatus* and, in the light of these results, assess impact of organotin in the field, particularly in Ria Formosa. In this context, the main objectives of this work are:

- to assess sublethal effects of TBT exposure on the early life stages (larvae and juveniles) of the clam *R. decussatus*
- to determine the relative importance of different routes of TBT uptake in this species
- to assess levels of TBT contamination in water, sediments and bivalves from the southern coast of Portugal, in particular the Ria Formosa lagoon.
- to observe the degree of imposex in indigenous populations of neogastropods from this coastal area, as an additional indication of TBT contamination.

The present document is organised in different chapters each corresponding to a distinct goal. The current Chapter (1) provides a general overview of the TBT problem in the marine environment together with the aims of this study.

A summary of the analytical methods employed to analyse organotins in water, sediments and biological tissues is presented in **Chapter 2**. The influence of low, environmentally realistic, concentrations of TBT on growth and development of *R. decussatus* planktonic larvae is described in **Chapter 3**. **Chapter 4** describes an assessment of TBT effects on growth of *R.*

decussatus juveniles, during long-term exposure to sublethal levels of this compound. **Chapter 5** describes an attempt to evaluate the importance of water, sediments and phytoplankton as vectors of TBT uptake for this clam species. Clams were exposed to environmentally realistic concentrations of TBT in (i)water, (ii)sediments and (iii)bound to phytoplankton (*Isochrysis galbana*) and uptake was followed during a long term experiment, in the laboratory.

A field survey carried out to determine concentrations of TBT in water, sediments and *R. decussatus* tissues from the southern coast of Portugal is described in **Chapter 6**. In addition, a survey on TBT effects, particularly imposex, in different neogastropod species along the coast is presented in **Chapter 7**.

The final discussion (**Chapter 8**), brings together data obtained for the effects of TBT on the different life stages of *R. decussatus* and factors governing TBT uptake and relates them to the field survey, predicting long-term effects of TBT in clams *R. decussatus*. Furthermore, TBT effects observed in neogastropods in the field are compared with those described in other coastal areas world-wide, to put into context the scale of the TBT problem in southern Portugal. Considering the TBT effects studied in clams *R. decussatus*, measures are proposed to decrease long-term deleterious effects of this compound in clam populations.

Chapter 2

ANALYTICAL METHODS

In the current chapter a description of the methods of extraction and analysis of organotins in sea water, sediments and tissues of clams *Ruditapes decussatus* and gastropods is presented. These methods were used in all the experimental work.

Methods for measuring TBT concentrations include an initial step involving a solvent extraction from a homogenate dispersed in HCl. Various techniques have been described to analyse extracts; the simplest method comprise chemical separation followed by atomic absorption spectrophotometry (AAS) analysis. This technique was originally described by Ward *et al.* (1981); modified by Bryan *et al.* (1986) and Langston *et al.* (1987) and used in the present work.

2.1. Organotin extraction

All the glassware used in the laboratory during extraction and analysis was previously washed with detergent (Decon-90) and left in *aqua regia* for, at least, 24 hours, to avoid TBT contamination, rinsed with distilled water and dried.

2.1.1. TBT extraction in Seawater

Several methods have been described to determine TBT in seawater, including derivatization by generation of volatile organotin hydrides, separation by gas chromatography (GC) and determination of Sn by atomic absorption in a quartz furnace (Bailey & Davies, 1988; Batley & Scammell, 1991; King *et al.*, 1989; Ritsema *et al.*, 1991).

The methods used in the present work to measure TBT in seawater involved the determination of TBT in 1 litre HCl-acidified sea water samples, extracted with hexane, with detection limits in the order of 1 $ng\,L^{-1}$ TBT (Sn) (Bryan *et al.*, 1986; Stroben *et al.*, 1992). Seawater samples were collected in one litre glass stoppered bottles. Sampling material was previously rinsed with diluted HCl (10%) and left filled with distilled water and HCl ($1mL\,L^{-1}$) (BDH-Aristar) until subsequent sampling. Collection of water samples was

performed submerging the bottle neck 50 cm under the surface, in order to avoid contamination from the surface microlayer, where high levels of organotins may be found (Cleary & Stebbing, 1987). Immediately after collection, samples were acidified with 5 mL of concentrated HCl (BDH-Aristar) (to pH ≈1), to avoid loss of TBT due to adsorption to the flask walls (Donard *et al.*, 1986; Weber, 1985). Until subsequent extraction, all the samples were kept in the dark to prevent decomposition of TBT by light (Donard *et al.*, 1988).

Extraction of butyltins was performed in unfiltered seawater in order to include particles normally available to suspension feeding organisms.

As illustrated in Figure 2.1, one-litre water samples were divided in two aliquots: 500 mL for the determination of organotin in the sample and the remaining 500 mL for a standard addition of 25 ng TBTO as Sn, representing a concentration of TBT of 50 ng L^{-1} Sn^a (Table 2.1) (Figure 2.1). In each aliquot, organotins were extracted with 5 mL of hexane (Sigma-HPLC grade) by hand-shaking, for 4 minutes, in 1L glass separating funnels. The two phases were left to separate and the hexane extracts were transferred to 20 mL glass vials and kept frozen (-20°C) until analysed.

Hexane extraction, after hydrochloric acid treatment, removes not only TBT but also other organotin compounds including DBT and very little monobutyltin (MBT). The subsequent addition of NaOH, to hexane extracts, removes DBT and MBT, allowing the determination of the TBT fraction of organotins when analysed (McKie, 1987). Thus, prior to analysis, an aliquot of 1 mL of the hexane extract was transferred to a glass centrifuge tube and 1 mL of NaOH (1M) (Primar) was added; it was briefly hand shaken and centrifuged at 1000 rpm, for 2 minutes (Figure 2.1). Determination of TBT concentrations using a graphite furnace atomic absorption spectrophotometer (GF-AAS) was carried out in the hexane extracts as described bellow (section 2.2.).

For each set of extractions a blank was prepared using the same procedure.

2.1.2. TBT extraction in Tissues

The method employed to determine TBT concentrations in biological tissues involved extraction of HCl acidified homogenates with hexane, followed by back-extraction of dibutyltin with sodium hydroxide solution (Bryan *et al.*, 1986; Langston *et al.*, 1987; Ward *et al.*, 1981), with a detection limit of 0.01 $\mu g\,g^{-1}$ Sn (dw).

Organotin extraction was performed in the tissues of clams *R. decussatus* and gastropods. After collection, organisms were kept frozen (-20°C), until extraction and for clams, their maximum shell length was measured. To perform organotin extraction, clams and gastropods were thawed and organisms dissected. Each pooled sample consisted of the whole soft tissues of 4-6 specimens, which were homogenised[b] (homogeniser Ultra-turrax T25).

An aliquot (≈ 2g) of the homogenate was used to determine wet : dry ratio, by weighing it before and after drying for 24h, at 80°C.

For each sample, three aliquots of 0.5 g of the homogenate were put in 3 glass stoppered tubes, which were previously weighted (Figure 2.1). Tube 1 contained only a sample homogenate and tubes 2 and 3 contained sample homogenates plus standard additions of TBT (0.2 µg TBTO as Sn) and DBT (0.2 µg DBTCl as Sn), respectively (Table 2.1). Subsequently, tubes were refrigerated (5°C), for 1-2 hours.

To decompose tissues and take tin into solution, 5 mL of concentrated HCl (BDH-Aristar) were added to each tube, shaken briefly and refrigerated (5°C) for a further 1 hour. After this period, 5 mL of hexane (Sigma-HPLC grade) were added to extract organotins, and tubes shaken for 15 minutes and centrifuged, at 3000 rpm, for 4 minutes. Five millilitres of distilled water were then added to each tube and, after swirling briefly, the tubes were re-centrifuged at 3000 rpm for 5-10 minutes. After this procedure the clear hexane top layer, containing the organic tin compounds, was transferred to a 20 mL glass vial and kept at -20°C for further analysis. This extract was used

[a] 500 mL water were spiked with 25 µL of a TBT standard solution (1 mg L^{-1}) (Sigma -96% purity)
[b] If homogenates were too thick a small amount (few mL) of distilled water was added

to determine the (DBT+TBT) fraction (tubes 1,2 and 3) (Figure 2.1). For each extraction a blank was prepared using the same procedure.

Prior to analysis, and in order to separate the TBT fraction of organotin in these samples, an aliquot (1 mL) of the hexane extracts from tube 1 and 2 was placed in glass centrifuged tubes (tubes 1A and 2A - Figure 2.1). The same volume (1 mL) of NaOH (1M – Primar) was added to the aliquots, hand shaken and centrifuged at 1000 rpm for 2 minutes. After this procedure the clear hexane top layer, containing TBT, was transferred to a glass tube for posterior analysis. Determination of the TBT fraction was carried out in this hexane extract (Figure 2.1).

Determination of TBT and DBT concentrations in biological tissue samples using GF-AAS (tubes 1; 1A; 2; 2A and 3), was carried out as described later (Section 2.2.).

2.1.3. TBT extraction in Sediments

Sediments were collected from the surface layer (≈ 1st cm), by scrapping with a spatula, stored in polyethylene bags and kept frozen (-20°C), until extraction. Organotin content in the sediments is best determined in sieved fractions (<100 µm), rather than in the whole sediments, in order to achieve better homogeneity and comparability of data and also because butyltins are likely to be preferentially absorbed onto the finest particles of the sediments (Donard *et al.*, 1986; Langston *et al.*, 1987; Quevauviller & Donard, 1990).

In order to separate the referred fraction, sediment samples were sieved through a 100 µm polypropylene mesh using 'TBT-free' sea water (<1 ng L^{-1} Sn) and left to settle for a minimum of 24 hours to allow particles to deposit. In the current experiment virtually all the sediment passed through a <100 µm mesh. The overlaying water was then drained and the sieved sediments were homogenised. From this homogenate, three sediment aliquots of approximately 0.5 g (wet weight) were put through the same extraction procedure as described above for biological tissues (2.1.2.). The described method presented a detection limit of about 0.005 µg g^{-1} Sn (dw).

An aliquot of approximately 2g was also removed to determine the wet/dry ratio, weighing the aliquots before and after drying (80°C for 24h).

Table 2.1- Standard additions of TBT and DBT in water, tissue and sediment samples.

Type of sample	Compound	standard sol. concentratior ($mg\ l^{-1}$ Sn)	added volume (µl)	addition per sample (TBT as Sn)
Water (500 ml)	TBTO (Sigma)	1	25	25 ng
tissues/sediments (0.5 g)	TBTO (Sigma)	10	20	0.2 µg
tissues/sediments (0.5 g)	DBTCl (Sigma)	10	20	0.2 µg

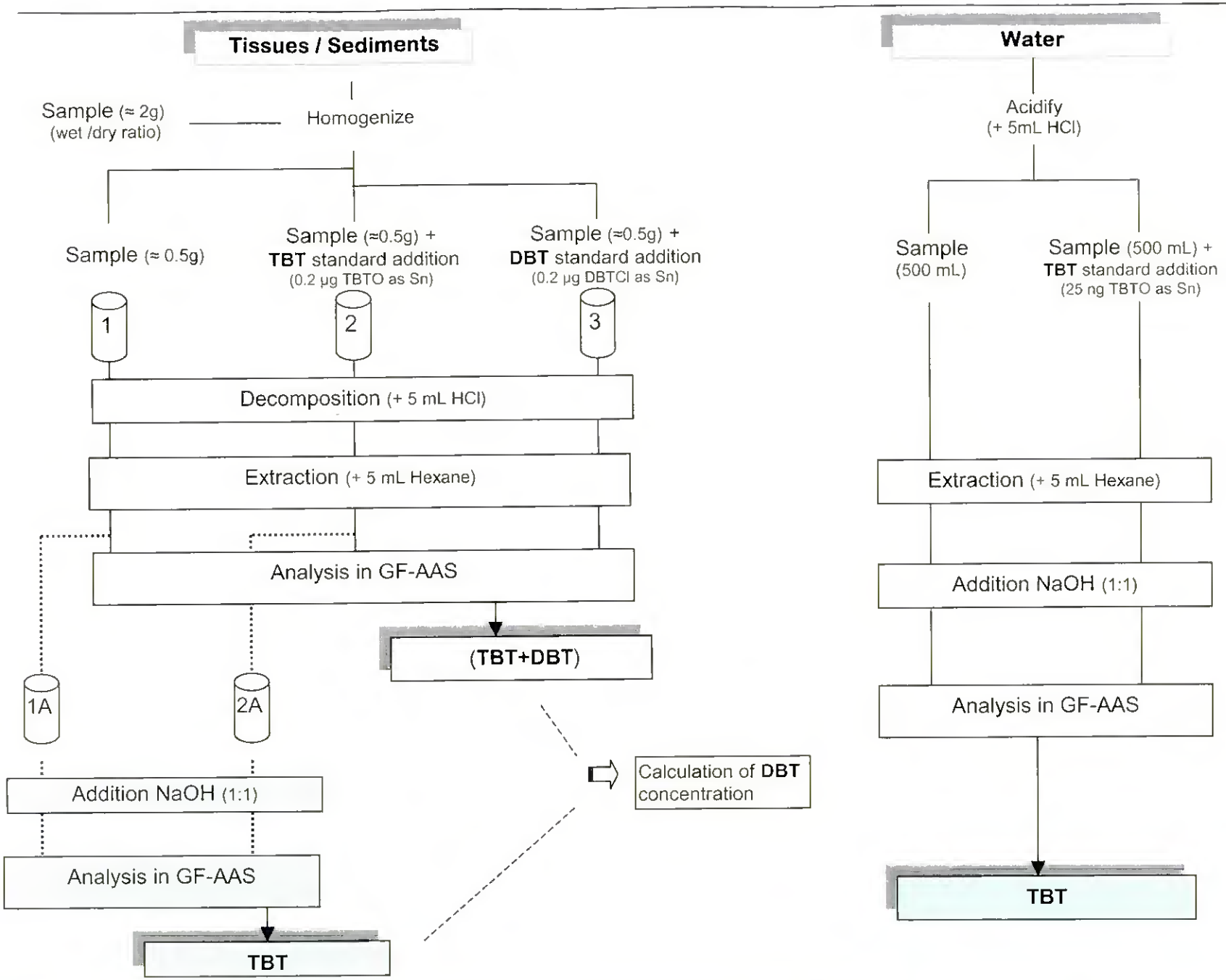

Figure 2.1- Outline of the methods employed to extract and analyse TBT and DBT in tissues, sediments and water samples.

2.2 Organotin analysis using atomic absorption spectrometry

In the HCl-acidified hexane extracts obtained from biological tissues and sediment samples, concentrations of total organotins (TBT+DBT) were measured in aliquots (50 µL) of the extracts (tube 1 and 2 – Figure 2.1). After back-extraction with NaOH solution (tubes 1A and 2A – Figure 2.1), only the TBT fraction was determined, as described earlier (2.1.2.). The DBT concentrations were subsequently calculated by subtraction (Figure 2.1). In the case of water samples, only the TBT concentrations were determined.

In the hexane extracts obtained from water, tissues and sediment samples, organotins (as Sn) were analysed using a graphite furnace atomic absorption spectrophotometer (GF-AAS) (GF-Perkin-Elmer 603) (AAS-Perkin-Elmer 76B). All analysis were performed in the Plymouth Marine Laboratory (U.K.).

Tin was analysed in 50 µL aliquots of hexane extracts, injected manually in the furnace by means of a micropipette (Finnpipette). The experimental conditions employed in the GF-AAS furnace to analyse tin are described in Table 2.2.

Table 2.2.- Experimental GF-AAS conditions employed to analyse Sn in water, biological and sediment sample extracts.

Process	time (s)	temperature (°C)
dry	30	90
ash	25	1000
atomise	5	2600

An electrodeless discharge (EDL) tin lamp was employed at a wavelength of 286.3 nm to measure tin concentrations. EDL discharge lamps provide radiant intensities usually one to two orders of magnitude greater than hollow-cathode lamps, thus providing higher sensitivity and lower detection limits (Skoog & West, 1982).

Electrothermal atomisation determinations are subjected to significant interferences from matrix effects, especially with samples containing high

organic loads or high concentrations of salts, such as the samples analysed in the present work. These interferences can be reduced by sample preparation or by the addition of matrix modifiers (Gonçalves, 1983). Since the graphite tubes used in the furnace contained a loosely-fitted platform, so called L'vov platforms, a treatment with tantalum pentoxide was applied to the platforms to minimize matrix interferences. This compound is a matrix modifier that binds organic tin strongly, allowing the use of a higher ashing temperature, thus eliminating much of the organic matrix from the sample, prior to atomisation. In addition, matrix interferences were minimized using the method of internal standard additions (Gonçalves, 1983; APHA, 1998).

A temperature ramping in the atomisation process was used to decrease background interferences (APHA, 1998) and background correction was not required.

Under the described conditions, detection limits were 1 ng L^{-1} Sn for water samples, 0.01 µg g^{-1} Sn (dry weight) for tissue samples and 0.005 µg g^{-1} Sn (dry weight) for sediment samples. All values in the current work are reported as Sn. To convert to TBT, a factor of 2.5 should be applied to the Sn ion values.

The described methodology was validated in an interlaboratory comparison between the Plymouth Marine Laboratory and the Lake Research Laboratory (Switzerland) using a gas chromatograph with flame photometric detection (GC-FPD) (Langston *et al.*, 1994).

Chapter 3

EFFECTS OF TBT ON CLAM LARVAE

Coelho, M.R.; Fuentes, S. & Bebianno, M.J. (2001). TBT effects on the larvae of *Ruditapes decussatus*. *J.mar.biol.Ass.U.K.* **81**: 259-265.

ABSTRACT

The effects of sublethal tributyltin concentrations on the growth and development of *R. decussatus* larvae were studied. Veliger larvae of *Ruditapes decussatus* were exposed to TBT nominal concentrations of 25, 50, 75 and 100 ng L^{-1} Sn in the water for a period up to 13 d. Larval growth and development were chosen as endpoints for evaluation of TBT toxicity.

Growth of *R. decussatus* larvae was severely affected by TBT concentrations (25-100 ng L^{-1} Sn). A 3- to 6-fold reduction growth was observed in these early larval stages. Furthermore, *R. decussatus* larvae exposed to TBT did not develop further than D-larvae while the unexposed ones that reached the umbonated stage.

3.1. INTRODUCTION

TBT has adverse effects on several species of marine invertebrates which are not the target organisms of antifouling paints. Several studies indicated that among marine organisms, molluscs are the most sensitive *taxa* to chronic, low level exposure to TBT (see reviews by Bryan & Gibbs, 1991; Hall & Pinkney, 1984; Maguire, 1987). For each species the sensitivity to pollutants, including TBT, usually varies according to the life-stage period and embryos and larvae are indicated to be the stages of greatest concern for effects of exposure to TBT (Laughlin *et al.*, 1989; Lapota *et al.*, 1993). Deleterious effects caused by organotins on early-life stages of bivalves include reductions in larval growth, recruitment and settlement rates (Langston *et al.*, 1987; Langston *et al.*, 1990; Minchin, 1987), post-settlement growth (Lawer & Aldrich, 1987; Nell & Chvojka, 1992; Salazar & Salazar, 1988; Stromgren & Bongard, 1987) and reductions both in feeding rates and oxygen consumption of juveniles (Lawer & Aldrich, 1987).

The limited number of studies performed on TBT effects on bivalve larval stages is due, in part, to obstacles found in culturing larvae during chronic (long-term) toxicity experiments. Studies focused mainly on effects on larval growth; a significant reduction on *Mytilus edulis* larval growth was observed at concentrations as low as 50 ng L^{-1} TBT, in a long-term exposure

(25 days) (Lapota *et al.*, 1993). Exposure to 100 ng L^{-1} TBT also reduced *M. edulis* larval growth significantly, after 15 days (Beaumont & Budd, 1984). Work carried out with *Mercenaria mercenaria* larvae, showed consistent growth reductions with increasing TBT concentrations from 10 to 500 ng L^{-1} TBT; in addition larvae were not able to undergo metamorphosis upon exposure to 100 ng L^{-1} TBT (Laughlin *et al.*, 1988). Levels of 125 ng L^{-1} TBT (Sn) in water reduced the growth rate, activity and normal shell development of the pediveliger larvae of clams *Scrobicularia plana* (Ruiz *et al.*, 1995a). For the pacific oyster *Crassostrea gigas*, a concentration of 50 ng L^{-1} TBT in the water reduced larval growth and 200 ng L^{-1} TBT induced the formation of abnormal larvae (His *et al.*, 1983).

As mentioned in Section 1.8 (Chapter 1) of the present work, the clam *Ruditapes decussatus* is one of the more exploited and valuable species of bivalves, especially in lagoon areas like he Ria Formosa, in the south coast of Portugal. Spawning occurs naturally in the lagoon from May to September, and planktonic larvae live in the water column, where they may be exposed to TBT released from antifouling paints. The purpose of the present study was to determine the effects of sublethal concentrations of TBT on the growth and development of *R. decussatus* larvae.

TBT concentrations were chosen based on results from a previous experiment where clam larvae were exposed to TBT levels in water ranging from 50 to 500 ng L^{-1} (Sn), for a period of 2 weeks. Severe mortality occured in cultures with concentrations higher than 200 ng L^{-1} TBT (as Sn) while for lower levels no significant mortality was observed during this period (S. Fuentes, pers.com.). Thus, during the present experiment veliger larvae of *R. decussatus* were exposed to a range of sublethal concentrations of TBT in water, nominally 25, 50, 75 and 100 ng L^{-1} TBT (Sn), for a period up to 13 days. These concentrations are representative of contaminated sites in estuaries and maricultures (see Table 1.2 – Chapter 1). Larval growth and development were chosen as endpoints for evaluation of TBT toxicity.

3.2. MATERIALS AND METHODS

3.2.1. Details of broodstock maintenance and fertilisation procedure

The following experiments were performed in an Experimental Aquaculture Station, in Huelva, southern Spain (Centro de Investigación y Cultivos Marinos) where several species of fish and bivalves, including *R. decussatus*, are cultured. In the Station adult specimens of *R. decussatus* are kept, throughout the year, under controlled conditions, in the laboratory. Conditioning of adult clams is intended to produce healthy individuals which are sexually mature (a broodstock) and capable of producing gametes and larvae in good condition and sufficient number to be cultured over the whole year (Camacho,1979).

The conditioning system for adult clams included an open circuit of sea water with a permanent flux of 1 to 2 L min^{-1}, running at constant temperature (20 ± 2 °C) and salinity (35). Organotin levels in the water circuit were regularly monitored and TBT levels were 2 ng L^{-1} Sn. The water circuit was enriched with a food supplement of phytoplankton, which was delivered into the circuit with a peristaltic pump for 12 hours a day. Phytoplankton supplement consists of a mixture of species which included *Isochrysis galbana*, *Chaetoceros gracilis* and *Tetraselmis suecia*. Food quantity was proportional to the weight of animals to be fed. Maturation of the broodstock was followed by macroscopic and microscopic observations of gonad samples. A mature individual is characterised by well developed gonads and, in the males, by the presence of mobile sperm; in females, by the observation of oocytes with a diameter larger than 60 µm. Groups of adult *R. decussatus* showing these characteristics could be artificially induced to spawn.

In the present experiment, release of male and female gametes was induced in a communal spawning container by cyclical temperature changes between 20 and 28 °C, coupled with sperm introduction in the water (Loosanoff and Davis, 1963). Clams were carefully observed and when gamete release begun, clams were sexed and transferred to separate containers so that egg emission could occur in the absence of sperm to prevent polyspermia (Gruffyd & Beaumont, 1972). Sperm from a few males

was kept in a beaker with gentle aeration. Each female was allowed to spawn in a glass beaker (500 ml). For this species a single female can release up to 1 million eggs with an average diameter of 70 µm (Camacho, 1979). Following egg emission, fertilisation was attempted by adding a few microlitres of sperm suspension to oocytes in order to obtain a proportion of up to 5 spermatozoids per oocyte (Camacho, 1979). After allowing 10 to 15 minutes for fertilisation to be accomplished a sample was taken and the proportion of fertilised eggs counted. When the percentage of fertilised eggs where >95% the gamete suspension was sieved through a 30 µm pore mesh to retain eggs and prevent contact with decomposing sperm (Loosanoff and Davis, 1963). Retained eggs were rinsed with sea water and re-suspended in a 1 litre glass beaker with very gentle aeration and left for 24 hours. The suspension was then homogenised by means of a perforated plastic plunger, and an aliquot (50 µl) of suspension was placed on a glass slide and counted. This procedure was repeated 6 times and the mean of the 6 determinations used to estimate the total number of larvae in the 1 litre beaker. The desired number of larvae, corresponding to a known volume of suspension, was finally distributed into each of the culture containers, as described below.

3.2.2. Exposure of clam larvae to TBT

In order to obtain a successful larval culture, extremely clean conditions and excellent water quality are required (Walne, 1964; 1974). To fulfil these needs, the sea water used for culturing larvae was pumped directly from the sea (TBT concentrations <2 ng L^{-1}), filtered (0.45 µm), UV sterilised to avoid the presence of bacteria and run at a constant temperature of 21 °C. In small tanks, where the area of solid surface is high compared with the volume of water, bacteria readily develop and their control is essential for successful larval culture (Walne, 1964; 1974). UV sterilisation and regular water changes, during the experimental period, help to keep the bacterial population down (Walne, 1974). Although the addition of antibiotics is recommend (Camacho, 1979; CNEXO, 1983; Walne, 1964; 1974) to prevent

bacteria development in bivalve larval cultures, in the current experiment the use of antibiotics was not considered necessary.

All the glassware used in the experiment was treated as described in section 2.1.

Exposure to TBT began within 48 hours of fertilisation, using D-stage larvae which had an average length of 90 µm. Larvae were cultured in rounded-bottom glass flasks (Figure 3.1), each containing 5 litres of sea water and, initially, 30 000 larvae (6 larva ml^{-1}).

In order to obtain good homogeneity as well as oxygenation of the culture medium, constant aeration was maintained in each flask by means of a glass pipette. Cultures were kept in a controlled temperature room (21 °C) and a static renewal protocol was adopted. Thus, all the cultures were changed daily by sieving the content of each flask through a 40 µm pore mesh, washing the culture flask and refilling it with sea water. Following this procedure larvae were returned back to the flask and finally food and contaminant were added.

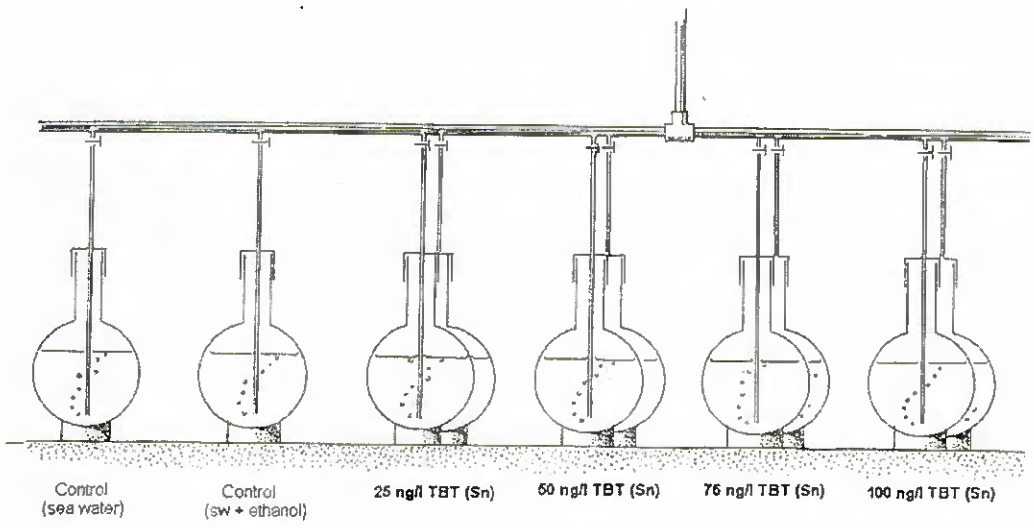

Figure 3.1 - Glass flasks used for culturing larvae of *R. decussatus*, exposed to different concentrations of TBT (ng L^{-1} Sn) in the water , with an initial density of 6 larvae per ml.

Although information concerning larvae diet in natural conditions is scarce, it is now generally accepted that live unicellular algae are ideal as food for bivalve larvae. Phytoplankton species cultured to feed larvae during this experiment were *Isochrysis galbana* and *Chaetoceros calcitrans* in equal proportions. Algae were maintained in a separate room at a constant temperature of 22 °C and cultured with filtered sea water (25 µm), 35 ‰ salinity, enriched with f/2 medium (Guillard & Ryther, 1962) under constant light and aeration. Microalgae were added to the larval cultures, daily, in order to obtain a density of 100 cells per µl in each culture flask (Camacho,1979; Walne,1974).

Nominally concentrations of TBT used in exposure experiments were 25, 50, 75 and 100 ng L^{-1} TBT (Sn). Because TBT presents low solubility in sea water, absolute ethanol was used as a solvent. Different TBT stock solutions were previously prepared in absolute ethanol, so that a constant volume of 50 µl (contaminant + solvent) could be added to each larval culture flask. A control culture was run with sea water only and, in order to study possible effects of the solvent, a further control with 50 µl (10 µl L^{-1}) ethanol was also tested. All spiked cultures were run in duplicate.

The experiment was run for 13 days, which corresponds to the typical planktonic larval phase for this species (Figure 3.2).

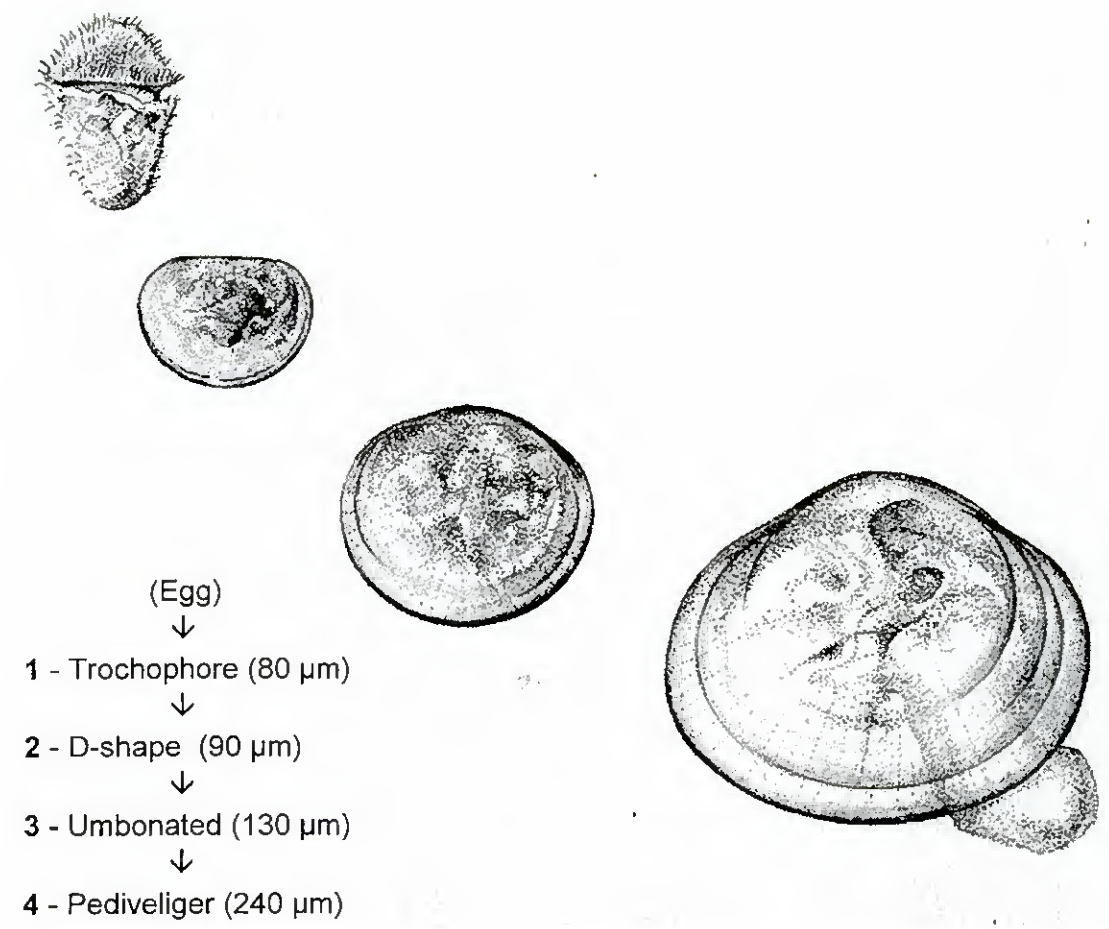

Figure 3.2- Different larval phases of the clam *Ruditapes decussatus*

Samples were taken every other day and at least 30 larvae from each flask were measured, to the nearest 10 µm, using a microscope (400 x) fitted with a micrometer eyepiece. In order to obtain larval length the maximum shell length in the antero-posterior axis was measured (Figure 3.3). In addition to this measurement, the developmental stages and eventual larvae deformations were registered at each observation.

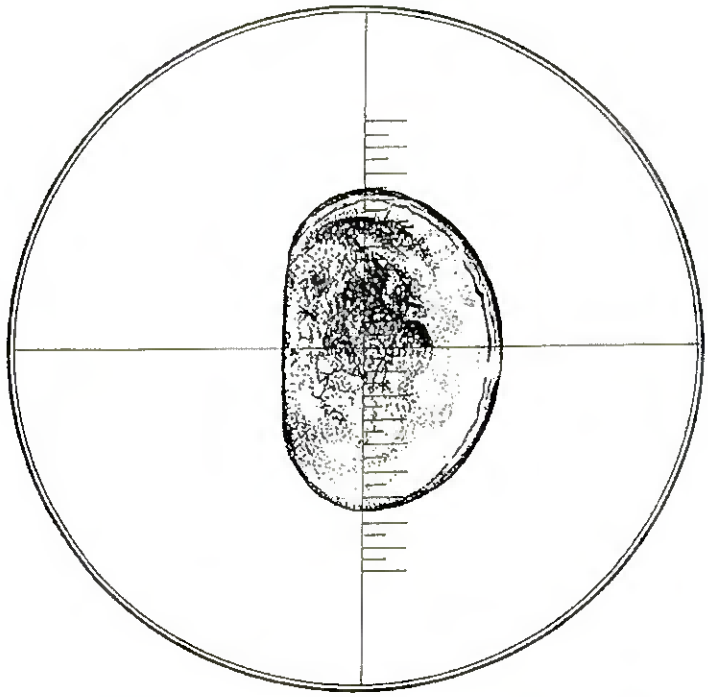

Figure 3.3 - *Ruditapes decussatus*. Measurement of larvae shell length in the antero-posterior axis, in a microscope fitted with a micrometer eyepiece

3.2.3. Statistical analysis

Several statistical tests were applied to data, including a Student *t*-test for paired means (Cochran & Snedecor, 1980) and an analysis of variance, using two-way factor ANOVA, Model I (fixed effects) (Zar, 1996). All tests were performed with a confidence interval of 95%.

3.3. RESULTS

Results for clam growth in the control culture (sea water only) are shown in Figure 3.4A. Relative frequency of individuals (%), per size-class (10 µm class) was plotted for the experimental period of 13 days. Larvae grew from an average length of 100 µm in the first day to 175 µm after 13 days, attaining a regular increase in length of 6 µm per day which is the expected growth rate for this species (Vilela, 1950). Concerning developmental stages, all control larvae passed from D-shape to umbonated larvae.

Growth of larvae in the ethanolic control culture is shown in Figure 3.4B; larvae grew similarly to the sea water control - increasing from an initial length of 100 µm to 175 µm after 13 days and achieving the same growth rate of 6 µm per day. Developmental stages were similar to sea water control. A student-t test for paired means, with a confidence interval of 95%, showed that larval growth was not significantly different in either sets of controls. Thus, ethanol used as carrier for TBT does not seem to affect R. *decussatus* larvae growth, at the concentrations (10 µl L^{-1}) and in the culture conditions described here.

Results obtained from larvae cultured in the presence of TBT, at different concentrations, are shown in Figure 3.4 C-F. At the lowest concentration, 25 ng L^{-1} TBT as Sn (Fig. 3.4C), larvae grew from an average initial length of 100 µm to 127 µm, after 13 days which corresponds to a growth rate of 2 µm/day. In this culture larvae did not display a regular development and by the end of the experiment the great majority of larvae were still D-shaped and only a few reached the umbonated stage. Larvae exposed to 50 ng L^{-1} TBT as Sn (Fig. 3.4D) increased their length from 100 µm to only 119 µm, after 13 days, with a growth rate of 1.4 µm/day. As in the 25 ng L^{-1} culture, most larvae were D-shape by the end of the experiment.

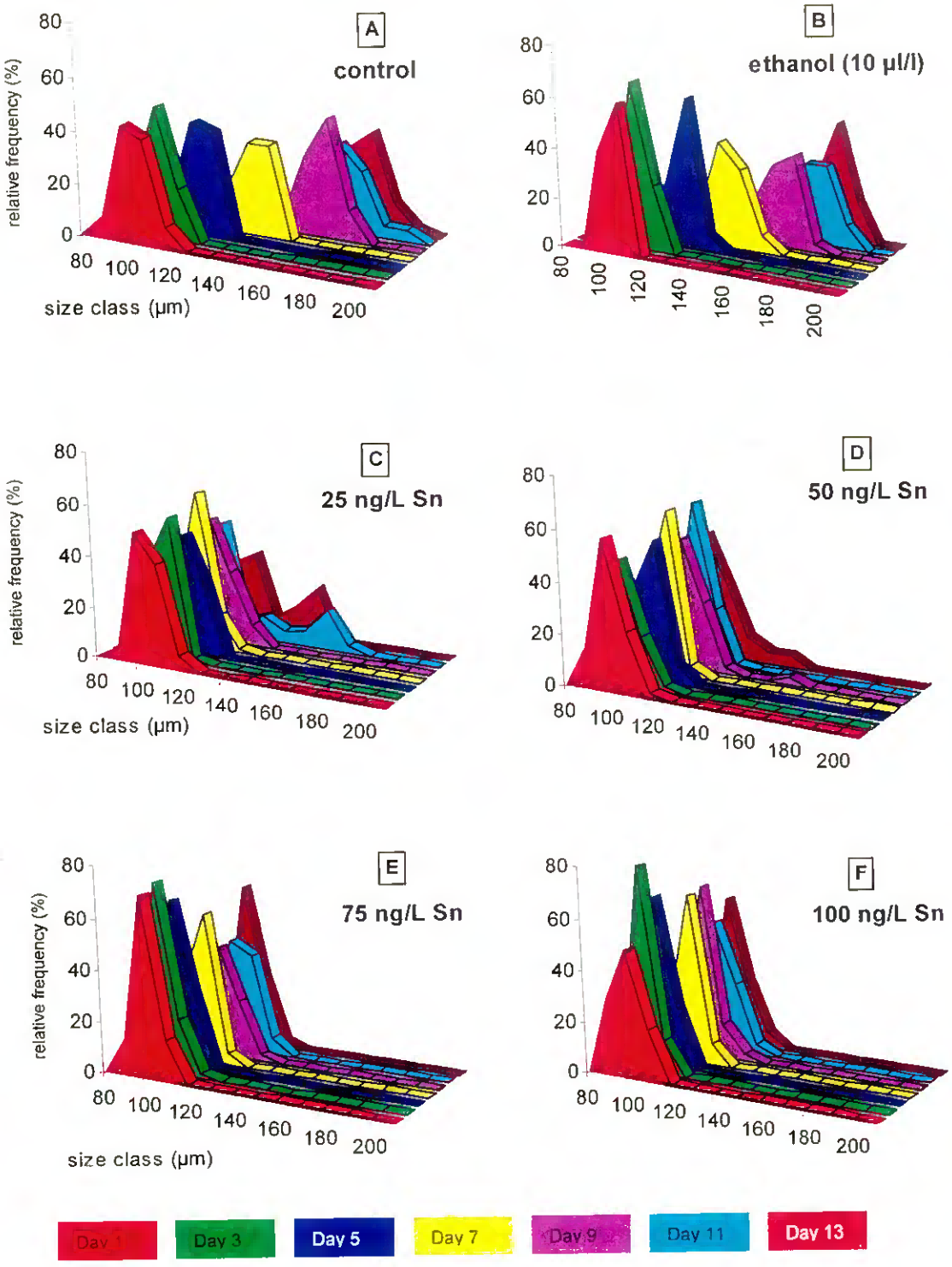

Figure 3.4 (A-F) - *R. decussatus*. Relative frequency (%) of larvae in each size class (20µm), when exposed to different concentrations of TBT in the water, for a period of 13 days.

In those cultures spiked with 75 and 100 ng L^{-1} TBT as Sn (Figure 3.4 E-F), further inhibition of larval growth and development was observed. In both treatments larvae grew from an initial length of 100 µm to only 113 µm by the end of the experiment, exhibiting a reduced growth rate of 1 µm/day. In these cultures none of the observed larvae developed further than D-shape stage, during the whole experimental period. A student-t test for paired means, with a confidence interval of 95%, applied to the final lengths, showed that there was a significant difference in larval growth between treatments with and without TBT. All cultures contaminated with TBT exhibited a reduced growth and a lack of development. No significant difference was detected among TBT treatments (ANOVA, $p<0.05$).

The growth curves exhibited by larvae subjected to the different treatments are shown in Figure 3.5.

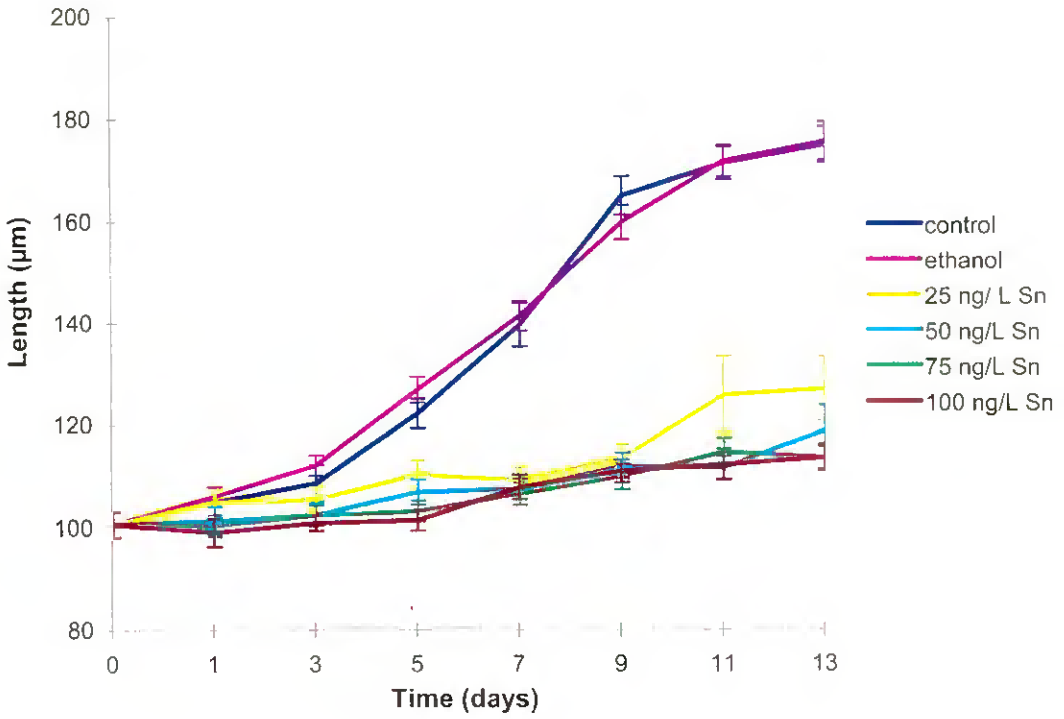

Figure 3.5 - *Ruditapes decussatus*. Total length (mean ± std.dev.) of control larvae and larvae exposed to different concentrations of TBT (25; 50; 75 and 100 ng L^{-1} Sn) in the water, for 13 days.

Growth rates (total increment/exposure period) for each treatment are shown in Table 3.1.

Table 3.1 - *R. decussatus*. Growth rates (µm/day) and maximum length (µm) exhibited by larvae after 13 days of exposure, in sea water (control 1), ethanol control (2) and different TBT treatments.

	TBT treatments					
	control (1)	control (2	25 ng/L*	50 ng/L*	75 ng/L*	100 ng/L*
Growth rate (µm/day)	6	6	2	1.4	1	1
Maximum length (µm)	175	175	127	119	113	113

*TBT concentrations are expressed as Sn.

Only larvae from the control and ethanol cultures exhibited the expected growth rates for this species (Vilela, 1950). Some reduction in growth, resulting from TBT exposure, can be observed from as early as day 3 of the experiment (Figure 3.4).

Maximum shell length displayed by larvae from different treatments, after 13 days is shown in Figure 3.6.

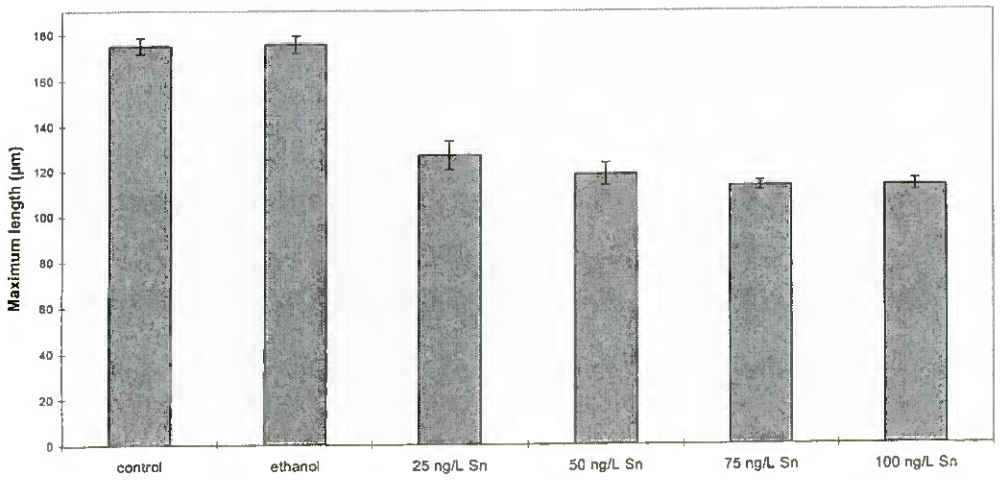

Figure 3.6 - *R. decussatus*. Maximum shell length of larvae after 13 days of exposure to different concentrations of TBT (Vertical lines shown are standard deviations)

Larvae from the sea water control and ethanol control cultures attained a shell length of 170 µm - an increase of 70 % of their initial length - while individuals from contaminated cultures obtained maximum shell lengths of

113-127 µm, during the same period. Thus, larvae growth rates (i.e. total length increment (µm)/13 days) showed a considerable variation between the control cultures and TBT contaminated cultures (Table 3.1).

Exposure to TBT resulted in a 3 to 6 fold reduction in growth of these early larval stages (see Table 3.1 and also for comparison of increments, Figure 3.7).

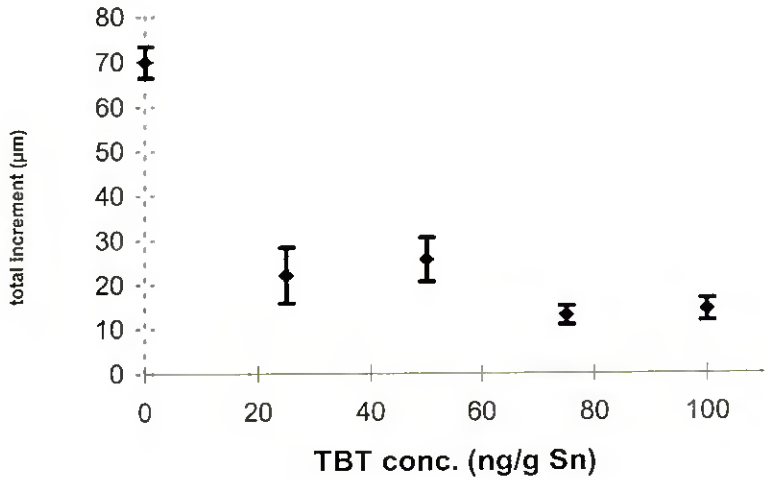

Figure 3.7 - *R. decussatus*. Relationship between total increment of larvae and TBT concentration in the water, after 13 days.

Several abnormal larvae, approximately 2 weeks old, were observed within all contaminated cultures (25, 50, 75 and 100 ng L^{-1} TBT (Sn) (Figure 3.8). These exhibited soft tissues extruded from the shells, although they were able to swim.

Figure 3.8 - *R. decussatus*. Abnormal larva, with tissues extruded from the shell, observed in all TBT contaminated cultures (25, 50, 75 and 100 ng L^{-1} TBT (Sn)).

3.4. Discussion

Growth of *R. decussatus* larvae was severely affected by low concentrations of TBT (25-100 ng L^{-1} Sn) in the water (Figure 3.4 A-F).

During the experiment ethanol was used as a TBT solvent and thus added to all the treatments (except the sea water control), but it did not seem to affect larval growth significantly (Figure 3.4B). Similarly, ethanol (also used as TBT solvent) did not reduce growth in larvae of *Scrobicularia plana* (Ruiz *et al.*, 1995a). However, other solvents such as acetic acid, used in similar concentrations, affected *M. edulis* larval growth (Beaumont & Budd, 1984).

Ruditapes decussatus D-shape larvae in control (seawater + ethanol) cultures (Figure 3.4 A&B) were shown to develop to umbonated stage at 20 °C, after 13 days and displayed a growth rate of 6 µm/day. In contrast, larvae exposed to nominal concentrations of 25, 50, 75 and 100 ng L^{-1} TBT (Sn) in the water did not develop further than D-shape and exhibited a reduced growth rate by a factor of up to 6, when compared to control.

Similarly, other studies on sublethal effects of TBT on bivalve larvae refer a reduction on shell growth over various time periods. For example, *S.*

plana larvae showed a reduction in growth when exposed to ≥50 ng L^{-1} TBT for 10 days (Ruiz *et al.*, 1995a), *M.edulis* larvae exhibited a reduced growth rate over 15 days at ≥100 ng L^{-1} TBT (Beaumont & Budd, 1984) and in other studies comparable effects were detected at ≥50 ng L^{-1} TBT, over a period of 33 days (Lapota *et al.*, 1993) and 5.4 ng L^{-1} Sn over 15 d (Stenalt *et al.*, 1998), for the same species. Oysters and soft shell clam larvae were shown to be among the most sensitive to TBT exposure: *Crassostrea gigas* larvae exhibited a reduced growth rate at 20 ng L^{-1} TBT after 8 days (His *et al.*, 1983) and *M.mercenaria* suffered a growth reduction when exposed to ≥10 ng L^{-1} TBT for 14 days (Laughlin *et al.*, 1988). Besides this effect these workers also noted that larvae of *M. mercenaria* did not develop the pediveliger stage at concentrations of ≥100 ng L^{-1} TBT (approximately 40 ng Sn L^{-1}), which is comparable to the results reported here. It is possible that a link exists between growth and metamorphosis and if growth is inhibited, as with TBT, metamorphosis may not occur (Laughlin *et al.*, 1988). Moreover, a possible delay in metamorphosis may produce larvae in a poor physiological condition (degeneration of velum and loss of ability to feed) and ultimately lead to a failure in settlement (Stenhalt *et al.*, 1998).

Results from this experiment showed a significant reduction in growth for larvae exposed to nominal concentrations of 25, 50, 75 and 100 ng L^{-1} TBT (Sn) (Figure 3.4 C-F) but no values for real concentrations of TBT in the water were determined. Nevertheless, it is important to emphasise that probably, due to the static renewal protocol used in this experiment, the '*dissolved*' TBT concentrations to which larvae were exposed were considerably lower than the nominal concentrations, due both to sorption of TBT to the containers walls and binding of this substance by microalgae. Initial concentrations of TBT were probably close to nominal but the average over 24 h may have been closer to half the nominal concentrations as indicated in similar studies by Laughlin *et al.* (1988) and Ruiz *et al.* (1995a). If TBT levels could have been kept constant throughout the experimental period, D-shape larvae would have been exposed to higher TBT burdens and possibly deleterious effects on growth and development would have been greater. Nominal values described in this paper may therefore underestimate

effects thresholds for *R. decussatus*. Furthermore, it is likely that part of the TBT in solution was bound to phytoplanktonic cells (Avery *et al.*, 1993; Coelho *et al.*, 2002b - Chapter 5,). However, presence of microalgae was unavoidable and clearly more representative of natural exposure conditions.

Abnormal larvae were observed in all TBT treatments. Similar abnormalities, as well as a reduced growth rate, were noted in *M. mercenaria* larvae exposed to nickel (Ni) in the water (Calabrese *et al.*, 1977). Thus, nickel appears to induce similiar toxic effects to TBT in clam larvae.

Exposure concentrations used in the current experiment are environmentally relevant, since TBT concentrations within the same order of magnitude has been measured in different mariculture sites worldwide (Table 1.2- Chapter 1) (Batley & Scammell, 1991; Gabrielides *et al.*, 1990; Langston *et al.*, 1997; Lau, 1991). Presumably, in the field clam larvae can be severely affected by the presence of TBT in the water which causes reductions in growth rates and may lead both to a delay in development and a lack of metamorphosis. Taking into account that, in the field, predators are the main cause of larval disappearance, it is accepted that any factors which tend to slow development and hence prolong larval life, will increase the chances of predation and will lead, ultimately, to a decrease in population recruitment. Subsequentely, the distribution pattern of adult populations can also be affected, since the planktonic larval stages are the major dispersive agents of benthic populations (Day & McEdward, 1984).

Another concern arises from the fact that in the marine environment, TBT concentrations are enhanced in the surface microlayer where organotin levels can be up to 27 times higher than in subsurface waters (Battley & Scammell, 1990; Cleary & Stebbing, 1987). Thus, bivalve larvae which are part of the plankton may come into contact with the surface microlayer (Hardy *et al.*, 1987) and may therefore be seriously affected by TBT, resulting in reductions in larval survival growth and development (McFadzen & Cleary, 1994).

In toxicity studies, field validation of laboratory results should always be performed. Nevertheless, in the case of *R.decussatus* larvae, and in lagoons like the Ria Formosa, validation is difficult because effects observed in the

field are the cumulative result of not only TBT contamination, but also of exposure to many other pollutants including heavy metals and other organic compounds (Bebianno, 1995). In addition, other factors such as fluctuations in temperature, pH or other potentially stressful environmental parameters may contribute to the cumulative effects observed in the field.

For bivalve larvae the mode of action of pollutants like TBT, as well as its metabolic pathway, is not well known. With respect to the route of exposure of clam larvae to TBT, Laughlin *et al.* (1988) suggested that, for *M.mercenaria,* larvae were mainly exposed through the consumption of contaminated microalgae because effects on larval growth were only apparent after several days of exposure. In case TBT uptake occured directly from water, TBT toxicity would be observed sooner because these organisms could quickly come to a steady state with dissolved TBT. From the results obtained in the current experiment it seems possible that the dominant route of TBT uptake in *R. decussatus* larvae was the aqueous phase since effects on larval growth were noticed from as early as the third day of exposure. Taking into account the difference in larval growth response between the control culture and the lowest TBT concentration tested, it seems important to develop further studies on the effects of TBT at concentrations lower than 25 ng L^{-1} Sn. For another clam species, *M.mercenaria*, veliger larvae presented a significantly reduced growth when exposed to a nominal concentration of TBT 10 ng L^{-1} (approximately 4 ng Sn L^{-1}) (Laughlin *et al.*, 1988). *M. edulis* post-larvae also exhibited significant reductions in growth at extremely low TBT concentrations (2.3 ng Sn L^{-1}) (Stenalt *et al.*, 1998).

In the case of Ria Formosa, if TBT water concentrations continue to exceed the threshold of 25 ng Sn L^{-1} in some parts of the lagoon (Coelho *et al.*, 2002c - Chapter 5), there will probably be damaging effects on the recruitment of *R. decussatus*, by preventing successful larval growth and development. A possible reduction in recruitment may lead to a population decline, and thus to a decrease in *R. decussatus* production, in the long run.

Chapter 4

EFFECTS OF TBT ON CLAM JUVENILES

ABSTRACT

The effects of sublethal concentrations of tributyltin (TBT) on growth of clam juveniles, *R. decussatus*, were determined in clams exposed to nominal TBT concentrations of 50, 100 and 250 ng L^{-1} Sn in sea water, for a period up to 2 years. *R. decussatus* juveniles increased regularly in length and weight over the whole experimental period, but their final length and weight decreased with the increasing in TBT exposure. Growth rates (in length and weight) although decreasing with the increase of TBT concentrations, were not significantly different between all treatments, after 2 years of TBT exposure. Thus, under the described experimental conditions, although a decrease in growth (length and weight) of *R. decussatus* juveniles was observed with the increase in TBT concentrations, growth was not significantly affected by TBT exposure.

4.1. INTRODUCTION

Growth represents the integrated response of internal biological processes. Significant reductions in growth rates are the result of adverse conditions and may indirectly affect the population dynamics (Bayne *et al.*, 1985). If juvenile growth is reduced, it will signify extended periods before reaching the adult stage, reducing chances of survival since juveniles will be exposed for longer periods to adverse conditions. Thus, in the long-term, population's dynamics may be modified. Moreover, it is generally believed that, in any given environment, the added stress of pollutants may reduce animal growth and that, for a given species, juveniles are more sensitive than adults (Salazar & Salazar, 1987). Thus, assessment of pollutant effects on growth of juvenile clams is important due to its ecological significance.

Several studies have demonstrated the deleterious effects of TBT on the growth of bivalves, including the post-settlement phase of different species. In chronic toxicity tests, significant growth reductions were observed when juvenile mussels, *M. edulis*, clams *R. decussatus* and oysters *Crassostrea gigas* and *Saccostrea commercialis* were exposed to levels of TBT in water (< 250 ng L^{-1}) over periods from 4 weeks to six months (Nell &

Chvojka, 1992; Salazar & Salazar, 1987; Thain & Waldock, 1986). Similar results were obtained in field tests where juvenile *M. edulis*, exposed to TBT concentrations of less than 500 ng L^{-1}, also showed significant reductions in their growth rates (Salazar & Salazar, 1988). Nevertheless, there is no consensus in the literature regarding this subject since juveniles of other bivalve species (*Ostrea edulis*; *R. semidecussatus*) exposed to moderate concentrations of TBT in the water (240 ng L^{-1}TBT) did not present significant growth reductions (Thain & Waldock, 1986).

The growth juvenile of oysters, *C. gigas* and *S. commercialis*, was reduced when exposed to a concentration of TBT as low as 5 ng L^{-1} in sea water (Nell & Chvojka, 1992). In the particular case of oysters, some studies have documented, not only growth reductions, but also the appearance of shell chambering in individuals exposed to concentrations of 150 ng L^{-1} TBT (Alzieu *et al.*, 1980; Thain, 1983). In the field, oyster juveniles transplanted from a "clean" site to several TBT contaminated places, exhibited chambering even in waters characterised as containing less than 30 ng L^{-1}TBT (Smith *et al.*, 1987). An additional study using oyster spat, *C. gigas*, determined that the no observed effect concentration (NOEC), for the shell thickening response to TBT, was between 2-20 ng L^{-1}, for a 49 day period (Thain *et al.*, 1987). For mussels the no effect concentration (growth) was estimated to be of a similar magnitude - 25 ng L^{-1} TBT in sea water (Salazar & Salazar, 1991).

Among the numerous shellfish species found along the coast of Portugal, the clam *R. decussatus* is farmed extensively in lagoon areas, like Ria Formosa. As previously referred (Sections 1.8 and 3.1), the spawning season extends from March to September. After the planktonic stage, larvae settle mainly in natural sand banks, near to sea entrances, where they grow until reaching the juvenile stage (10-15 mm). Juveniles are also known as 'seed' clams. At this stage, 'seeds' are harvested and transplanted to farming areas, in other parts of the lagoon. After attaining a marketable size of approximately 35-40 mm, clams are collected for commercial purposes. Good acclimation and growth of transplanted juveniles is a crucial part of the

clam farming process. However, during the acclimation and on-growing period seed clams can be exposed to a number of pollutants, including TBT released from antifouling paints, especially in the inner parts of the lagoon where ports and harbours are located.

The purpose of this chapter is to report the observed effects of sublethal concentrations of TBT on the growth and survival of clam juveniles, *R. decussatus*, during a long-term exposure experiment. Juveniles were exposed to nominal concentrations of 50, 100 and 250 ng L^{-1} TBT (as Sn) in sea water, for a period up to 2 years. These TBT concentrations were selected considering they represent moderately contaminated sites in estuaries and maricultures (see Table 1.2).

4.2. Materials and Methods

Juveniles of *R. decussatus* were obtained from an Experimental Aquaculture Station, in Huelva, southern Spain (Centro de Investigación y Cultivos Marinos -CICEM) where several species of fish and bivalves are cultured. Prior to the experiment, juveniles were acclimated in clean sea water (36 ‰ salinity; 20 °C) for 30 days.

The effects of TBT on clam growth and survival were determined in clams exposed to nominal concentrations of 50, 100 and 250 ng L^{-1} TBT (as Sn) in sea water, for a period up to 2 years.

Batches of 50 clams were exposed to TBT in aquaria with 25 L of aerated sea water, with a salinity of 36 ‰, maintained at a constant temperature of 20 °C. In each aquaria density of clams was approximately 2000/ m^2 as recommended for *Ruditapes* of this size (Saint-Felix *et al.*, 1984). At the start of the experiment, clams had an average maximum shell length of 10.76 ± 0.49 mm and an average wet weight of 0.234 ± 0.029 g.

Clams were kept in the middle of the aquaria, inside a glass "box" with the base covered with a net (Figure 4.1). This "box" was designed for this experiment and built to minimise handling of individuals, since water renewal and cleaning could be carried out without manipulating the clams. Another

advantage of this devise was that aeration, which was placed below the "box", could ensure good water circulation.

Considering its hydrophobic nature, TBT concentrations in solution are likely to be depleted due to adsorption onto test container walls. Thus, a static renewal system was used and sea water together with the contaminant was changed every 48 hours. Absolute ethanol was used as a carrier and different TBT stock solutions were prepared in ethanol so that a constant volume of 250 µl of stock solution and solvent would yield the desired exposure concentrations. A sea water control and a solvent control (+250 µl of absolute ethanol) were run together with TBT treatments. .

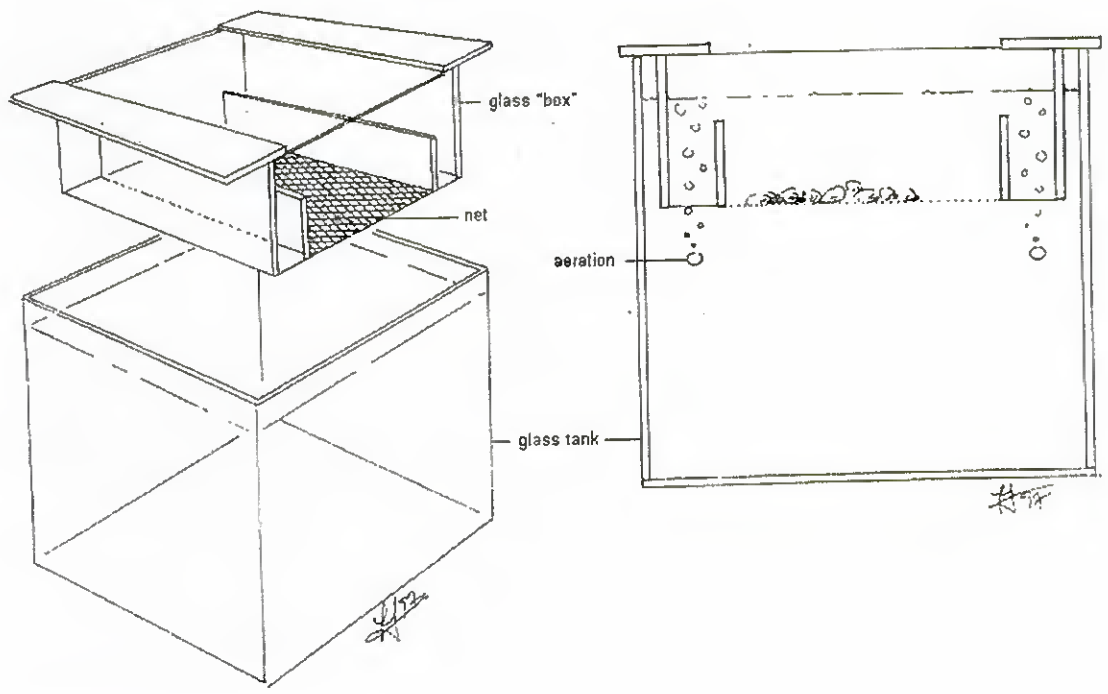

Figure 4.1- Static renewal system devised for culturing clam *R. decussatus* juveniles in the laboratory

During the whole experimental period microalgae, *Isochrysis galbana* were cultured to feed clam juveniles. Diet is probably one of the most important factors regulating culture of bivalves in the laboratory. From eight species of phytoplankton commonly used in aquaculture, *I. galbana* was shown to promote the highest growth rate for juveniles of *R. decussatus*

(Laing *et al.*, 1987; Walne, 1976). Thus, this species of phytoplankton was selected as the diet during the present experiment. Algal culture was performed with autoclaved (120 °C for 30 min) sea-water, 36 ‰ salinity, enriched with medium F/2 (Guillard & Ryther, 1962) under constant temperature (20 °C), light and aeration. Microalgae were harvested after reaching the lag phase of growth, usually after 5-6 days of inoculation. Algae were permanently added to aquaria by means of a peristaltic pump, in order to obtain a daily amount of algal tissue equivalent to 7% of the clam's whole soft tissues, in each aquarium (Morales, 1983).

Every week aquaria were thoroughly washed to remove vestiges of clam pseudofeces as well as bacteria and microalgal slimes.

In order to assess clam growth, all individuals from each aquarium were measured every month. Measures included maximum shell length, in the antero-posterior axis, and total wet weight. Shell length was measured using a digital calliper (Mitutoyo - 150 mm), to the nearest 0.01 mm, which was connected to a PC and a printer (Figure 4.2). Total weight (ww) of individuals was determined, to the nearest 0.001 g, in a precision balance (Precisa 1212M). For bivalves, total wet weight is not the best parameter to characterise growth because it includes the shell, soft tissues and water that may exist within the valves, all of which may vary considerably. Thus, the dry weight of soft tissues is preferable to total wet weight. However, this parameter could not be measured in the present work because repeated sampling was used.

In addition to growth, survival was also registered every month.

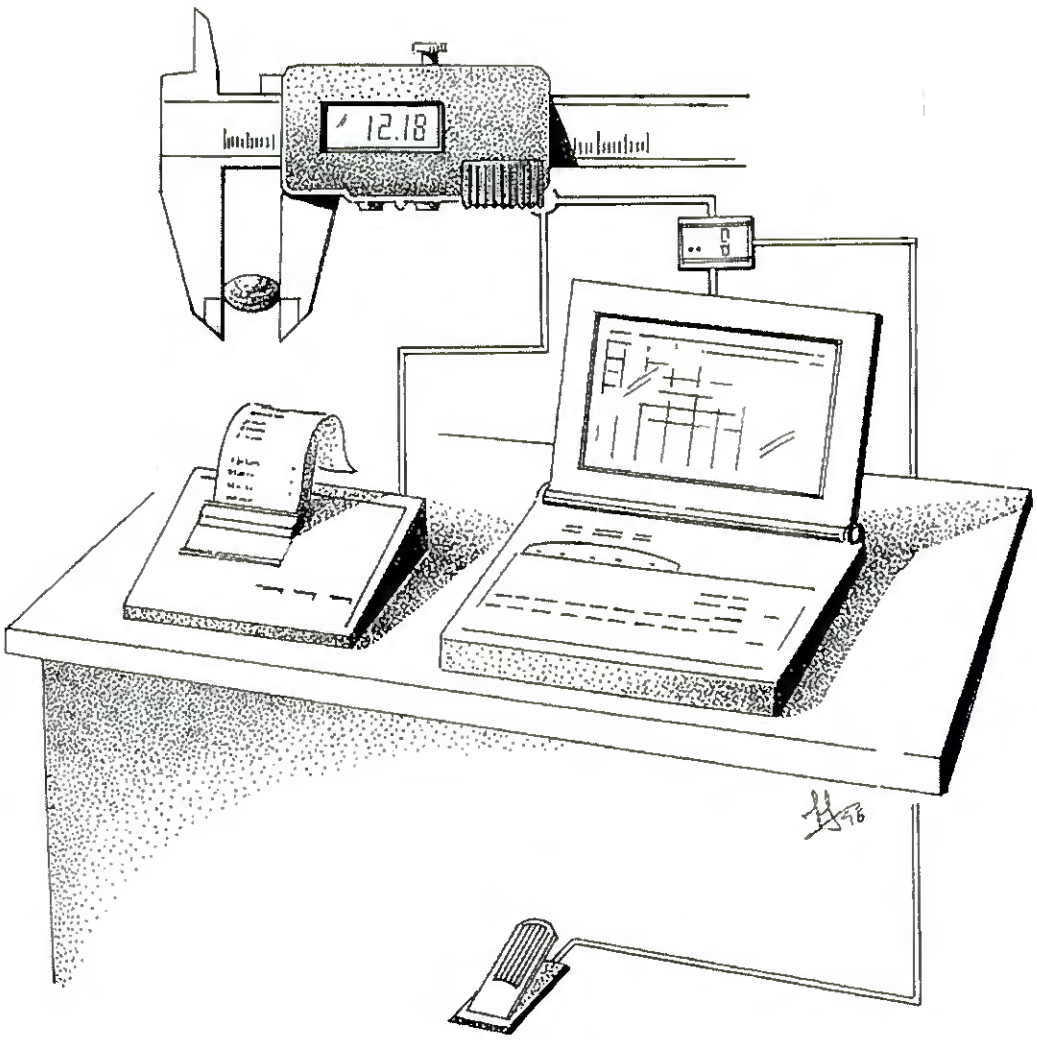

Figure 4.2- Measurement of *R. decussatus* juveniles using a digital calliper connected to a PC and a printer.

4.3. RESULTS

Data for average maximum shell length (mm) of *R. decussatus* juveniles exposed to TBT (50-250 ng L^{-1} Sn), over the two-year period are plotted in Figure 4.3.

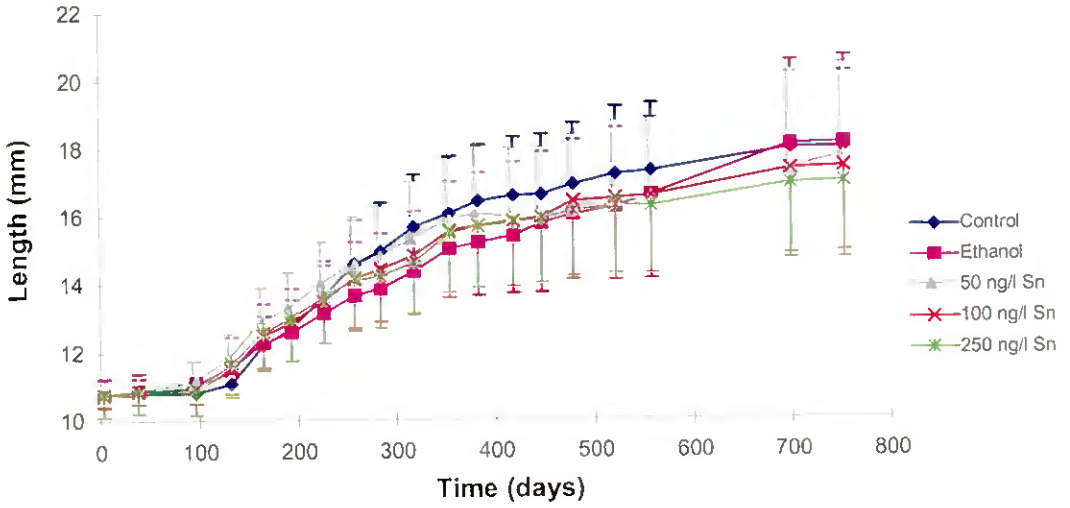

Figure 4.3 - *R. decussatus*- Increase in length (mm) of juveniles exposed to different concentrations of TBT: 0, 50; 100; 250 ng L^{-1}Sn, during the experimental period of approximately 2 years (Vertical lines shown are standard deviations)

Average values for initial and final maximum length (mm), as well as growth rates (mm/month), of *R. decussatus* juveniles from different treatments are presented in Table 4.1.

Juveniles from all treatments and controls presented a similar regular increase in length, during the whole experimental period. Nevertheless, after five months of exposure (day 190) slight differences in maximum length were observed, although these were not significant ($p<0.05$). After 10 months (day 350), control juveniles presented slightly higher values of maximum length while lower values were observed in individuals from the ethanolic control. After 18 months of exposure an alteration occurred, with higher values of maximum length corresponding to individuals from both controls and lower values observed in juveniles exposed to 250 ng L^{-1} TBT (Sn) (Figure 4.3).

Table 4.1- Initial and final average length (mm) and growth rates (mm/month) of juveniles in two control treatments and exposed to different TBT concentrations, after an exposure period of 2 years.

Treatment ($ng\ L^{-1}$ TBT)	Initial length (mm) (average ± st.dev.)	Final length (mm) (average ± st.dev.)	Growth rate** (mm /month)
Control 1	10.74 ± 0.46	18.00 ± 2.24	0.30
Control 2 (ethanol)	10.77 ± 0.52	18.16 ± 2.56	0.31
50 ng/L TBT *	10.73 ± 0.45	17.69 ± 2.77	0.29
100 ng/L TBT *	10.80 ± 0.37	17.46 ± 2.51	0.28
250 ng/L TBT *	10.76 ± 0.64	16.99 ± 2.26	0.26

* Concentrations of TBT expressed as Sn.

** Growth rate = (final length - initial length) / total No. of months

Concerning the maximum length of individuals, final length of juveniles decreased slightly with increasing TBT concentrations. Growth rates obtained over the two-year period were similar in all treatments, although the controls present slightly higher rates than TBT treatments (Table 4.1). A non-parametric test Kruskal-Wallis Anova, by ranks (Rice, 1995), applied to the maximum length data, at the end of the experiment, showed that there was no significant difference ($p<0.05$) between all treatments, after 2 years of exposure to TBT.

Data for average weight increases in clam juveniles over the two-year period, in different treatments, are plotted in Figure 4.4.

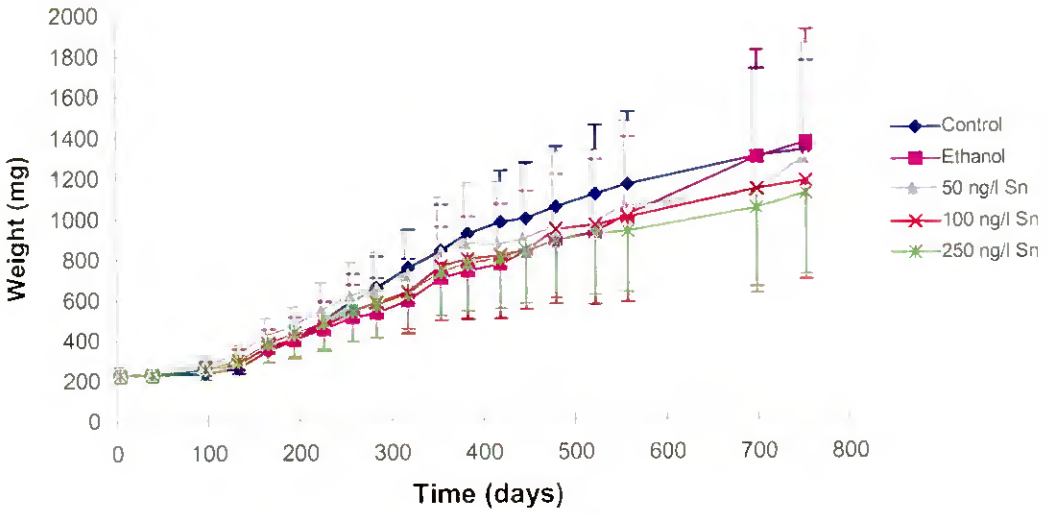

Figure 4.4 - *R. decussatus*- Increase in weight (mg) of juveniles exposed to different concentrations of TBT: 0; 50; 100; 250 ng L^{-1} Sn, during the experimental period of approximately 2 years (Vertical lines shown are standard deviations)

Average values of initial and final weight (mg) of individuals, as well as growth rates (mg/month) are presented in Table 4.2.

Table 4.2- Initial and final average weight (mg) and growth rates (mg $month^{-1}$) of juveniles in two control treatments and different TBT concentrations, after exposure for 2 years.

Treatment	Initial weight (mg) (average ± st.dev.)	Final weight (mg) (average ± st.dev.)	Growth rate** (mg $month^{-1}$)
Control	236 ± 34	1334 ± 439	46
Control 2 (ethanol)	232 ± 28	1371 ± 558	48
50 ng/L TBT *	236 ± 30	1284 ± 565	44
100 ng/L TBT *	233 ± 19	1183 ± 485	40
250 ng/L TBT *	234 ± 36	1121 ± 401	37

* Concentrations of TBT expressed as Sn.
** Growth rate = (final weight - initial weight) / total No. of months

Ruditapes decussatus juveniles presented a regular increase in weight, during the whole experimental period. In the period between 10 and 18 months of exposure (days 350-550), highest weight values were observed in

control individuals while juveniles exposed to 250 ng L^{-1} TBT (Sn) showed the lowest average weights.

The growth rates of juveniles, expressed as weight increases, were similar in all treatments, although controls (sea water and ethanol) achieved slightly higher growth rates. A non-parametric test Kruskal-Wallis Anova, by ranks (Rice, 1995), applied to the weight data at the end of the experiment, showed that there was no significant difference ($p<0.05$) between all treatments, after 2 years of exposure to TBT.

Thus, under the described experimental conditions, growth of *R. decussatus* juveniles was not significantly affected by the exposure to TBT.

Linear regressions of the allometric length/weight relationships, for each treatment, are plotted in Figure 4.5. *R. decussatus* juveniles presented similar length/weight relationships - an expression of bivalve's condition (Newman & Heagler, 1991) - in all treatments. These relationships are in accordance with other allometric relationships obtained for this species in a field study (Furtado, 1991). A statistical analysis - test on the coefficient β of simple linear regressions (Fonseca, 1992; Shrisagar, 1983;) - applied to the data showed that there was no significant difference ($p<0.05$) in allometric relationships between treatments, thus indicating that exposure to TBT did not affect the bivalves condition and confirming the previous results obtained for growth rates.

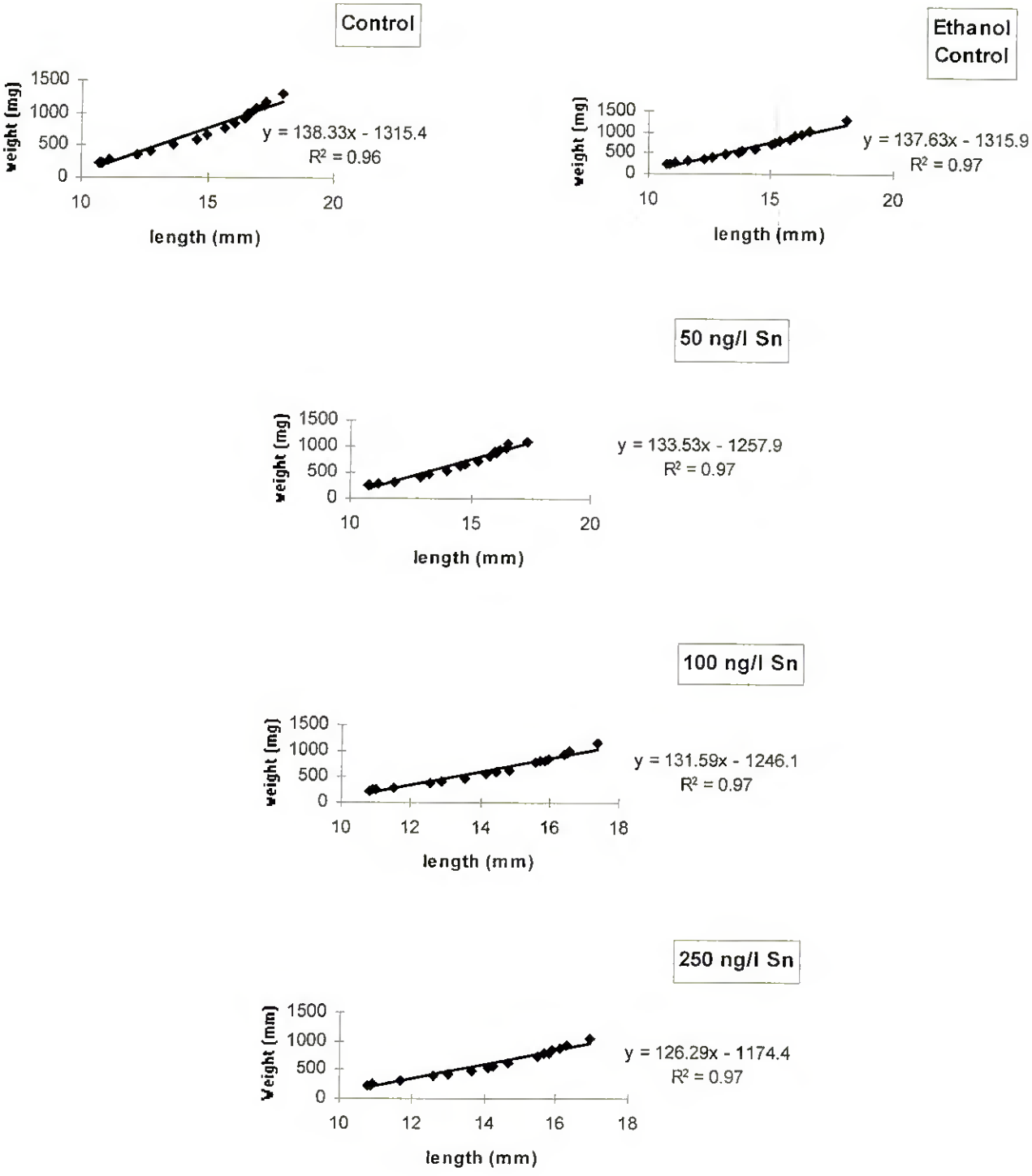

Figure 4.5 - *R. decussatus* - Weight/length relationship (and linear regressions) of juveniles exposed to different concentrations of TBT (0; 50; 100; 250 ng L^{-1} Sn) in the water, for a period of approximately 2 years

Survival (%) of *R. decussatus* juveniles during the exposure time of 2 years is shown in Figure 4.6.

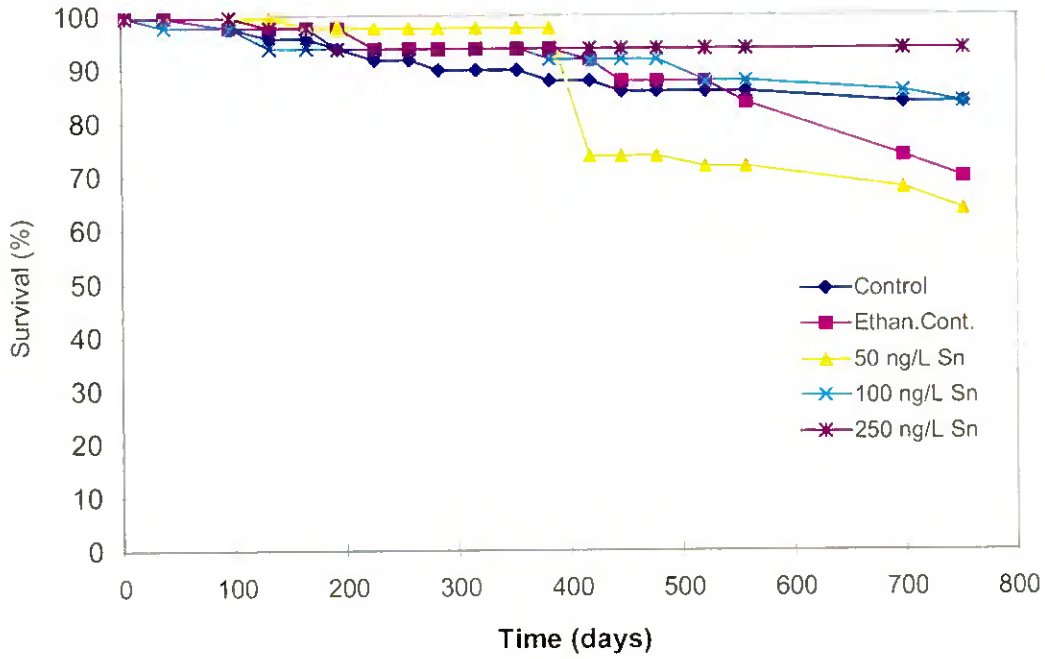

Figure 4.6- *R. decussatus* - Survival (%) of juveniles exposed to different concentrations of TBT: 0; 50; 100; 250 ng L^{-1} Sn, during the experimental period of approximately 2 years

Highest survival was observed in the highest exposure regime (250 ng L^{-1}) and lowest survival was obtained at the 50 ng L^{-1}TBT (Sn) concentration. Therefore, no relationship was observed between survival and TBT exposure. Considering the length of the exposure period (2 years) and the frequent handling during water renewal, survival of individuals was relatively high (Figure 4.6).

It is worth noting that some juveniles exposed to TBT develop an abnormal shell growth, compared with juveniles from the control cultures. In malformed shells, growth was more evident not on a sagittal plane but perpendicular to it, changing the typical flattened shape of clams in to a more "rounded" form. This characteristic was more visible in the anterior margin of valves than on the posterior one and was mainly observed in clams exposed to TBT at 50 ng L^{-1} (Sn).

4.4. Discussion

Under the described experimental conditions, growth of *R. decussatus* juveniles was not significantly affected by the exposure to TBT (50-250 ng L^{-1} Sn).

Similar results were obtained for other bivalve species such as O. *edulis* and *R. semidecussatus*, which presented no significant growth reduction when exposed to 240 ng L^{-1} TBT in water for seven weeks (Thain & Waldock, 1986). In contrast, growth of *C. gigas*, *M. edulis* and *R. decussatus* spat was severely reduced after exposure to TBT under the same conditions (240 ng L^{-1} TBT for 7 weeks) (Thain & Waldock, 1986). Juvenile mussels, *M. edulis*, exposed to low levels of TBT in water (70 - 200 ng L^{-1}), also exhibited significant growth reductions after a period of 196 days (Salazar & Salazar, 1987).

Results obtained in our experiments with *R. decussatus* may be related to the exposure protocol adopted. A static renewal protocol was used hence, although initial TBT concentrations were close to nominal, levels of contaminant probably decreased until the next water renewal. After 48h, concentrations were probably less than 50% the nominal concentrations (Laughlin *et al.*, 1988; Ruiz, 1993). Conversely, in some of the experiments with juvenile bivalves where growth rates were reported to be affected by exposure to TBT, a flow-through system was utilised ensuring a continual supply of contaminant - keeping TBT concentrations at constant levels (Salazar & Salazar, 1987). Generally bivalves are more affected by a continuous exposure to a contaminant than to a discontinuous regime of exposure (Davenport, 1977), since in the latter, loss of contaminant can occur, producing lower average concentrations of the contaminant.

Field tests where juvenile *M. edulis*, were exposed to ambient TBT concentrations between 7 and 500 ng L^{-1} showed significant growth reductions at TBT concentrations between 200-500 ng L^{-1} (Salazar & Salazar, 1988). As in the previous case, in field tests, juveniles were probably exposed to roughly constant concentrations of TBT in the water, since they were

placed near harbours and marinas that are a permanent source of TBT from antifouling paints.

In the present work, stress due to laboratory conditions may also have contributed to "mask" the possible deleterious effects of TBT on juveniles, in both experimental and control bivalves. It is likely that stress due to excess of handling may have affected juveniles, although much care was taken to minimize this effect. In addition, lack of sediment may also have contributed to stress juveniles (Furtado, 1991). In fact, growth rates of individuals even in the control cultures were smaller than observed for *R. decussatus* in the natural environment (Furtado, 1991), confirming the stressing conditions of laboratory culture.

Constant supply of low amounts of phytoplankton to test containers is recommended to mimic environmental conditions. On the other hand, phytoplankton is known to bind lipophilic compounds in short periods of time (minutes) (Avery *et al.*, 1993; Coelho *et al.,* 2002b - Chapter 5) thus, TBT could have been "removed" from solution through binding to algal cells. If this was the case, then TBT would be incorporated in clams not from water but through food. Accumulation of TBT from food is probably lower than from water and in this case TBT accumulation may have been insufficient to cause deleterious effects in juvenile growth.

Shell deformities in bivalves exposed to TBT is a well reported effect for oysters *C. gigas*. TBT induces the shell thickening producing "ball shaped" oysters at concentrations as low as 2 ng L^{-1} Sn (Alzieu *et al.*, 1986; Waldock & Thain, 1983). Nevertheless, malformation of shells in clam juveniles is an uncommon effect only reported for post-larvae of *S. plana* (Ruiz, 1993). For these post-larvae deformation was more evident at a concentration of 50 ng L^{-1} TBT (Sn) as in our experiments. Ruiz (1993) postulates that deformities may be linked with growth of less rigid shell layers, which would deform as a result of friction with sand, as post-larvae crawl and bury. However, for *R. decussatus* no substrate was utilised in clam rearing, thus although clams may have deposited "softer" layers due to a malfunction of the calcification mechanism, friction with substrate could not have caused shell malformation.

It is possible that shell deformities in *R. decussatus* happen in the natural environment, however its ecological relevance is unknown. Considering the results presented in this work, further studies should be performed in order to clarify the origin of the phenomen

Chapter 5

ROUTES OF TBT UPTAKE IN CLAMS

Coelho, M.R.; Bebianno, M.J. & Langston, W.J. (2002a). Routes of TBT uptake in the clam Ruditapes decussatus. I. Water and sediments as vectors of TBT uptake. *Mar.Environ.Res.* **54**: 179-192.

Coelho, M.R.; Bebianno, M.J. & Langston, W.J. (2002b). Routes of TBT uptake in the clam Ruditapes decussatus. II. Food as vectors of TBT uptake. *Mar.Environ.Res.* **54**: 193-207.

ABSTRACT

Marine bivalves are exposed to pollutants *via* the aqueous phase, sediments and food. Nevertheless, the relative importance of these phases as uptake vectors of contaminants in these marine organisms has not been well studied.

I. The first part of this study assessed the relative importance of water and sediments as vectors of TBT uptake in the sediment-dwelling suspension feeder, *Ruditapes decussatus*. Accumulation of TBT was determined in *R. decussatus* exposed for 60 days to, moderately high but environmentally realistic levels of TBT dissolved in water (100 ng L^{-1} Sn) and sediments (0.8 µg g^{-1}Sn dw), separately or in combination, using constant-flow systems. The results indicate that this species accumulates TBT predominantly from water. Although some accumulation from sediments does occur, the processing of large amounts of water needed to sustain the filter-feeding habits of this species is a prime determinant of TBT uptake. The route of exposure is reflected in tissue distributions of TBT in *R. decussatus.* However, gills are the most important site for accumulation of TBT from water, irrespective of whether contaminated sediments are present or not.

II. Phytoplankton concentrate contaminants from seawater and given their position at the base of most marine food webs, these algal cells may play critical roles in the transfer of contaminants to higher trophic levels.

The second part of the present work assessed the relative importance of microalgae as a vector of TBT uptake in the infaunal, suspension-feeding bivalve, *Ruditapes decussatus*. Accumulation of TBT via the algal diet was determined by experimental exposure of *R. decussatus* to ^{14}C-TBT labelled phytoplankton *Isochrisis galbana*, for a period up to 60 days. The digestive tract of these clams initially accumulated TBT preferentially from food. After a few weeks of exposure, internal remobilization resulted in a more widespread partitioning of TBT amongst tissues.

From the results of the reported experiments, in which *R. decussatus* were exposed to TBT in water, sediments and food partitioned realistically, it seems clear that this species accumulates TBT predominantly from water. Although some accumulation from sediments and food does occur, the contribution from these phases was negligible when compared to water, at least under the conditions and concentrations used in this experiment.

5.1 INTRODUCTION

In the marine environment, bivalves are exposed to pollutants in the water and benthic sediments (see Section 1.5). But, since pollutants may accumulate in phytoplankton they may be transferred to herbivores through dietary intake, implying food as another route of uptake in benthic organisms. Nevertheless, the relative importance of these phases as uptake vectors in these marine bivalves has not been well characterized.

5.1.1. Uptake of TBT

Uptake of the major classes of organic contaminants by marine organisms is generally considered to be dominated by passive diffusion from solution, although there is evidence that carrier-mediated uptake may also occur for some metal and organometallic forms (Rouleau *et al.*, 1995). Once incorporated across the cellular boundaries organic contaminants are often translocated to other tissues, by either passive or active processes, where they will be accumulated, metabolised or eventually eliminated (Fowler, 1982).

In attempting to evaluate accumulation of TBT in marine organisms, most studies have used exposure directly from seawater. This is probably highly appropriate for organisms at the lowest trophic level. Uptake from sediments and food could, however, be important for organisms at higher trophic levels and these pathways are likely to be particularly relevant for filter feeding organisms that inhabit benthic muds and sands. TBT is readily adsorbed to sediments (Kp values are typically in the range 4-30 L g^{-1} for

estuarine fines; (Langston & Pope, 1995)) and may also be present as minute paint particles within the sediment matrix, especially close to boatyards and maintenance facilities. This potential route of TBT uptake for benthic filter-feeders has frequently been inferred as a threat to benthic organisms, but it is based largely on correlative evidence. For example, *Mya arenaria*, collected from contaminated sediments, accumulate extraordinarily high TBT burdens (Langston *et al.*, 1987); similarly, TBT concentrations in freshwater mussels, *Elliptio complanata*, may exceed those in surronding sediments by 18-fold (Chau *et al.*, 1989).

One of the few studies to specifically address the relative importance of water and sediments as sources of TBT to benthic organisms involved the infaunal clam *Scrobicularia plana*. Body burdens in this species are dominated by assimilation of sediment-bound TBT, as a result of the bivalve's deposit-feeding habit (Langston & Burt, 1991). For suspension feeding clams, it is not clear which TBT pathway prevails (Gomez- Ariza *et al.*, 1999).

In the case of TBT, reported Bioconcentration factors (BCF) for bivalves varied between 1 000 and 540 000, as indicated earlier (Table 1.6), showing that this compound readily accumulates in bivalves. Steady state levels, if ever reached, will usually take days to weeks (Guolan & Yong, 1995; Laughlin & French, 1988; Waldock *et al.*, 1983). Differing contributions from particulate (dietary) sources are thought to account for some of this inter-specific variation. Unfortunately, however, detailed knowledge of assimilation routes across a range of taxonomic groups and feeding types is not available.

Despite the number of studies reported on TBT accumulation, data for BCF in *R. decussatus* is scarce. Thus in order to understand, more fully, the implications and risks of TBT impact in this species, a study of uptake rates and routes is required.

5.1.2. Phytoplankton as possible vector for TBT uptake

Because phytoplankton can concentrate some pollutants very extensively from sea water and because they lie at the base of most marine food webs, these plants can play critical roles in introducing pollutants into the marine food-chain (Fisher & Reinfelder, 1995).

Uptake of TBT from sea water by phytoplankton and bacteria is thought to occur over short time scales (minutes to hours) and two forms of uptake - simple single compartment and biphasic - have been distinguished to date. Cyanobacteria, *Synechocystis* PCC 6803, exposed to 0.5 mM TBTCl, reached steady state in only 5 minutes, with no subsequent accumulation, suggesting that a simple partitioning process (adsorption) was occurring (Avery *et al.*, 1993). In contrast, the microalgae *Chlorella emersonii*, exposed to the same TBT concentration displayed an initial rapid phase of uptake (5 min), followed by a second slower component between 5 min and 2 hours (Avery *et al.*, 1993). Differences in uptake patterns are thought to be related to the distinct structure and composition of cell membranes in cyanobacteria and microalgae .

Studying TBT uptake in three different species of microalgae, Chiles *et al.* (1989) demonstrated that each accumulated TBT during a 2h exposure to ^{14}C-labelled TBT (2 ng l^{-1} to 20 mg l^{-1}). However, whilst hydrophobic partitioning appeared to be the dominant mechanism of uptake for two of the algal species (*Nannochloris* sp. and *Chaetoceros gracilis*) metabolically-driven processes controlled accumulation in a third species (*Isochrysis galbana*).

Although some work has been performed on the accumulation of TBT by phytoplankton, data on bioconcentration factors (BCF) for phytoplankton are limited. Work with *Chlorella vulgaris*, *Duniella salina* and *Duniella vivides* suggests BCFs in the range of 10^3 - 10^5. (Guolan & Yong, 1995; Laughlin *et al.*, 1986).

Published information on transfer factors to higher organisms are also scarce. Mussels, *M. edulis*, fed on phytoplankton *Dunaliella* sp., previously exposed to TBT (20-500 ng l^{-1}), have been shown to assimilate dietary TBT (Guolan & Yong, 1995). In addition, differential tissue accumulation has been observed in *M. edulis* exposed to phytoplankton (*Isochrysis galbana*) contaminated with TBT: highest levels of this compound were detected in the viscera (Laughlin *et al.*, 1986; Page *et al.*, 1995; Zoulian & Jensen, 1989). Working further up the food chain, Rouleau *et al.* (1995), studied the distribution kinetics of TBT in the starfish *Leptasterias polaris*, fed with TBT-

contaminated mussels. Using whole-body autoradiography, they indicated that TBT was absorbed in the stomach and transferred to the pyloric caecae (where absorption of food takes place) and from this tissue to the rest of the body.

Thus, whilst aquatic primary producers such as microalgae derive TBT almost exclusively from the aqueous phase, this may not be the case for many marine fauna. In view of the variety of possible uptake pathways it is important to acquire a quantitative appreciation of the relative importance of each possible source (water, sediments and food), as well as the kinetics of TBT accumulation, in different groups of organisms.

The study reported here was undertaken to assess the relative importance of water, sediments and food as vectors of TBT uptake in the sediment-dwelling suspension feeder, *R. decussatus*. Accumulation of TBT was studied in *R. decussatus* exposed to environmentally realistic levels of TBT dissolved in water (100 ng L^{-1} Sn) and/or bound to sediments (0.8 µg g^{-1} Sn dw), using constant-flow systems for a period up to 60 days. In addition, accumulation of TBT from food was evaluated through exposure of *R. decussatus* to ^{14}C-TBT labelled phytoplankton *I. galbana*, for a period up to 60 days. The study of phytoplankton as a possible vector of TBT uptake for clams included preliminary experiments performed to determine the kinetics of TBT accumulation in *Isochrisis galbana* exposed to TBT in water (100 ng l^{-1} Sn) for a period of up to 48 hours.

The TBT concentration used (100 ng l^{-1} Sn) represents a moderately high level of contamination but is environmentally realistic and ensures ecotoxicological relevance.

5.2. MATERIALS AND METHODS

Clams, were exposed to varying combinations of TBT-contaminated water, sediment and food, in order to determine their relative importance as vectors of TBT uptake. Two separated sets of long-term exposure experiments were designed to identify the principal accumulation pathways for the clam *R. decussatus*. In the first experiment (described in 5.2.1.) water

and sediment were treated with TBT (equilibrated at concentrations representative of moderate contamination in the field) to study the relative importance of these pathways in TBT uptake. In the second experiment (described in 5.2.2.), phytoplankton were labelled with ^{14}C-TBT and were used to investigate dietary assimilation of organotins in *R. decussatus*.

5.2.1. Water and sediment as vectors of TBT uptake in *R. decussatus*

A series of long-term experiments (60 days) were conducted exposing suspension-feeding clams, *R. decussatus*, to varying combinations of TBT-contaminated water and sediments. These systems were equilibrated at concentrations representative of those in areas influenced by commercial shipping, such as major estuaries and ports. Exposure of clams to dissolved and particulate forms, separately or in combination, was designed to identify the principal accumulation pathway for TBT.

TBT concentrations in sea water were generated and maintained by immersing a perpex rod coated with TBT-containing self-polishing antifouling paint into a constant-flow tank (Figure 5.1) to a depth sufficient to produce the required TBT concentration in the outflow (nominal concentration of 100 ng L^{-1} Sn- measured value of 102 $\pm$ 28 ng L^{-1} Sn). The salinity and temperature of the sea water used in the experiment were 35 ‰ and 15- 16° C, respectively.

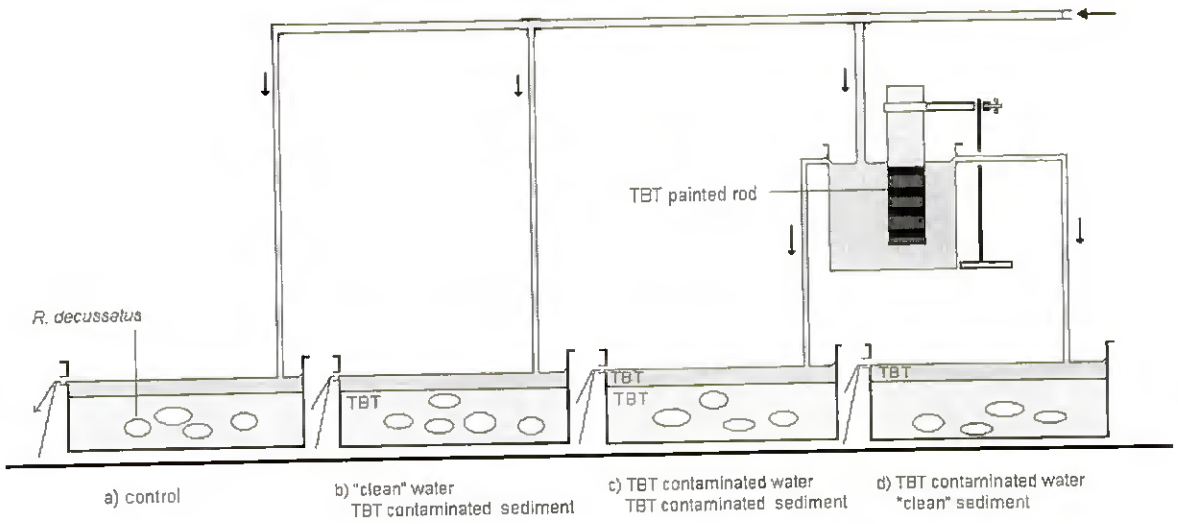

Figure 5.1- Flow-through system used for exposure of *R. decussatus* adults to TBT in the water (100 ng L^{-1} Sn) and sediments (0.8 µg g^{-1}), either combined or separately.

For sediment exposure, surface sediment was collected from the Plym estuary (UK) and batches of 1 kg of fresh material were slurried with sea water and spiked with TBT (dissolved in small amounts of ethanol), under continuous mixing for 24 hours. The amount of TBT added was calculated to give nominal TBT concentrations of approximately 0.8 µgg^{-1} Sn (dw) in the sediment. The TBT levels in sediments and water used here were based on the partioning studies of Langston & Pope (1995) and are considered representative of steady-state proportions.

TBT concentrations in sea water and sediments used in the present study, were chosen considering to represent a moderate level contamination in the field (see Table 1.3 and 1.4 - Chapter 1). Spiked sediments were allowed to settle for 24 hours before re-suspension in clean sea water. The slurry was transfered to experimental tanks (5L) for a further conditioning and settlement period of 7 days. Organotin concentrations in sediments were measured periodically throughout the experiment (Table 5.1).

Table 5.1 - TBT and DBT concentrations (µg g^{-1} Sn dw) in spiked sediments (see Figure 5.1), during the whole experimental period (60 days); treatment (b) TBT contaminated sediments and 'TBT -free' seawater; treatment (c) TBT contaminated seawater and TBT contaminated sediments.

DAYS		0	10	20	40	60	
Treatments							Mean ± st. dev.
b) Contam. sediment	TBT (µg g^{-1} Sn)	0.741	0.677	0.718	0.788	0.705	0.754 ± 0.051
	DBT (µg g^{-1} Sn)	0.080	0.070	0.048	nd	0.011	
c) Contam. water + sedim.	TBT (µg g^{-1} Sn)	0.741	0.730	0.702	0.756	0.839	0.726 ± 0.042
	DBT (µg g^{-1} Sn)	0.080	0.016	0.006	nd.	nd.	

nd - not detected

Batches of 20 adult *R. decussatus,* (~35 mm shell length) previously acclimated in clean sea water for 7 days, were exposed to TBT in water and sediments, either combined or separately (Figure 5.1). Four flow-through treatments were established:

a) Control: 'TBT-free' sea water (<1 ng L^{-1} Sn) and acid washed sand as substrate;

b) TBT-contaminated sediments: sediments spiked with TBT at 0.8 µg g^{-1} Sn dw (for measured values see Table 5.1); overlying sea water 'TBT-free' (<1 ng L^{-1} Sn);

c) TBT-contaminated water and sediments combined: TBT in sea water 100 ng L^{-1} Sn (measured value of 102±28 ng L^{-1} Sn). Sediments spiked with TBT at 0.8 µg g^{-1} Sn dw (for measured values see Table 5.1);

d TBT-contaminated water: TBT in sea water 100 ng L^{-1} Sn (measured value of 102±28 ng L^{-1} Sn); acid washed sand as substrate.

Four *R. decussatus* and an aliquot of spiked sediments were taken from each treatment at days 10, 20, 40 and 60. An additional batch of clams was left for another 30 days (until day 90) to assess the re-distribution of TBT

within tissues. All clams were allowed to depurate for two days in clean seawater (to eliminate any contaminated sediment in the gut) prior to being frozen (-20°C) for analysis.

Water, sediments and clam tissue samples were extracted and analysed for TBT and DBT in accordance with the methods first described by Ward *et al.* (1981), as modified by Bryan *et al.* (1986) and Langston *et al.* (1987; 1994), and described in Chapter 2.

5.2.2. Food as a source of TBT to *Ruditapes decussatus*

i) ^{14}C-TBT uptake by phytoplankton *Isochrysis galbana*

As a preliminary experiment, it was necessary to determine TBT uptake rates and equilibration times in the unicellular flagellate alga, *Isochrysis galbana*, to ensure that this phytoplankton species acts as dietary source of TBT when feed to clams. To help follow kinetics more rapidly *I. galbana* were exposed to ^{14}C-labelled TBT.

The flagellate unicellular alga, *I. galbana*, was selected for this study because it is an appropriate food source for this clam species (Jeffrey *et al.*, 1994; Laing *et al.*, 1987; Walne, 1976). Unialgal cultures of *Isochrysis galbana* from the Marine Biological Association's culture collection were grown in sea water (35‰ salinity) and enriched with f/2 medium (Guillard & Ryther, 1962). Sea water used in the experiments was obtained from Plymouth Sound and autoclaved (120°C for 30 min) before starting the cultures. All glassware used in the experiment was also autoclaved. Phytoplankton cultures were maintained in 10L pyrex flasks and harvested for these experiments during the stationary phase of growth. Density of phytoplankton cells was determined using a Coulter Counter. Algae were sub-cultured into 2.5 L pyrex conical and kept in a controlled temperature room at 15-16 °C, with permanent light and aeration. The density of cells in sub-cultures of *I. galbana* used throughout the experimental period was $5.84 \times 10^6 \pm 1.87 \times 10^6$ cells ml^{-1}.

Working solutions of ^{14}C-TBT were prepared in absolute ethanol from stock solution (specific activity 21 mC $mM^{-1;}$ radiochemical purity 95%; Amersham International). The initial TBT concentration in the water used for algal exposures was 100 ng L^{-1} Sn, obtained by spiking the 2.5 litre flasks containing the algae with ^{14}C-TBT, under active mixing. This concentration was chosen because it represents a moderate to high level of contamination, comparable to that used used in the previous experiment (described in 5.2.1). It also represents a sublethal concentration to *Isochrysis galbana* .

To measure organotins in *Isochrysis galbana* during the course of the experiment, two aliquots of 30 ml were sampled from each of the ^{14}C-TBT spiked algal flasks, after 10, 20, 40, 60 minutes, and also after 2.5, 4 and 48 hours. Fractions containing radiolabelled TBT and its degradation product DBT, together with total ^{14}C, were determined in algal and aqueous phases in each of these samples. Aliquots were centrifuged for 10 min at 3000 rpm. Overlying water was separated from the algal pellet and transferred to a 100 ml conical flask. Five ml of HCl (BDH-Aristar) were added to the aqueous samples, which were kept in the dark, at 4°C until subsequent extraction. Each acidified water sample (approximately 30 ml) was extracted with 5 ml of hexane by hand-shaking for 4 minutes, in a separating funnel and the aqueous and solvent phases left to separate.

One milliliter of the hexane extract was used directly for ^{14}C-organotin determination (TBT+DBT fraction). A further 2 ml of the hexane extract were transferred to a centrifuge tube and subjected to an alkali wash with a 1:1 mixture with NaOH (1M) in order to remove DBT from the hexane extract. A 1 ml aliquot of NaOH-washed hexane were then used to determine ^{14}C-TBT concentrations. ^{14}C-DBT concentrations were calculated by difference between the two previously described fractions, allowing for the difference in sensitivity caused by the absence of one ^{14}C-butyl group in the DBT molecule. To determine radioisotope activity, 14 ml of scintillation cocktail (Optiphase-MP) was added to the 1 ml hexane extracts. ^{14}C determinations were performed using a LKB 1215 Liquid Scintillation Counter, calibrated against previously prepared standards, blanks and quench curves.

The algal pellet from one of the samples obtained after centrifugation was acidified with 5 ml of HCl (BDH-Aristar) and left for one hour, in order to extract TBT and DBT from algal tissues. Subsequently, 5 ml of hexane were added and the tubes were placed on an automatic shaker for 15 minutes to extract the labelled organotins. After centrifugation at 3000 rpm for 5 minutes, one milliliter of the separated hexane extract was used to measure ^{14}C-butyltin concentrations (TBT+DBT fraction), and, following alkali washing, the TBT fraction on its own, as described above.

The algal pellet from the other sample in the pair, was used to determine the total ^{14}C activity in the sample (i.e. ΣTBT, DBT + other ^{14}C-containing metabolites and breakdown products, including MBT). This pellet was transferred to a scintillation vial and digested with 1 ml of tissue solubilizer (Optisolv) overnight. Fourteen milliliters of scintillation cocktail (Optiphase-MP) were added and samples were counted for total ^{14}C activity.

A further sample of algal culture was taken at each sample interval in order to determine the wet/dry ratio, by weighing the centrifuged pellet before and after oven drying for 24h, at 80°C.

ii) TBT uptake by clams *R. decussatus* fed with TBT contaminated *I. galbana*

Based on the results from the preceding experiment (i), a protocol was established in which *R. decussatus* was fed with ^{14}C-TBT labelled phytoplankton. *I. galbana,* was cultured according to the conditions described above and harvested during the stationary phase of growth. Density of phytoplankton cells was determined periodically, using a Coulter Counter. Microalgae were transferred to 2.5 L conical flasks and kept under constant illumination and aeration. *Isochrysis galbana* cultures were spiked with a nominal concentration of 100 ng L^{-1} ^{14}C-TBT (as Sn), at least 1 hour prior their transfer to the clam tank (this was established in the previous experiment (i) as sufficient time for an almost complete assimilation of ^{14}C-TBT by algal cells).

Adult *R. decussatus* used in the feeding experiment were the same population as those used in the water/sediment experiment (described in 5.2.1) .

Clams were acclimated in clean sea water, for 7 days Prior to feeding on ^{14}C-TBT labelled *Isochrysis galbana*. As shown in Figure 5.2, 60 clams were introduced into a tank (35x45x24 cm) with 10 litres of sea water subjected to constant aeration. The experiment was coducted in a temperature controlled room at 15-16° C. At the start of the experiment, clams were measured with a calliper, to the nearest 0.01 mm, presenting an average maximum shell length of 34.9 ± 3.2 mm. In order to provide continuous feeding to the clams, a peristaltic pump (LKB 2132), set to a flow rate of approximately 20 ml/ hour, was used to transfer the ^{14}C-TBT spiked culture to the experimental tank.

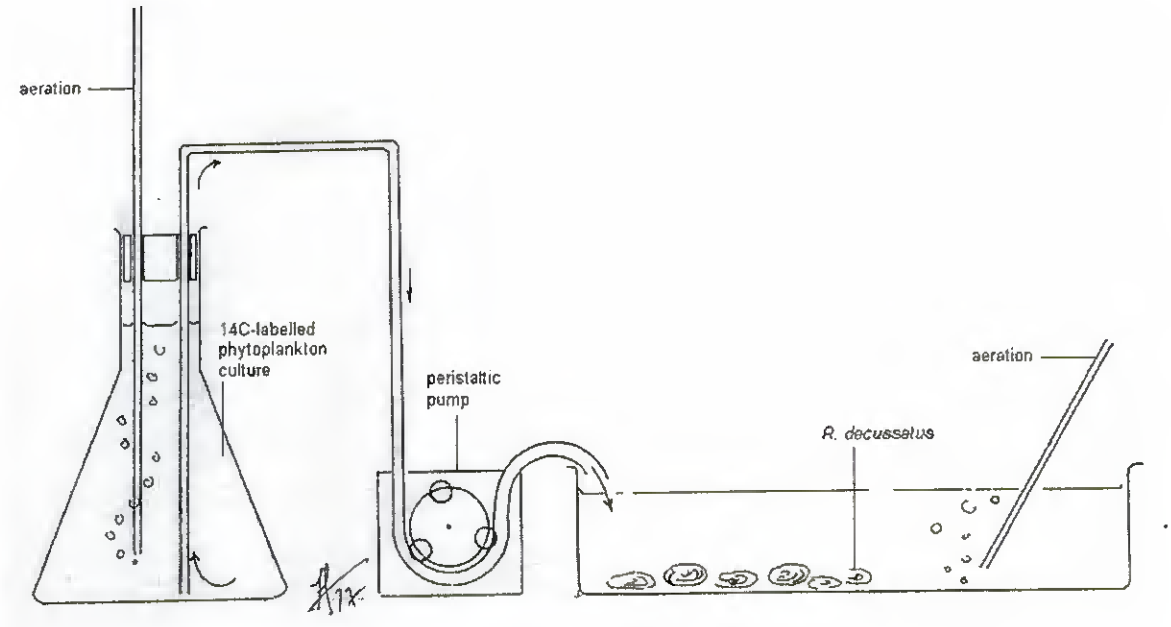

Figure 5.2- System utilised to feed adult clams *R.decussatus* with ^{14}C-labelled phytoplankton (*Isochrysis galbana*), using a peristaltic pump to provide continuous transfer of phytoplankton from the culture flask to the clam tank.

The daily amount of algae used as food for the clams, - equivalent to 1% of the clams dry weight (calculated on a dry weight basis for both algae

and clams), was considered an optimal cell concentration for adult clams (Widdows, pers.comm.).

Seawater from the tank was changed every 24 hours. Algal cultures and ^{14}C-TBT labelling of algal stocks were also maintained on a daily basis.

A set of 12 clams was sampled from the tank at days 10, 20, 40 and 60 and allowed to depurate for a further 24 h (to remove labelled material from the gut). Clams were dissected into gills, digestive gland and 'remaining tissues'. Similar tissue-types from 3 clams were pooled (usually 3 replicates) and homogenized. ^{14}C-organotin extraction and fractionation were carried out according to the scheme previously described for algae. The remaining clams at each sampling interval were analysed whole, according to the same procedure.

For total ^{14}C activity, an aliquot of approximately 0.2 g of homogenate was transferred to a scintillation vial and digested overnight with 1ml of tissue solubilizer (Optisolv). Subsequently, 14 ml of liquid scintillation cocktail (Optiphase-MP) were added before counting. For TBT/DBT fractions, another aliquot of the homogenate of approximately 0.5 g, (equivalent to 0.2 dry weight) was transferred into a glass stoppered centrifuge tube and 5 ml of HCl (37%) (BDH-Aristar) added. The tube was shaken and left for one hour, in order to release organotins from the tissues. Following the addition of 5 ml of hexane, the tubes were placed on an automatic shaker for 15 minutes and then centrifuged, at 3000 rpm, for a few minutes; five ml of distilled water were added to each tube and, after swirling briefly, the tubes were re-centrifuged, at 3000 rpm, for 5-10 min. This procedure results in a clear upper hexane layer (containing TBT and DBT). One milliliter of the hexane extract was transferred to a scintillation vial, 14 ml of scintillation cocktail (Optiphase-MP) were added and ^{14}C concentrations (TBT+DBT fraction) were determined. A further one ml of the hexane extract was washed with NaOH to remove DBT, allowing determination of ^{14}C-TBT, as described above.

Disintegrations per minute have converted to organotin concentrations based on the specific radioactivity of the compound (1 ng ^{14}C-TBTCl = 128 dpm). All ^{14}C-TBT and ^{14}C-DBT concentrations in algal and clam tissues, are subsequently expressed as tin equivalents, on a dry weight basis. Similarly,

^{14}C-TBT and ^{14}C-DBT concentrations in sea water are expressed as ng L^{-1} Sn. These conversions were made to allow direct comparisons with experimental exposures (*via* water and sediments) which employed stable organotin compounds (described in 5.2.1). Validation of the technique is described elsewhere (Langston *et al.*, 1994; Langston & Pope 1995).

5.3. RESULTS

5.3.1. Water and sediment as vectors of TBT uptake in *R. decussatus*

Organotin concentrations measured in the spiked sediments used in these experiments are presented in Table 5.1. There were no major changes in either TBT or DBT concentrations in sediments in either of the treatments, over the 60d period. The presence of TBT in the overlying water (at a concentration of 100 ng Sn l^{-1} - treatment c) appears to cause only minor increases in the TBT concentration of the contaminated sediment (nominally 0.8 µg Sn g^{-1}). This confirms anticipated predictions of partitioning behaviour, and supports the rationale behind the choice of experimental concentrations. The persistence of TBT in sediments is also demonstrated by the maintenance of constant TBT concentrations measured in treatment b) over 60 days, despite the fact that sediments were subjected to a continual exchange of overlying 'TBT-free' seawater. Throughout the experimental period, DBT represented a low proportion (<10%) of the total organotin burden (ΣTBT+DBT), indicating a low TBT degradation in sediments in both treatments (Table 5.1).

TBT accumulation patterns (µg g^{-1} dw) in the whole soft tissues of *R. decussatus*, exposed to nominal concentrations of 100 ng L^{-1} TBT (as Sn) in water and 0.8 µg g^{-1} TBT (as Sn) in sediments, separately or in combination, over 60 days, are presented in Figure 5.3.

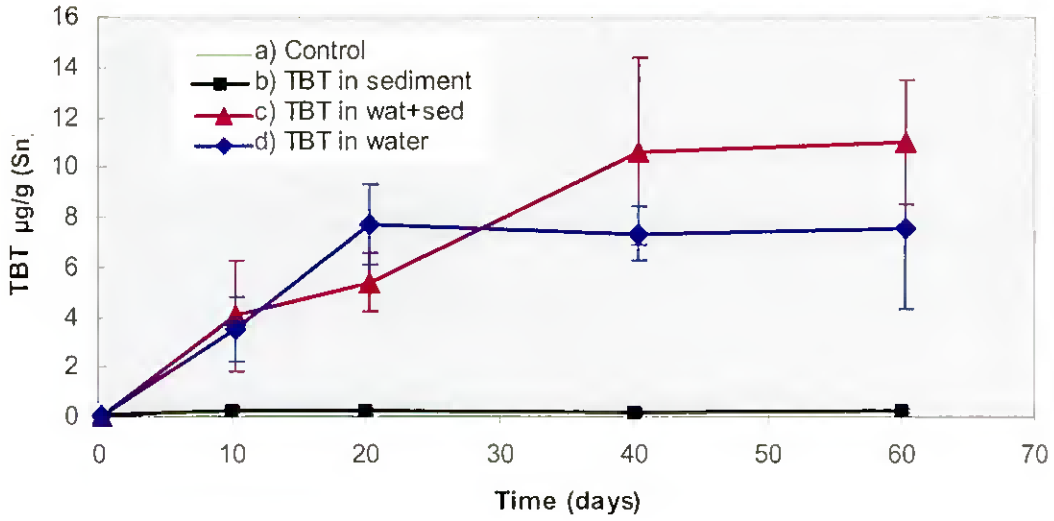

Figure 5.3 - TBT (µg g^{-1} Sn) in whole soft tissues of *R. decussatus*, exposed to this compound through the water (100 ng L^{-1} Sn) and sediments (0.8 µg g^{-1} Sn), either combined or separately (Data are means ± st.dev.)

Concentrations of TBT in clams from all TBT treatments were significantly different (p<0.05) than in the controls (< 0.15 µg Sn g^{-1}) indicating that at least some uptake occurs from both dissolved and solid phases.

Throughout the experiment, TBT accumulation in the whole soft tissues of clams exposed to TBT in water and in water and sediments combined, was significantly higher (p<0.05) than in the other two treatments (TBT in sediments only (b) and controls (a)), during the whole experimental period. This indicates that the water-column is the major source of TBT uptake to these suspension-feeding clams (Figure 5.3).

The rate of TBT uptake in clams exposed to TBT *via* water only was approximately 0.4 µg g^{-1} day^{-1} Sn. Thereafter body burdens approached steady state at ≈8 µg g^{-1} Sn. The Bioconcentration Factor ($BCF_{tissue÷water}$) for TBT in clams exposed to the dissolved contaminant, at steady state, i.e.,

$$BCF = \frac{\text{TBT concentration in the whole soft tissues of } R.decussatus \text{ (µg g}^{-1}\text{)}}{\text{TBT concentration in water (mg L}^{-1}\text{)}}$$

was calculated to be 9 x 10^4.

R. decussatus exposed to TBT *via* water and sediments in combination (treatment c), accumulated only slightly higher TBT concentrations in the whole soft tissues than those exposed to TBT *via* water only (Figure 5.3). Furthermore, clams in the former treatment appear to take slightly longer time to achieve steady state (30-40 days), perhaps reflecting a more protracted accumulation of the particulate TBT component (albeit a minor fraction). The rate of TBT uptake in these clams, up to day 40, was 0.25 $\mu g\, g^{-1}\, day^{-1}$ Sn.

The small contribution to body burdens from particle-bound TBT is confirmed for clams exposed to TBT *via* sediments only (Figure 5.3): *R. decussatus* exposed to TBT in sediments (treatment b) accumulated < 0.27 $\mu g\ g^{-1}$ TBT (Sn) during the whole exposure period - 30 times less than from contaminated water. Thus, clams exposed to TBT in sediments did not bioconcentrate TBT from this source ($BCF_{\text{tissue} \div \text{sediment}} < 1$).

DBT concentrations ($\mu g\ g^{-1}$ dw) in the whole soft tissues of *R. decussatus*, exposed to the various TBT treatments are presented in Figure 5.4.

Body burdens of DBT generally reflected accumulation patterns described for TBT, above, suggesting that the source of DBT was internal metabolism of the parent compound rather than accumulation from external media. DBT concentrations were highest in clams exposed to TBT in dissolved form, irrespective of the presence of TBT contaminated sediment. DBT levels in clams exposed to TBT in water (treatment d) and in water and sediments combined (treatment c), increased until day 40, to reach levels of 2.55 and 2.37 $\mu g\ g^{-1}$ Sn, representing rates of increase of 0.066 and 0.059 $\mu g\ g^{-1}\, day^{-1}$ Sn, respectively.

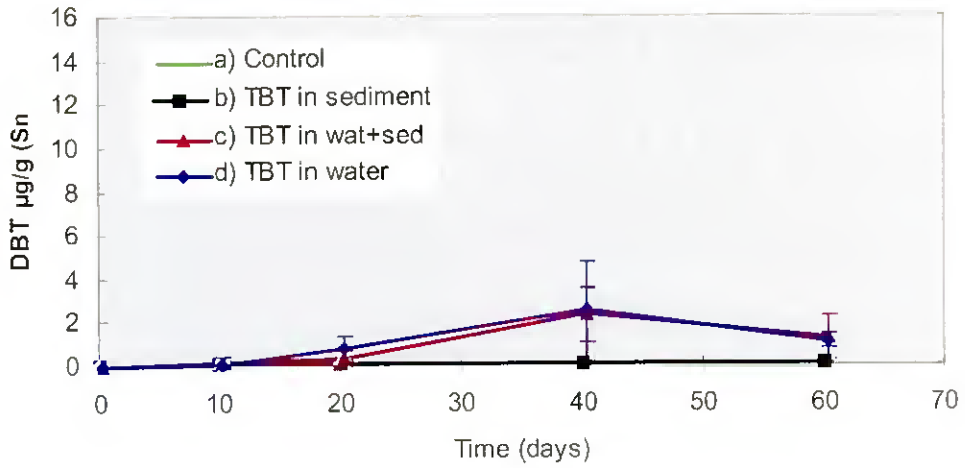

Figure 5.4- DBT (µg g^{-1} Sn) in whole soft tissues of *R. decussatus*, exposed to TBT *via* water (100 ng L^{-1}) and sediments (0.8 µg g^{-1}), either combined or separately. (Data are means ± st.dev.)

After day 40, DBT concentrations in clams from these two treatments, decreased to levels of about 1.2 µg g^{-1} Sn. DBT levels in clams exposed to TBT *via* sediment only were generally an order of magnitude lower than in those exposed *via* water, and were not significantly different from controls (p> 0.05) (Figure 5.4).

Throughout the 60 day experiment, DBT concentrations in the whole soft tissues represented a relatively small proportion (18±11%) of the total accumulated organotin burden (Σ TBT+DBT), across all treatments (Figure 5.5), indicating a consistently low rate of transformation from TBT. Thus, following the initial increase in TBT (and corresponding lowered proportion of DBT) in the early stages of the exposure (day 10), proportions of DBT returned to levels that were comparable to controls (17%) during the remainder of the experiment.

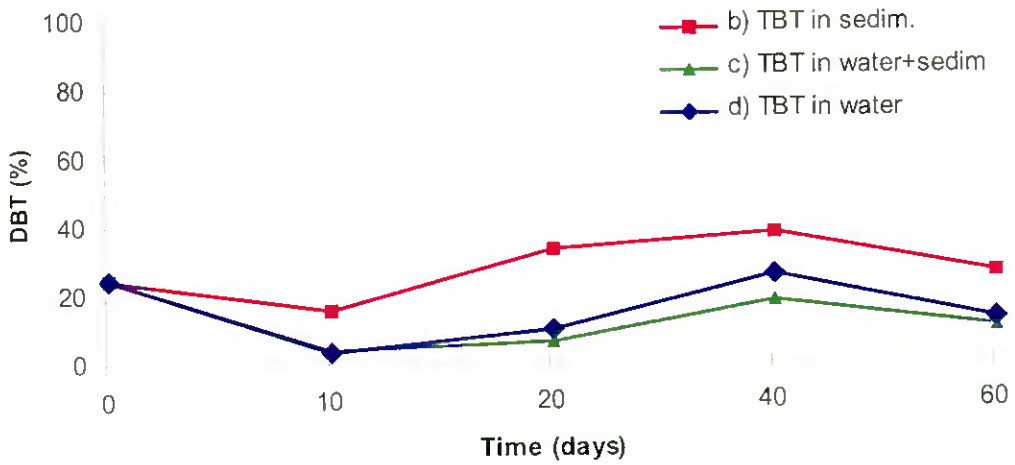

Figure 5.5 - Fraction of DBT (%) from the total organotin burden (Σ TBT+DBT), in the whole soft tissues of *R. decussatus* during exposure to TBT in b) sediments; c) water and sediments and d) water only (see Figure 5.1)

TBT concentrations measured in gills and remaining tissues of clams held in the different treatments, for 90 days, are shown in Figure 5.6.

The gills of clams exposed to TBT *via* water only, and *via* water and sediments simultaneously, (treatments d and c) accumulated significantly higher TBT concentrations than remaining tissues ($p<0.05$). The affinity for gills is consistent with the proposal that water is a major vector for TBT uptake in *R. decussatus* The fact that the TBT levels accumulated in the two tissues did not differ significantly ($p>0.05$) between treatments d) and c) indicates, again, that sediments have little influence on burdens when clams are exposed to both dissolved and particulate forms of contamination (as would normally be expected in the field). This is supported by results from clams exposed to TBT solely *via* sediments (treatment b), where tissues contained markedly lower TBT burdens than the other two exposures ($p<0.05$) (Figure 5.6).

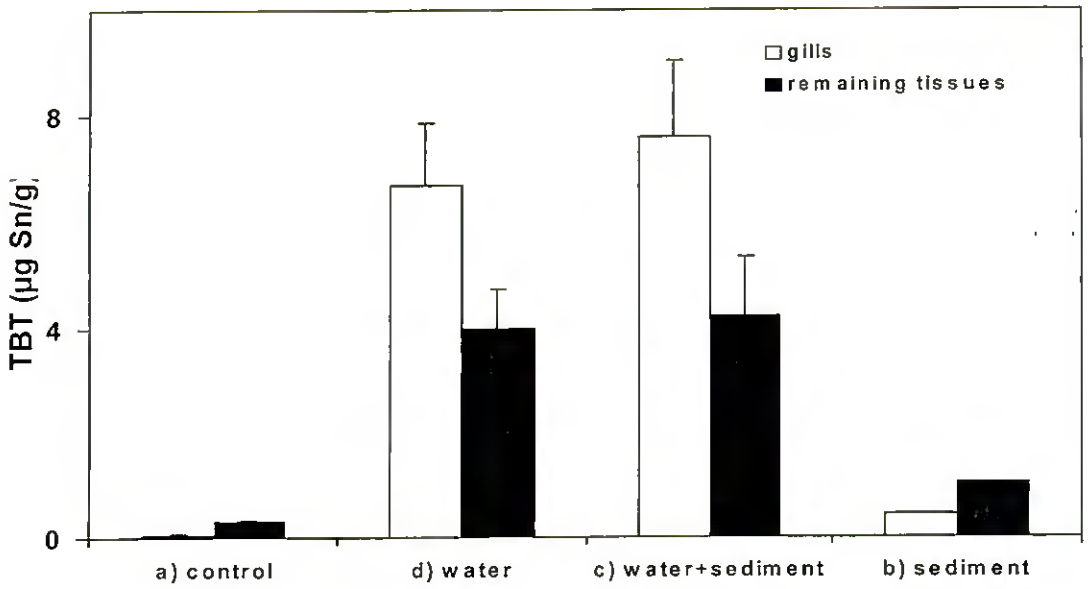

Figure 5.6 - TBT concentrations (µg g^{-1}Sn dw) in different tissues of *R. decussatus* exposed to TBT through distinct routes of exposure, for a period of 90 days. (Data are means + st.dev.)

Furthermore TBT concentrations in gills of these clams were if anything slightly lower than in remaining tissues, reflecting the absence of an aqueous uptake route.

The following section addresses the question of the relative importance of food (phytoplankton) as vector of TBT uptake, to *R. decussatus*.

5.3.2. Food (phytoplankton) as a source of TBT to *Ruditapes decussatus*

i) ^{14}C-TBT uptake by phytoplankton *Isochrysis galbana*

Uptake of TBT (ng g^{-1} dw) by the microalgae, *Isochrysis galbana*, exposed for 48h to 100 ng L^{-1} TBT (Sn) in water, is presented in Figure 5.7 (note logarithmic time scale).

After 10 minutes of exposure, a significant proportion of TBT was already associated with *Isochrysis*, representing an uptake rate of 13 ng g^{-1} min^{-1} Sn.

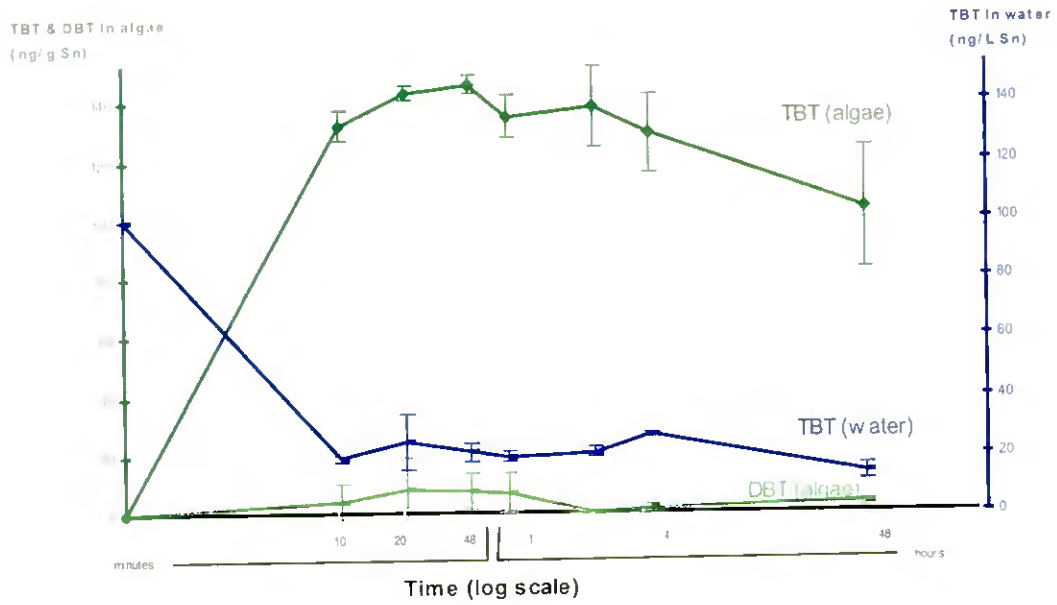

Figure 5.7- TBT and DBT in the algae *I. galbana* (ng g^{-1} Sn) and culture water (ng L^{-1} Sn) after exposure to ^{14}C- labelled TBT (100 ng L^{-1} Sn), for a period up to 48 hours (log scale) (Data are means ± st.dev.).

TBT bound to the algae continued to increase during the subsequent 30min period, reaching a mean concentration of 145 ng g^{-1} Sn. After this period, TBT levels in algae decreased slightly with an apparent 'loss' rate of 0.013 ng g^{-1} min^{-1} Sn (possibly due to metabolism), reaching a mean concentration of 100 ng g^{-1} Sn, after 48 hours of exposure.

Tributyltin concentrations in the water, during the experimental period, were inversely related to TBT burdens accumulated by *Isochrysis*, (Fig. 5.7). This result confirms that most of the TBT introduced into the tank (81%) became associated with algae, even after only 10 minutes.

The bioconcentration factor for TBT in *Isochrysis galbana*, calculated from water and algal tissue concentrations, at steady state (20 min), was;

$$BCF = \frac{\text{TBT concentration in algae } (\mu g\ g\text{-}1)}{\text{TBT concentration in water } (\mu g\ mL^{-1})} = 7 \times 10^{3}$$

Concentrations of DBT bound to the algae (Fig. 5.7) increased slightly during the first 20 min to reach a mean level of 8 ng g^{-1} Sn. Thereafter, DBT

did not increase further and remained at a consistently low level, compared with TBT concentrations (Fig. 5.7).

ii) *TBT uptake in the clams* R. decussatus *fed with* ^{14}*C-TBT labelled* I. galbana

^{14}C-TBT concentrations (µg g^{-1} dw) in the whole soft tissues of *R. decussatus*, fed continuously with ^{14}C-TBT labelled phytoplankton (^{14}C-TBT concentration in algae of approximately 140 ng g^{-1} Sn), over 60 days, are presented in Figure 5.8.

Ruditapes decussatus exhibited increasing TBT burdens during a period up to 40 days, equivalent to an uptake rate of 1 ng g^{-1} day^{-1} Sn. After 40 days, an apparent steady state was achieved at a mean ^{14}C-TBT concentration equivalent to 40 ng g^{-1} Sn (Fig. 5.8). Thus, the time taken for clams to reach steady state was similar to that observed in the water/sediment experiment (see 5.3.1.) following exposure to TBT in the water and sediment. Compared with the water route however, the rate of ^{14}C-TBT uptake from labelled algae was approximately 400 times slower. The BCF for ^{14}C-TBT (Bioconcentration Factor: (TBT in clams)/(TBT in algae)) of approximately 0.3 indicates that food chain biomagnification, from phytoplankton to whole clams, did not occur.

^{14}C-DBT concentrations in the whole soft tissues of *R. decussatus* (Fig. 5.8) increased throughout the experimental period, with an apparent "accumulation rate" of 0.4 ng g^{-1}·day^{-1} Sn - approximately 2.5 fold slower than TBT uptake. However, the relative contributions arising from: 1) TBT degradation within the clams and 2) assimilation of DBT from the small concentrations present in the diet (algae) during the experimental period, are not known .

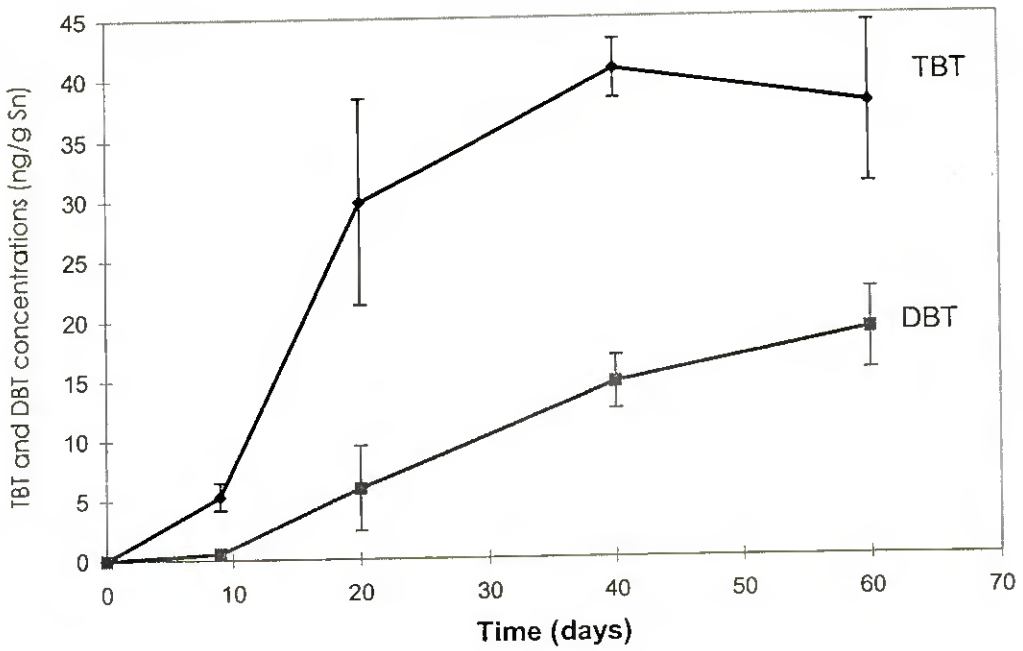

Figure 5.8 - TBT and DBT concentrations (ng g^{-1} Sn) in the whole soft tissues of *R. decussatus* exposed to TBT contaminated phytoplankton, for a period up to 60 days. (Data are means ± st.dev.)

Data for ^{14}C-TBT accumulation in individual tissues (gills, digestive gland and 'remaining tissues') of *R. decussatus*, throughout the 60d experiment are presented in Figure 5.9. ^{14}C-TBT concentrations in the gills increased up to 70 ng.g^{-1} at day 40 (uptake rate of 1.9 ng g^{-1}·day^{-1} Sn) and decreased thereafter to a mean burden of 45 ng g^{-1} Sn (Figure 5.9A). ^{14}C-TBT concentrations in the digestive gland increased most rapidly - up to 130 ng g^{-1} Sn at day 20 (uptake rate of 6.5 ng g^{-1} day^{-1} Sn) - but also decreased, subsequently, to a mean level of 40 ng g^{-1} Sn (an apparent 'loss rate' of 2.2 ng g^{-1}·day^{-1} Sn) (Figure 5.9B). In the remaining tissues TBT concentrations increased regularly during the whole experimental period with an uptake rate of 0.6 ng.g^{-1}·day^{-1} Sn. At the end of the experiment the mean concentration of ^{14}C-TBT in these tissues was 36 ng g^{-1} Sn (Figure 5.9C).

Clearly, the digestive gland presented higher ^{14}C-TBT burdens than the other tissues, until day 20 (Figure 5.9B). After 60 days of exposure, ^{14}C-TBT concentrations in the gills were comparable to those in the digestive gland.

The TBT increase in the pooled 'remaining tissues' (Figure 5.9C) was slower than in gills or digestive gland although this increase was maintained throughout the whole experimental period, such that after 60 days TBT concentrations were very similar in all tissues (Fig. 5.9A-C).

Nevertheless, an analysis of variance, using two-way factor ANOVA, Model I (fixed effects) (Zar, 1996) applied to ^{14}C-TBT data, showed a significant difference (p<0.05) in ^{14}C-TBT accumulation between all analysed tissues.

These results suggest different kinetics from other pathways (water and sediments). ^{14}C-TBT was, probably, first accumulated in the digestive tract, and may then be remobilized to other tissues. ^{14}C-TBT lost from the digestive gland between day 20 and 40 (58 ng g^{-1} Sn) was equivalent in scale to that accumulated in the gills and remaining tissues (54 ng g^{-1} Sn) over the same period (Fig. 5.9), possibly confirming this hypothesis.

^{14}C-DBT levels in the gills, digestive gland and remaining tissues, although lower than TBT, reflected similar patterns, generally, to that of ^{14}C-TBT accumulation (Figure 5.9). Thus, DBT in the digestive gland increased to a concentration of 19 ng.g^{-1} Sn, at day 40 (equivalent to an 'uptake' rate of 0.53 ng g^{-1} day^{-1} Sn) (Figure 5.9b). Subsequently, no significant changes in DBT levels were observed in this tissue. DBT in the gills increased linearly throughout the exposure period (rate of 0.23 ng g^{-1} day^{-1} Sn), reaching a concentration of 13 ng g^{-1} Sn, after 60 days (Figure 5.9A). A similar linear trend was observed in 'remaining tissues' (0.38 ng g^{-1} day^{-1} Sn) (Figure 5.9C).

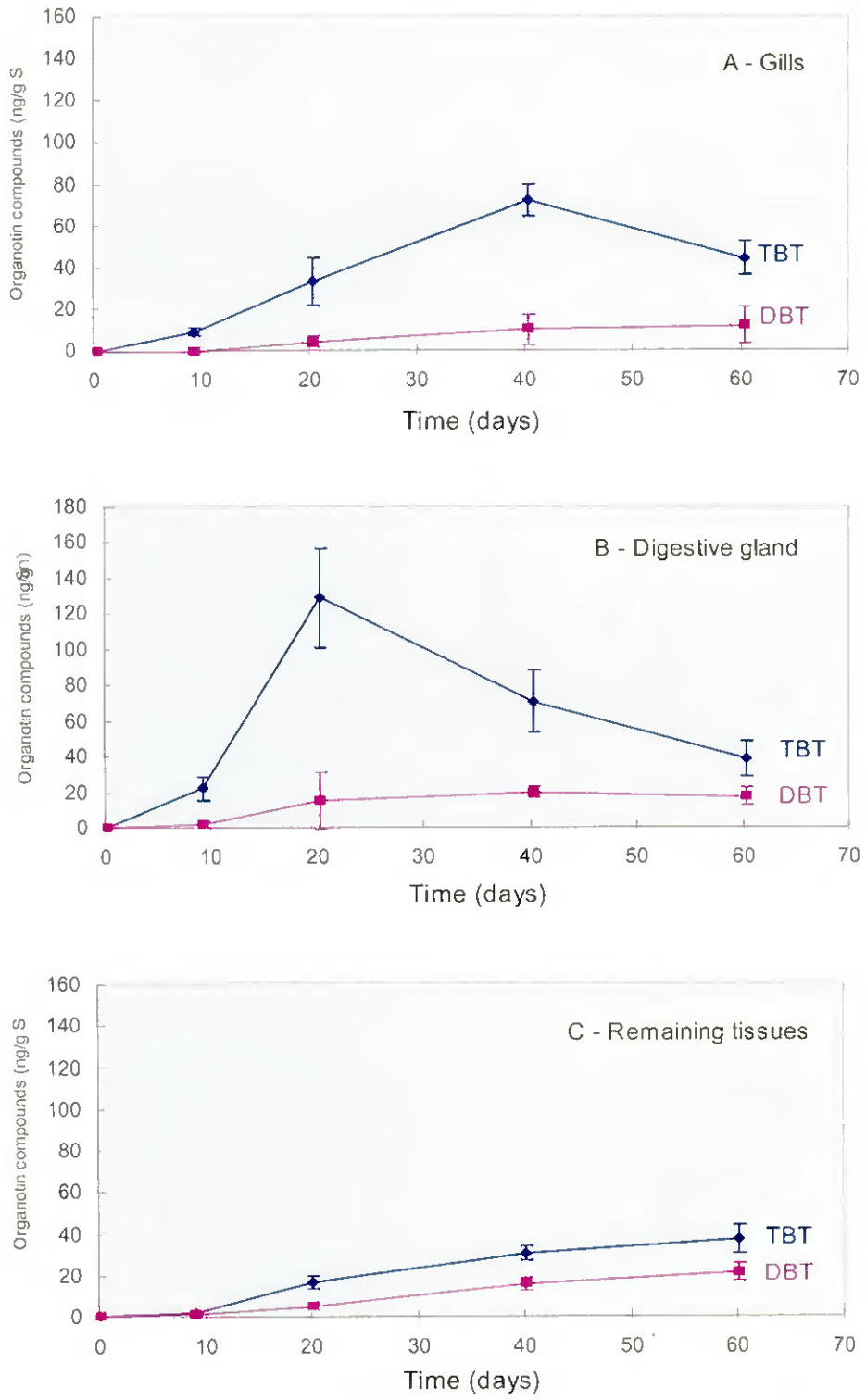

Figure 5.9 - TBT and DBT concentrations (ng g^{-1} Sn) in: (A) gills, (B) digestive gland and (C) remaining tissues of *R. decussatus*, exposed to contaminated phytoplankton, for 60 days (Data are means ± st.dev.).

Throughout the experiment, DBT and TBT concentrations in 'remaining tissues' increased proportionally, with the former perhaps reflecting metabolism of the latter (Fig. 5.10).

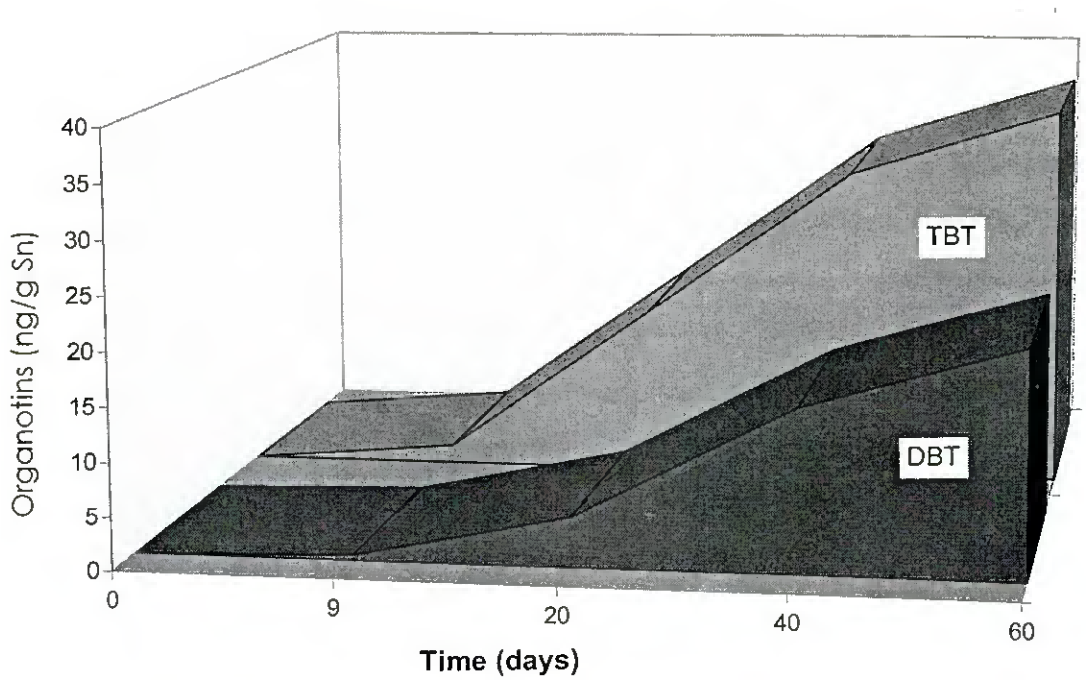

Figure 5.10 - TBT and DBT concentrations (ng g^{-1} Sn) in remaining tissues of *R. decussatus*, exposed to contaminated phytoplankton for a period up to 60 days.

Combining data from the water/sediment (5.3.1.) and the food (5.3.2.) experiments, a comparison of the TBT accumulated in *R. decussatus via* the three different routes of TBT uptake studied - water, sediment and food - is presented in Figure 5.11.

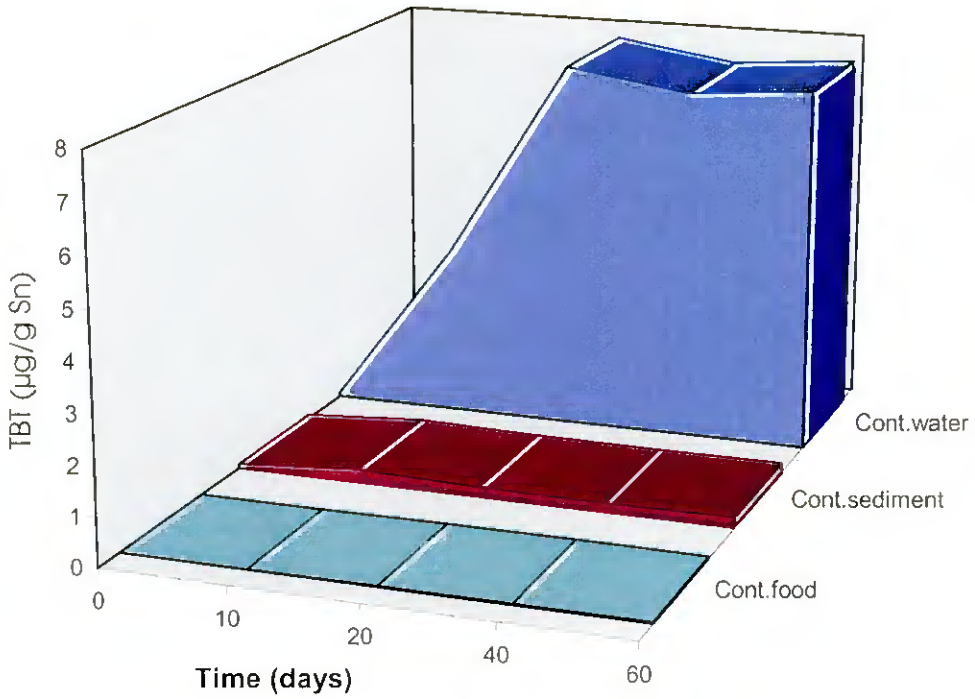

Figure 5.11 - TBT (µg g^{-1} Sn) in whole soft tissues of *R. decussatus*, exposed to this compound in water (100 ng L^{-1}), sediment (0.8 µg g^{-1}), and food, for 60 days.

A non-parametric Friedman Test (p<0.05) (Sokal and Rohlf, 1990), applied to the data, showed a significant difference in TBT accumulation among all exposure routes.

It seems clear that, under these experimental conditions, water is the dominant route of TBT uptake, although concurrently some bioaccumulation from sediment and food also occurred. Possible explanations for these results and how they might differ under natural conditions are discussed below.

5.4. DISCUSSION

From the results of the reported experiments, in which *R. decussatus* were exposed to TBT in water, sediments and food partitioned realistically, it seems clear that this species accumulates TBT predominantly from the water-column. Although some accumulation from sediments and food does occur, the contribution from these phases was negligible when compared to water, at least under the conditions and concentrations used in these experiments. In nature, provided that TBT partitioning between benthic sediments and overlying water are in quasi-equilibrium, the dissolved phase will determine residues found in populations of these clams.

1. Water as vector of TBT uptake to *Ruditapes decussatus*

Organotin accumulation has been demonstrated, using aqueous exposure usually, in a number of bivalve species including oysters, *C. gigas* (Shim *et al.*, 1998; Waldock *et al.*, 1983), mussels *M. edulis* (Laughlin & French, 1988), scallops *Pecten maximus* (Davies *et al.*, 1986) and clams, *S. plana* and *Mya arenaria* (Kure & Depledge, 1994; Langston & Burt, 1991). Comparison of uptake rates in these studies indicates that the time to reach steady-state (between water and tissue concentrations) varies according to exposure conditions and species. In an experiment carried out with *R. decussatus* exposed to TBT in water at concentrations between 4 and 2460 ng L^{-1} TBT, steady state was achieved after 40 days of exposure (Gomes-Ariza *et al.*, 1999). In *M. edulis* steady-state is achieved within 12 - 25 days at TBT concentrations ≤100 ng l^{-1}. For higher TBT concentrations (500-670 ng l^{-1}) there is no sign of saturation, even after 60 days of exposure (Guolan & Yong, 1995; Laughlin & French, 1988; Zuolian & Jensen, 1989). In contrast, TBT concentrations in oysters *C. gigas* and *O. edulis* exposed to TBT in water at both 'low' (150 ng l^{-1}) and 'high' (1.25 µg l^{-1}) levels of contamination attained steady state within 14 days (Waldock *et al.*, 1983).

In the present study, TBT concentrations in *R. decussatus,* exposed via water only, reached maximum values after 20 days exposure to 100 ng l^{-1} TBT (Sn), representing a final TBT bioconcentration factor of 9 x10^4. This is

similar to values cited for certain other suspension feeders. The "equilibration time" is, coincidentally, also of the same order as that described for dissolved metals, Cd and Cu, in *R. decussatus* (Sobral & Widdows, 1997; Bebianno, 1995), suggesting similar uptake kinetics may apply to different contaminants.

Although *R. decussatus* reached a steady state, this fact does not mean that an impairment on the physiological functions is not occurring, as demonstrated in studies on the scope for growth (SFG) of clams of this species exposed to Cu (Sobral & Widdows, 1997).

At steady state, clams exposed to TBT dissolved in water displayed a bioconcentration factor of 9 x10^4. This result agrees with reported BCF values for other suspension feeding bivalves such as mussels and other clam species (Table 5.2).

Table 5.2- Bioconcentration factors (BCF) reported for marine suspension feeder bivalves, exposed to TBT in the water.

Species	BCF	TBT conc. in water (ng L^{-1})	Reference
M. edulis	1300	0.023 0.045	Laughlin & French, 1988
O. edulis	1 000 1 500	1.25 0.15	Waldock & Miller, 1983
C. gigas	2 000 6 000	1.25 0.15	Waldock & Miller, 1983
M. edulis	5 000 50 000	0.50 * <0.10	Zuolian & Jensen, 1989
C. gigas	25 000	* <20 - 88	Shim *et al.*, 1998
C. edule	60 528	* 0.17	Bryan & Gibbs, 1991
M. balthica	67 258	* 0.17	
R. decussatus	**90 000**	*** 0.25**	**this study**
M. mercenaria	126 818	* 0.17	Bryan & Gibbs, 1991

* Converted from concentrations originally expressed as Sn.

The concept that bioaccumulation of organic contaminants is largely controlled by partitioning behaviour in lipids is a widely held tenet which has been extended to include organotins. Nevertherless, scrutiny of TBT bioconcentration factors in different taxonomic groups, and even within the same grouping, often reveals considerable variability - casting doubt that

these organometals obey conventional dogma. BCF for bivalves are often much higher than predicted values based on the K_{OW} coefficient (Hawker & Connell, 1986; Langston *et al.* 1987; Maguire, 2000). This suggests that lipid-partitioning is not the only factor responsible for accumulated TBT body-burdens. Chemical binding of TBT to different proteins (Davies *et al.*, 1986; Kim *et al.*, 1996; Laughlin *et al.*, 1986), varying involvement with metabolic pathways - particularly the MFO system (Fent & Stegeman, 1991, 1993; Sole, 2000) - and the concept of multiple compartments within the body, could help to explain the variability and discrepancy between predicted and observed BCF's for TBT in bivalves.

2. Sediments as a vector for TBT uptake

TBT concentrations accumulated by *R. decussatus* from sediments were lower (by more than a factor ten) than from contaminated water 'equilibrated' to an equivalent level of contamination. This primarily reflects the feeding style of the species. However, low rates of bioaccumulation of sediment-bound TBT could also result from either; a) limited bioavailability of sediment-bound TBT, or, b) a high rate of TBT degradation in *R. decussatus*. TBT bound to sediments is certainly bioavailable to deposit-feeding clams. In a series of experiments with *S. plana*, similar to those described here, accumulation of TBT was shown to be dominated (>90%) by uptake from sediments (Langston & Burt, 1991). Concerning the second possibility (b), a high rate of TBT degradation in *R. decussatus* seems unlikely, given the low tissue concentrations of DBT determined throughout. The suspension-feeding habit of *R. decussatus* therefore seems the most likely explanation for preferential uptake of TBT from water (>90%) and its low accumulation rate from benthic sediments. It would be interesting to compare and contrast TBT accumulating behaviour in a wider range of suspension and deposit feeders to confirm this.

As a consequence of the low TBT accumulation from sediments, clams *R. decussatus* did not magnify TBT from this source ($BCF_{sediment} \approx 0.3$). Similar results were described for freshwater mussels, *Elliptio complanata*

exposed to low TBT concentrations (66-110 ng g^{-1} Sn dw) in sediments (Chau *et al.*, 1989).

In marine surveys of TBT in suspension feeding bivalves (mussels and oysters) a lack of correlation with TBT concentrations in sediments appears to indicate that other sources of TBT, besides sediments, are more important (Wade *et al.*, 1990). The current results for *R. decussatus* point to the aqueous phase as the most likely route of uptake, though, again, further experimental evidence is required to examine in detail all potential sources.

Few studies have compared the relative importance of sediments and water as vectors of TBT uptake in bivalves. Deposit feeding clams *S. plana*, exposed to TBT in water and sediments either combined or separately, with the same concentrations used in this experiment, showed preferential accumulation from the particulate phase (Langston & Burt, 1991). In contrast, our results suggest that for suspension feeders, *R. decussatus*, the most important route of uptake is the aqueous phase, supporting the view that mode of nutrition is determinant in TBT accumulation by bivalves.

The current results (Figure 5.6) indicate that tissue distributions of TBT are determined by route of uptake. *R. decussatus* exposed to TBT *via* water only (treatment d) accumulated TBT preferentially in gills, as did clams exposed to TBT in water and sediments simultaneously (treatment c). In contrast, absorption of TBT from sediments (and food) led to a different TBT tissue distribution pattern, not dominated by gills (Figures 5.6 and 5.9).

3 - Food (phytoplankton) as a source of TBT to *R. decussatus*

i) ^{14}C-TBT uptake by phytoplankton *Isochrysis galbana*

The preliminary experiment in which chrysophytes, *I. galbana*, were exposed to ^{14}C-labeled TBT in water demonstrated that most TBT uptake (~80%) occurred in a brief period of 10 minutes. Judging by the rapid, saturation-type kinetics displayed in this experiment, the dominant mode of TBT uptake in *I. galbana* appears to be by adsorption of TBT to the outer surface of cells, at least initially. This rapid uptake of TBT by *I. galbana* is

probably related with the microorganism's large surface area to volume ratio (Fowler, 1985). Similarly, TBT uptake by cyanobacteria was shown to occur within the first 5 min of exposure to this compound in the water (Avery *et al.*, 1993).

In the present study, algae *I. galbana* exposed to 100 ng L^{-1} TBT (Sn) in water displayed a bioconcentration factor of $7x10^3$. In a similar accumulation experiment, using other species of phytoplankton, *Duniella salina* and *Duniella vivides*, BCFs were in the order of 10^4, after 7 days of exposure to 100 ng L^{-1}TBT in the water (Guolan & Yong, 1995). At higher concentrations (1 µg l^{-1} TBT) BCF values in *Dunaliella* sp. were 10^3 (on a wet-weight basis) (Guolan & Young, 1995).

As previously discussed for bivalves, the BCF obtained for algae, *I. galbana*, was also much higher than predicted from the octanol-water partitioning coefficient (K_{ow}) (240-370) (Mackay, 1982; Fent, 1996), implying that lipid partitioning alone can not explain TBT accumulation in algae. In fact, it has been suggested that *I. galbana* accumulates TBT by a specific saturable binding mechanism, whilst in other phytoplankton species, namely *Chaetoceros gracilis* and *Nanocloris* sp., hydrophobic partitioning as dominant mechanism of uptake (Chiles *et al.*, 1989). Saturable binding in *I. galbana* is thought to involve interaction of TBT with cell membrane components, although the nature of these components has yet to be identified. Differences in phytoplankton cell coatings (Leadbeater, 1994) and specific lipid composition (Jeffrey *et al.*, 1994) may explain distinct modes of TBT uptake (and loss) in phytoplankton.

In the present experiment, TBT burdens in algae decreased slightly between 2.5 h and 48 h. *Dunaliella percei* exposed to dieldrin have also been shown to loose a small proportion of the contaminant burden after 24 h of exposure (Petrocelli *et al.*, 1975). It was suggested that such decrease could be related to a growth dilution factor, due to production of new phytoplankton cells. This could also be the case for TBT and *I. galbana* in the present study. O Alternatively, a decrease in TBT concentrations could also be linked to metabolism in the algae. Small amounts of DBT were detected in the algae after 10 min, which increased up to 20 min. Thereafter, DBT concentrations

appeared to have reached a steady state - at concentrations approximately 20 fold lower than TBT - an indication that perhaps formation and degradation were proceeding at similar rates. Direct assimilation of DBT from water is unlikely since very little radio-labelled DBT was detected in the water.

Thus, although TBT biodegradation does appear to be initiated rapidly (see also Mensink *et al.*, 1997), compared to other phytoplankton e.g. green algae (*Dunaliella* sp.), diatoms (*Skeletonema costatum*; *Chaetoceros curvisetus*) and dinoflagellates (*Prorocentrum triestinum*), *I. galbana* may have limited capability to break down TBT (Guolan & Yong, 1995; Lee *et al.*, 1989). Different mechanisms of uptake and binding, discussed above, may be part of the explanation though further experiments would be useful to characterize the nature and rates of organotin metabolism in phytoplankton. Nevertheless, in the context and time span of bivalve feeding experiments, it is clear that *I. galbana* cells are a much more important dietary vector of TBT - rather than DBT - for clams. TBT biodegradation does appear to be initiated rapidly in *Isochrysis* and may contribute to the lower BCF compared with clams.

ii) *TBT uptake in the clams* R. decussatus *fed with* ^{14}*C-TBT labelled* I. galbana

R. decussatus fed continuously with ^{14}C-TBT labelled phytoplankton, accumulated increasing TBT burdens in their tissues, over time. However, accumulation was estimated to be an order of magnitude lower than from TBT-spiked sediments (0.8 ng g^{-1} TBT as Sn) and two orders of magnitude lower than direct accumulation from water containing 100 ng l^{-1} TBT (as Sn).

Only a few studies have been performed previously on the assessment of food as vector of organotins uptake in marine bivalves. Mussels *M. edulis* fed TBT-dosed *I. galbana* for 30d, accumulated TBT continuously throughout the whole exposure period: however, the exposure concentration used to label algal cells (2.5 µg l^{-1} TBT) was exceptionally high (Laughlin *et al.*, 1986). Although using lower TBT exposure concentrations to label algae (100 ng l^{-1}),

our results with *R. decussatus* indicate the approach of steady-state in whole clams after 40 days exposure, though some individual tissues may take longer (Figure 5.9).

Despite lower tissue burdens, *R. decussatus* exposed to ^{14}C-TBT labelled phytoplankton displayed similar uptake characteristics as clams exposed to TBT *via* water – i.e. an initial linear uptake phase and a similar time to reach steady state. Since TBT-labeled algal cells were 'equilibrated' at 100 ng l^{-1} TBT - the same concentration used to evaluate TBT uptake in *R. decussatus via* water - it is presumed that the faster TBT uptake rate in the latter clams reflects the greater bioavailability of TBT in the aqueous phase.

However, although uptake of TBT from phytoplankton appears to be much slower than from water, it is possible that the microalgal food supply to the clams was limiting in these experiments. In nature, algal densities can be considerably higher in certain conditions and at specific times, and may, accordingly, contribute significantly more to TBT body burdens in clams. Further studies with different quantities and qualities of food supply should be performed to better understand the role of food as vector of TBT uptake in *R. decussatus*.

Our studies have demonstrated that dietary assimilation of TBT does occur, but probably this represents a small proportion of the TBT available to clams (compared with dissolved TBT), particularly in areas where algal productivity is low. However, at very high phytoplankton densities most of the TBT in the water column is likely to be bound to algal cells and could represent a larger vector for TBT uptake.

A limited food supply may also explain why TBT uptake rates in these experiments were lower than from benthic sediments. Slightly higher TBT concentration in sediments (0.8 µg Sn g^{-1}) and different binding characteristics, compared with those in phytoplankton (0.14 µg Sn g^{-1}) could also be contributory factors, though modes of uptake are expected to be similar for these two sources. According to Scharp (1991), if sediment particles represent a dietary source for the organism, these will be digested, as food, in the digestive tract and uptake will occur directly from particles. Data obtained in our experiments appears to confirm this hypothesis, since an

equivalent outcome in terms of bioconcentration factors was observed in clams exposed to TBT in sediments and food (both BCF ~0.3). The observation, at the end of the experimental period (day 90), that clams exposed to TBT in sediments presented lower TBT burdens in gills than in other tissues substantiates this hypothesis.

However, more evidence is needed concerning possible differences in TBT binding intensities and assimilation efficiencies from sediments and algae (Fisher & Wang, 1998), as well as distinct metabolisms involved in these processes.

At higher trophic levels, uptake of organotin compounds from diet has been demonstrated in marine organisms. Red sea bream, *Pagrus major*, fed with TBT-contaminated artificial food, accumulated TBT in tissues and reached a steady state after approximately 30 days of exposure (Yamada *et al.*, 1994). In a short term experiment, crabs, *Rhithropanopeus harrisii*, were exposed to TBT in water and food, although no steady-state was achieved, probably due to the brief period of exposure (6 days) (Evans & Laughlin, 1984). Dog-whelks *Nucella lapillus* fed on ^{14}C-TBT labelled *M. edulis* also displayed bioaccumulation of TBT, attaining a steady state after approximately 15 days of exposure (Bryan *et al.*, 1993a).

Clams exposed to TBT *via* contaminated phytoplankton did not exhibit biomagnification of this compound, confirming the results obtained for phytoplankton-*M. edulis*, *M. edulis*-*N. lapillus*, *M. edulis* – polar seastar and fish-Steller sea lion food chains (Bryan *et al.*, 1989; Laughlin *et al.*, 1986; Guolan & Yong, 1995; Kim *et al.* 1996; Békri & Pelletier, 2004). It would seem that dietary TBT residues are unlikely to biomagnify along marine food chains.

iii) *Accumulation of TBT within the tissues of* R. decussatus

The route of uptake may be reflected in tissue distributions of TBT. *R. decussatus* exposed to TBT in water contained higher TBT burdens in gills than in other tissues, as did clams exposed to TBT in water and sediments

simultaneously. In contrast, absorption of TBT from sediments and food led to a different TBT tissue distribution pattern. The ranking of tissue burdens in *R. decussatus* exposed for 40d to contaminated phytoplankton was; digestive gland > gills > remaining tissues. Similarly, exposure to contaminated sediments resulted in lower TBT accumulation in gills relative to other tissues.

The presence of elevated TBT levels in the digestive gland, following dietary exposure *via* contaminated food is to be expected. Similar results were observed in other molluscan species fed TBT contaminated phytoplankton (Laughlin *et al.*, 1986; Mensink *et al.*, 1997). Like other particles in suspension, microalgae enter the organism through the siphon, and are carried by ciliary filaments in the gills, towards the mouth, and, finally, into the digestive tract. Thus, if TBT associated with phytoplankton is assimilated, it will probably be desorbed, partly, from algal cells in the stomach, and subsequently accumulated in the digestive gland as a consequence of extra- and intra-cellular digestion (Mensink, 1996; 1997).

After 20 days of exposure to labelled phytoplankton, TBT concentrations in the digestive gland exhibited an apparent decrease, possibly due to a combination of: (1) saturation of uptake sites, (2) stimulation of degradation products, (3) remobilisation to other tissues. Scallops, *Pecten maximus*, exposed to TBT in water, also displayed a biphasic uptake pattern, with gills/mantle and digestive gland containing higher burdens than remaining tissues, initially, followed by a relative decrease thereafter (Davies *et al.*, 1986). Transfer between tissues is thought to influence kinetics, following the initial stages of uptake and is manifested in scallops, for example, by increasing levels of TBT in adductor muscle.

The same biphasic uptake pattern was also observed for the fresh water bivalve *Dreissena polymortha* (Van Slooten & Tarradellas, 1994). In the present study, TBT concentrations in the remaining tissues of *R. decussatus* increased gradually during the whole exposure period, perhaps confirming internal remobilisation of TBT from the digestive gland, and to a lesser extent from the gills.

Studying different TBT uptake pathways in mussels *M. edulis* and clams *Mya arenaria,* Laughlin *et al.* (1986) and Kure & Depledge (1994)

concluded that if uptake was mainly from water, concentrations in gills would be the highest of all tissues, followed by viscera (digestive tract and gonads) and other tissues (muscles and mantle). If TBT uptake was predominantly by ingestion of contaminated phytoplankton, then viscera would display the highest accumulation followed by gills and other tissues. Our experiments, with *R. decussatus*, led to similar tissue distributions, supporting this hypothesis.

Assimilation pathways and tissue partitioning have been studied in a variety of other marine organisms, and for several organotin compounds. Red sea bream *Pagrus major* exposed to triphenyltin (TPT) in food accumulated highest TPT concentrations in the liver and digestive tract, followed by gills and other tissues (Yamada *et al.*, 1994). With direct uptake from water, highest TPT burdens were observed in the liver followed by gills, digestive tract and other tissues. The major difference between assimilation routes was that the TPT concentration in the gills was elevated following direct uptake from water (Yamada *et al.*, 1994). As in *R. decussatus*, dietary uptake of organotins in these fish was less significant than from solution. Crabs, *Rhithropanopeus harrisii*, exposed to TBT in water and food (*Artemia salina*) assimilated TBT most rapidly from water. The gills and carapace accumulated TBT burdens preferentially, from the aqueous source, whilst ingestion of contaminated food led to a higher concentration of TBT in the digestive tract (Evans & Laughlin, 1984). Neogastropods *N. lapillus* fed on ^{14}C-TBT labelled *M. edulis* also absorbed dietary TBT more efficiently *via* the digestive gland; conversely, absorption of dissolved TBT in *N. lapillus* was observed primarily in the mantle and gills (ctenidium) - tissues in direct contact with the water (Bryan *et al.*, 1993a). Differences in tissue butyltin accumulation were also observed in the steller sea lion (*Eumetopias jubatus*). Individuals caught in the field - presumably exposed to organotins *via* food - showed a higher TBT accumulation in the liver and hair, confirming that uptake from food is linked to higher burdens in the digestive tract (Kim *et al.*, 1996).

Although clams, crabs, gastropods and fish present different modes of nutrition, there appears to be an analogous relationship between uptake from

water and a high gill burden as well as uptake from food and high digestive tract burdens.

Results for *R. decussatus* are therefore consistent with other marine organisms in that tissue distributions are a function of assimilation pathway. Gills and digestive tract will initially accumulate TBT, preferentially, from water and food, respectively. After a few weeks of exposure, internal remobilization assumes greater significance and, superficially, TBT distributions reflect the net result of a combination of partitioning processes. However, further studies are needed to fully understand the remobilization and degradation mechanisms of TBT in molluscs.

FINAL REMARKS

Although the present study clearly demonstrates that the predominant uptake route for TBT in *R. decussatus* is from water, it should be born in mind that in the environment many abiotic and biotic factors act together to modify the relative importance of different uptake pathways. Two of these factors should be emphasised: (1) the relative concentrations of TBT in different compartments of the environment and (2) the quality and relative abundance of food available for ingestion. Considering the non-homogeneous distribution of organisms in the sea (especially phytoplankton) and the wide range of TBT concentrations and partioning behaviour that occur in the environment, it is highly probable that the relative importance of each pathway will not be constant. The relative contribution of each route will depend upon the prevailing ecological and physiological conditions and will thus vary, spatially and temporally. Extrapolation of laboratory results to the natural environment should therefore be undertaken with caution.

Feeding habit is of obvious importance in terms of determining which TBT pathway poses most risk to bivalves. According to our results, water is shown to be the major vector of TBT uptake in *R. decussatus*, and thus the critical pathway to consider. In applying criteria such as Environmental Quality Standards (EQS) to TBT for management purposes, water appears to be the appropriate media to set regulations for the protection of *R. decussatus*. For

other species, such as deposit-feeding clams sediment quality guidelines would be more appropriate (Langston & Burt, 1991; Midorikawa *et. al.*, 2004). Future trends in contamination, whether the result of, for example, altered industrial practices, dredging and disposal of spoils, or legislative action, may well see changes in the relative importance of sediments, food and water, as media for bioaccumulation. A wider knowledge base on assimilation pathways would help environmental managers in the selection of the most relevant regulatory criteria.

Chapter 6

ASSESSMENT OF TBT LEVELS IN SOUTHERN PORTUGAL (Field survey)

Coelho, M.R.; Bebianno, M.J. & Langston, W.J. (2002c). Organotin levels in the Ria Formosa lagoon, Portugal. *Appl.Organometal.Chem.* **16**: 384-390

ABSTRACT

Organotin concentrations were measured in water, sediments and clams (*Ruditapes decussatus*) from 11 sites in the Ria Formosa lagoon, Portugal, in 1992-1993. Results showed a marked spatial pattern of tributyltin (TBT) and dibutyltin (DBT) concentrations. The highest organotin concentrations were observed at Olhão (site 5), where the most important fishing harbour of the Southern coast of Portugal is located.

Results indicated that fishing vessels, moored in the harbour at Olhão (site 5), were the major source of organotin contamination to the lagoon. No significant seasonal trend was observed, suggesting a continuous input of organotin compounds throughout the year. In several areas of the lagoon TBT burdens in *R. decussatus* could have deleterious developmental effects.

6.1. INTRODUCTION

Extensive assessments of TBT contamination in bivalves have been performed in several coastal areas worldwide; however data on TBT burdens in Portugal are scarce. Therefore a survey on TBT burdens seemed crucial to assessing the potential hazards and implications for human consumption in a commercially important species, such as the suspension-feeding clam *Ruditapes decussatus* (Linnaeus, 1758).

In order to assess the possible environmental risk from TBT in coastal waters of Southern Portugal (particularly to clams *R. decussatus*), a pilot monitoring programme has been undertaken in one of the most important and sensitive areas, the Ria Formosa Lagoon. The objective was to measure the contaminant in key components of the system, particularly water, sediments and biota (clams).

6.1.1. Study area : Ria Formosa

The Ria Formosa lagoon, also known as Ria de Faro, is a shallow coastal lagoon located in the south of Portugal, extending from 7° 32' W to 8° 2' W of longitude and from 36° 58' N to 37° 3' N of latitude.

Seaward, it is limited by a non-continuous belt of sandy dunes formed by two peninsulas (Ancão e Cacela) and five barrier islands, which are, from west to east : Barreta, Culatra (or Farol), Armona, Tavira and Cabanas. These islands separate the lagoon from the Atlantic Ocean. Six inlets allow exchange of the water with the sea - Barra do Ancão (or Barra de S. Luís), Barra de Faro, Barra da Armona, Barra da Fuzeta, Barra de Tavira e Barra de Cabanas (or Barra de Cacela). The mesotidal lagoon extends for about 55 km (E-W) and is about 6 km at its widest point, enclosing an area of approximately 84 km^2 (Andrade, 1990; Neves *et al.*, 1994). The entire water-body is sheltered with an average depth of 2m (Andrade, 1990). Ria Formosa includes different habitats, such as salt marshes, mud flats, sand banks and dunes interspersed by a branched system of channels, some of which are navigable (Bebianno, 1995). The tides are semidiurnal with amplitudes that range from about 0.7 m (neaps) to about 3.5 m (springs). Daily, in the outer regions of the Ria Formosa, most of water is exchanged between the lagoon and the ocean and there is a possibility of exchange of about 50 to 75% of the water between the lagoon and the ocean. Only a small fraction (14%) of the lagoon is permanently immersed and approximately 80% of the total area is uncovered, at least during spring tides (Andrade, 1990). The lagoon does not receive any significant freshwater input, except the Gilão river, and consequently salinities range between 35.5 to 36.9 all year round (Falcão & Vale, 1995). Water temperatures are in the range of 12 - 13° C in winter and 27 - 28° C in summer (Chicharo & Chicharo, 2001).

Owing to its significance as a wetland, conservation area and ornithological importance, the Ria was designated as a Portuguese Natural Park, in 1987 (CCRA, 1984).

Its high nutrient concentrations and productivity (Falcão & Vale, 1988; Mudge & Bebianno, 1997; Newton, 1995) give rise to an important diversity and abundance of flora and fauna. For many aquatic species it constitutes an important spawning and nursery ground owing to its sheltered conditions. Moreover, the combination of hydrographic factors and the nature of the

substrate (predominantly sand and silt), constitute ideal conditions for the development of benthic communities (Austen *et al.*, 1989).

Fisheries (bivalves and fish) are the main activities in the lagoon, which has great potential for aquaculture development. The productivity of the Ria is evident from the abundance and diversity of the flora and fauna, and the yields from fisheries. The Ria is a nursery and breeding ground for many aquatic species. The productivity of the Ria is evident from the abundance and diversity of the flora and fauna, and the yields from fisheries. It has a long tradition of bivalve harvesting, especially of *R. decussatus* (90% of Portugal's mollusc fishery is harvested here) (Chicharo & Chicharo, 2001). Other species of significance include *Ruditapes romboides*, the thick trough shell *Spisula solida*, the common cockle, *Cerastoderma edule* and oysters, *Crassostrea angulata* and *Ostrea edulis* (Muzavor, 1991).

Around 20% of the total area of Ria Formosa is occupied by on-growing banks of clams *Ruditapes decussatus* that are cultured throughout the entire lagoon, being the most important commercial species in the area (Cachola, 1996).

Records indicate that in 1891 a total area of 44 m^2 of the lagoon was licensed for clam exploitation and in 1899, this activity was already legislated. By 1996, a total of 1587 clam plots were identified, occupying around 47 Km^2. Most of the plots (75%) are located in the area of Olhão; 17% in the Faro region and approximately 8% in Tavira (Cachola, 1996). The annual harvest of this bivalve approached 8000 tons in 1993. However, more recently there has been a decrease in production, to about 3000 tons/year (DGPA/INE, 1998). Average bivalve mortality in the last decade has been estimated at ≈50%, although reliable mortality data are not available. The mean bivalve production is currently estimated in 0.5 kg/m^2 whereas some years ago it reached ≈3 to 4 kg/m^2 and even 7 kg/m^2 in places (Cachola, 1996; Mudge & Bebianno, 1997).

6.1.2. TBT survey in Ria Formosa

Water quality in the lagoon is thought to have deteriorated over the last years due mainly to uncontrolled economic development. Domestic sewage

discharges (treated and/or poorly treated) from a population of ≈150 000 people, industrial discharges, agricultural drainage and aquaculture effluents are the major direct (non atmospheric) pollution inputs along the Ria Formosa. The high number of boats present also has an important contribution to the poor water quality. Boat traffic is largely dominated by small leisure and fishing boats. However, large commercial and fishing vessels also call into the main harbours (Olhão and Faro) (Figure 6.1). In fact, the main channels between the mouth of the lagoon and these two ports are the only navigable channels for large vessels.

Considering the high boating activity in the lagoon, an assessment of the contamination of organotin compounds leached from antifouling paints and associated risks for bivalves seemed important. *R. decussatus* is a benthic suspension feeder, and thus is a potential bioaccumulator of pollutants, especially lipophilic compounds such as TBT. As an important edible species these clams may also constitute a potential risk for human consumption. Thus, as part of the assessment, the present chapter reports the results of a survey of organotin compounds in water, sediments and clams (*R. decussatus)*, carried out in the Ria Formosa during 1992-1993.

6.2. MATERIALS AND METHODS

6.2.1. Sampling program

Measurement of the degree of contamination by TBT required the establishment of a field data set, covering the whole Ria Formosa. Eleven sites in the lagoon were sampled on each of four occasions: in winter (1992), spring, summer and winter (1993). Sites were selected from the lagoon's western border at Praia de Faro (1) to its eastern end, at Cacela (11) (Fig. 6.2 and Table 6.1),

Table 6.1- Sampled sites in Ria Formosa lagoon (see Figure 6.1)

Site	Location
1	Praia de Faro
2	Ilha do Farol
3	Ilha da Culatra
4	Canal de Olhão
5	Olhão (next to port)
6	Ilha do Côco
7	Marim
8	Torre D'Aires
9	Tavira (4 Águas)
10	Tavira (Forte do Rato)
11	Cacela Velha

Most of the sampled sites were located in clam culturing plots. Additionally, Praia de Faro (1), Ilha da Culatra (3), Tavira (4 Águas) (9) and Cacela (11) are sheltered areas where a considerable number of small leisure vessels are usually moored. Sampling site Olhão (5) was located next to the most important fishing port in the region of Algarve, in terms of number of boats and fishing products delivered (approximately 13×10^3 tons per year). Although there are a considerable number of large fishing boats, the vast majority are small (< 25 m in length) vessels.

Figure 6.1- Map showing sampling sites in the Ria Formosa lagoon

6.2.2. Collection and treatment of samples

In order to assess possible seasonal trends in TBT contamination in the lagoon, sampling was carried out in winter, spring and summer conditions. In 1992-93, samples were collected in four different periods: November-December 1992 (winter 92), May 1993 (spring 93), August 1993 (summer 93) and December 1993 (winter 93).

Samples of water, sediment and molluscs (where available) were collected for TBT and DBT analysis on low spring tides, at each location. The methods used for sample processing were those described by Ward *et al.* (1981) and Bryan *et al.* (1986), addressed in Chapter 2 and summarised below.

i) Water

Water samples were collected in 1L glass stoppered bottles as described in Chapter 2 (2.1.1). Concentrations of TBT were determined in unfiltered sea water samples by the method described in Chapter 2 (2.2). Results of TBT concentrations in water are expressed as Sn.

ii) Sediment

Surface sediment samples were collected and kept frozen (-20° C), until subsequent extraction and analysis (see Chapter 2 (2.1.3)). TBT and DBT concentrations were determined by the methods described before (Chapter 2 (2.2)). Results of TBT and DBT concentrations in sediments are expressed as Sn, on a dry weight basis.

iii) Molluscs

Usually, exclusively shellfish farmers carry out collection of *R. decussatus* in clam beds. Thus, samples of approximately 20 clams were kindly offered by farmers at all sampled sites except, Tavira (Forte do Rato) (site 10) and Cacela Velha (site 11) where no clams were found. Bivalves

were transported alive to the laboratory in iceboxes. Bivalves were then depurated in filtered sea water, for 48 hours, to empty their gut contents and avoid interferences from sediment contamination (Bryan *et al.*, 1985). Three replicates of samples of 6 pooled animals were selected and kept frozen (-20°C) until further analysis. Organotin (TBT and DBT) extraction and determination was performed in the whole soft tissues of bivalves using the methods described earlier (see Chapter 2 (2.1.2 & 2.2)). Results of TBT and DBT concentrations in bivalve tissues are expressed as Sn, on a dry weight basis.

6.3. RESULTS

6.3.1. TBT in water

The TBT concentrations in water samples from the Ria Formosa are shown in Table 6.2 and Figure 6.2.

Table 6.2- TBT concentrations (ng L^{-1} Sn) in water samples from the field survey in Ria Formosa.

Site	Winter 1992	Spring 1993	Summer 1993	Winter 1993
1 - Praia Faro	6.2	5.4	<1	1.4
2 - Ilha Farol	3.4	5.8	5.3	n.s.
3 -Ilha Culatra	3.2	5	6.3	4.2
4 - Canal Olhão	5.4	8.6	7.0	3.1
5 - Olhão	33.8	5.8	3.1	12.1
6 - Ilha Côco	2.8	4	18.6	2.0
7 - Marim	8.4	3.4	2.0	<1
8 - Torre D'aires	1.2	4.8	<1	2.0
9 - Tavira (4 águas)	3	5.2	1.7	1.5
10 - Tavira (Forte Rato)	3.2	3	<1	2.5
11 - Cacela	2	5	2.3	2.2

n.s.- not sampled

A distinct spatial pattern in TBT concentrations in water from Ria Formosa was evident in winter and in summer (Fig. 6.2). Maximum concentrations were detected in Olhão (5) (33.8 ng L^{-1}) and Ilha do Côco (6) (18.6 ng L^{-1}), respectively. However, concentrations at these sites were not significantly different (p<0.05) from the remaining sampled sites, as shown by a non-parametric statistical test - Kruskal-Wallis Anova (Rice, 1995) - applied to the data.

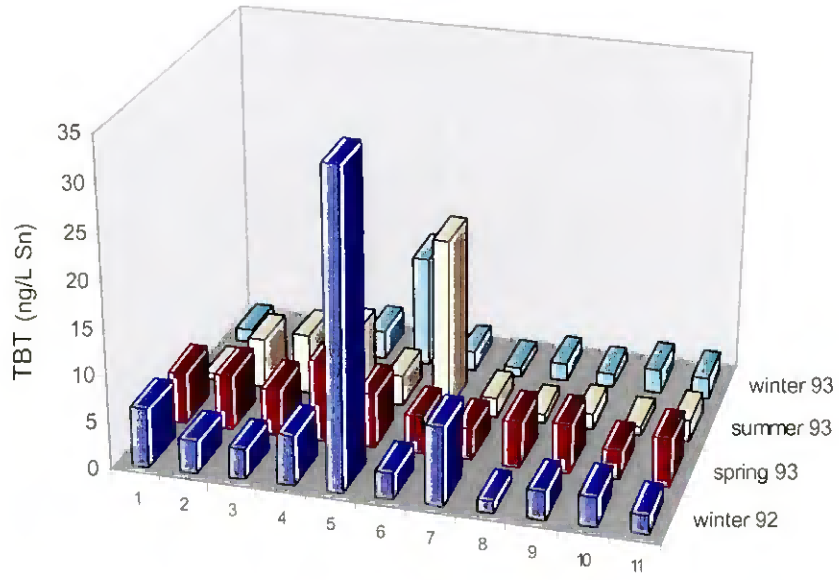

Figure 6.2- TBT concentrations (ng L^{-1} Sn) determined in water samples, collected during a 1-year period (winter 92-winter 93), in Ria Formosa.

When data from all the samples were combined, the highest mean TBT concentration in water was observed at Olhão (5) (13.7 ng L^{-1}) (Fig. 6.3). These relatively high TBT levels are probably related to the existence of a fishing port and a dockyard at Olhão and thus, to a higher density of small vessels in the area.

Considering the whole sampled period, more than 98% of the water samples presented TBT concentrations in excess of the UK EQS of 0.8 ng L^{-1} Sn (2 ng L^{-1} TBT) and the EPA water quality criterion (0.4 ng L^{-1} Sn), set for marine waters (Fig. 6.3).

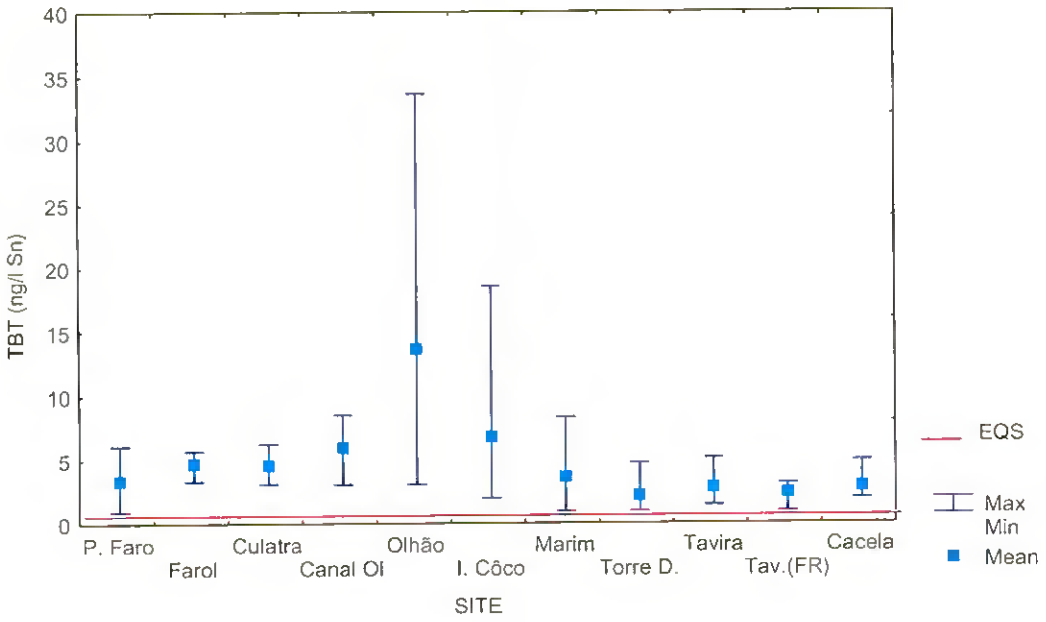

Figure 6.3- Mean, maximum and minimum (n=4) TBT concentrations (ngL^{-1} Sn) determined in water samples, collected during a one year period, in Ria Formosa. (EQS (0.8 ngL^{-1} Sn) – environmental quality standard).

Despite the localised contamination, results indicate that the TBT concentrations in the water of the Ria Formosa, although exceeding the EQS (UK) and the EPA water quality criterion (0.4 ng L^{-1} Sn), were generally low, with 93% of the samples presenting concentrations lower than 10 ng L^{-1} TBT (Sn) (Fig. 6.4).

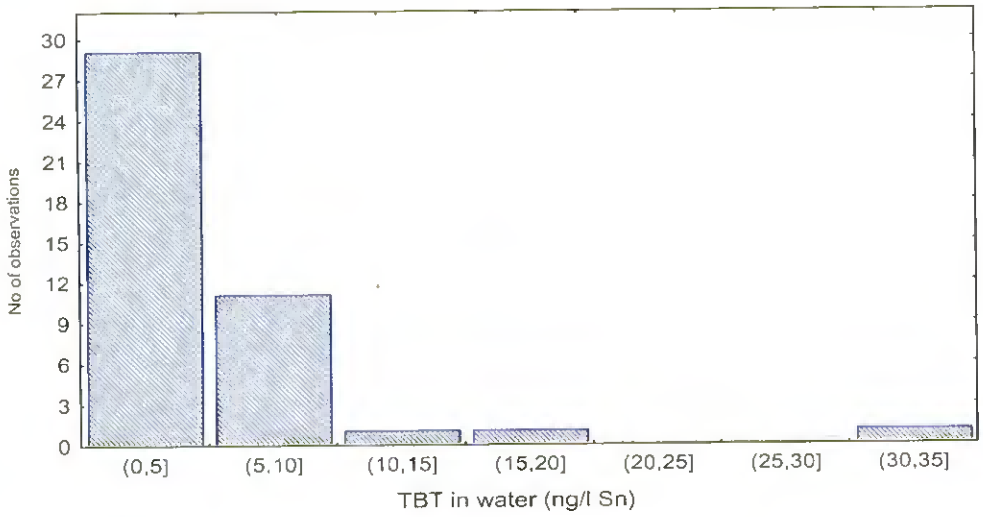

Figure 6.4 - Frequency distribution (class intervals = 5 ng L^{-1}) of TBT concentrations (ng L^{-1} Sn) in water samples, collected during a one year period, in Ria Formosa (n=43).

A marked temporal trend was not observed for TBT concentrations in water during the period of study (Fig 6.2). In fact, at each site TBT concentrations were not significantly different (p<0.05) among the seasons, in Ria Formosa, as shown by a non-parametric statistical test - Kruskal-Wallis Anova (Rice, 1995) - applied to the data.

6.3.2. TBT in sediments

Sediment samples were also collected throughout the year - winter 92 to winter 93 - concurrently with water samples. However, a problem arose in the organotin extraction procedures of sediments samples, due to contamination of the hexane used for extractions. TBT concentrations could not be accurately determined in two of the sets of sediment hexane extracts, specifically samples from winter 92 and spring 93. Consequently, TBT concentrations in sediments from these seasons will not be presented.

TBT and DBT concentrations in surface sediments collected in the summer 1993, in Ria Formosa are presented in Figure 6.5 and in Table 6.3.

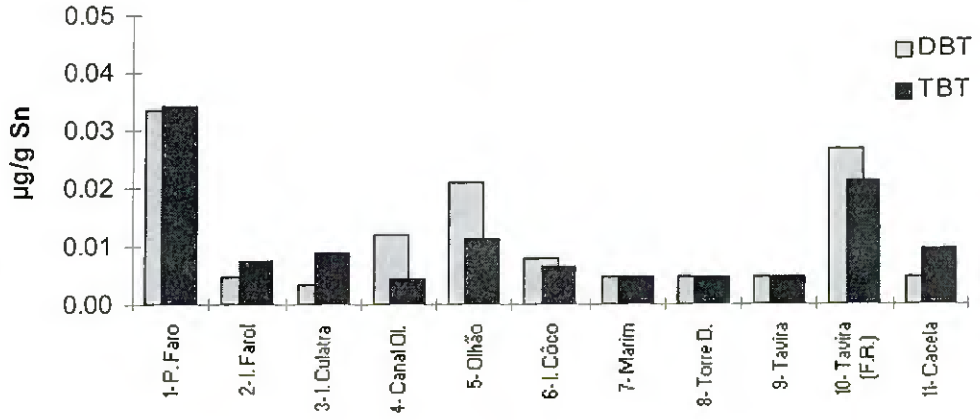

Figure 6.5 TBT and DBT concentrations (µg g^{-1} Sn-d.w.) in surface sediments sampled in the summer of 1993, in Ria Formosa.

Results obtained for organotin concentrations in sediments in summer, 1993 show a distinct spatial pattern. The highest TBT and DBT levels (both 0.034 µg g^{-1} Sn) were observed at Praia de Faro (1). Sediments from Tavira

(Forte do Rato) (10) and Olhão (5) also contained higher levels of TBT and DBT than other sites. The means and ranges for TBT and DBT (as Sn) were 0.010±0.009 μg g^{-1} Sn and 0.010±0.010 μg g^{-1} Sn, respectively.

TBT and DBT concentrations in surface sediments collected in the winter 1993, in Ria Formosa are shown in Figure 6.6 and Table 6.3.

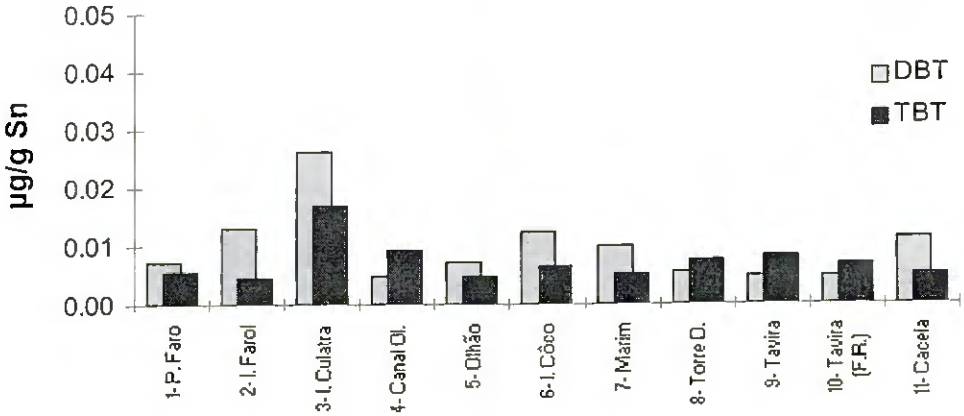

Figure 6.6- TBT and DBT concentrations (μg/g Sn-d.w.) in surface sediments sampled in the winter of 1993, in Ria Formosa.

TBT concentrations in sediments, in winter 1993, showed similar TBT levels at most sites (mean=0.007±0.004 μg g^{-1} Sn), with the exception of Ilha da Culatra (3), where higher organotin burdens were observed (0.017 μg g^{-1} Sn) (Figure 6.6 and Table 6.3).

Table 6.3- TBT and DBT concentrations (μg g^{-1} Sn-dw) in surface sediments from Ria Formosa, collected in 1993.

Site	Summer 93		Winter 93	
	TBT (μg g^{-1} Sn)	DBT (μg g^{-1} Sn)	TBT (μg g^{-1} Sn)	DBT (μg g^{-1} Sn)
1- Praia Faro	0.034	0.034	0.006	0.007
2- Ilha Farol	0.007	<0.005	0.005	0.013
3- Ilha Culatra	0.009	<0.005	0.017	0.026
4- Canal Olhão	0.005	0.012	0.009	<0.005
5- Olhão	0.011	0.021	<0.005	0.007
6- Ilha Côco	0.007	0.008	0.007	0.013
7- Marim	<0.005	<0.005	0.005	0.010
8- Torre D'Aires	<0.005	<0.005	0.008	0.006
9- Tavira (4 Águas)	<0.005	<0.005	0.008	<0.005

Site	Summer 93		Winter 93	
10- Tavira (Forte Rato)	0.022	0.027	0.007	<0.005
11- Cacela	0.010	0.005	0.005	0.011

Limited data from two seasonal samples is clearly insufficient to draw any conclusions about temporal trends on organotin contamination in sediments. Nevertheless, the variation observed in TBT concentrations in sediments may reflect sediments characteristics (Hoch & Schwesig, 2004). Sediment-water partition coefficients K_d, the ratio between TBT concentrations in sediments and the overlying water, calculated for all samples in the Ria Formosa ranged from 328 to $39x10^3$, perhaps reflecting these differences in sediment properties.

The spatial pattern for DBT was similar to that of TBT, with a mean value of 0.009±0.007 μg g^{-1} Sn and a maximum at Ilha da Culatra (3) (0.26 μg g^{-1} Sn).

The average proportion of extractable butyltin (Σ TBT+DBT, expressed as tin) in sediments present as DBT, was 51±17%. A positive and significant correlation was obtained between TBT and DBT concentrations in surface sediments ([DBT] = 1.024[TBT] + 0.001; r=0.826; p<0.01).), showing that at most sites DBT was present in proportion to TBT (Fig. 6.7), as should be expected if origin were from degradation of the parent compound.

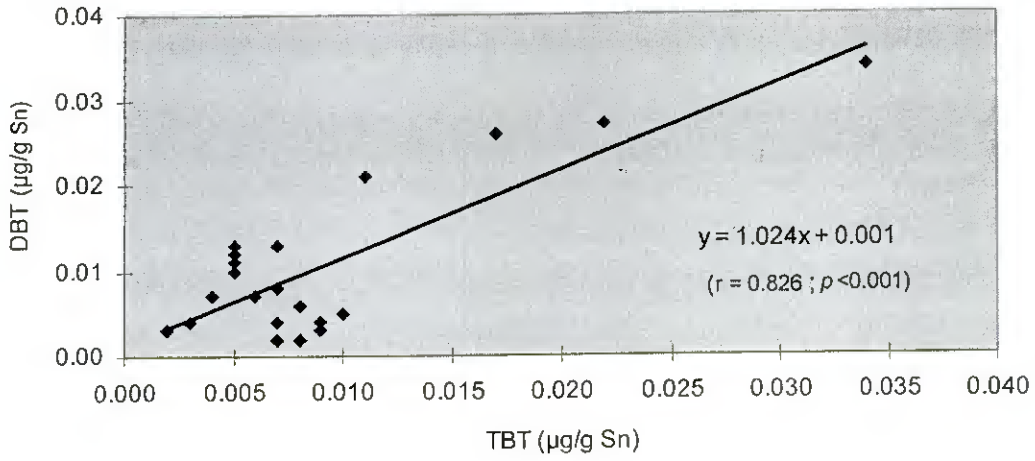

Figure 6.7- Relationship between TBT and DBT concentrations (μg g^{-1} Sn - dw) in sediments from Ria Formosa. (r=correlation coefficient for p<0.001).

6.3.3. TBT in *Ruditapes decussatus*

TBT and DBT concentrations in the whole soft tissues of clams *R. decussatus* collected in winter 1992, in Ria Formosa, are shown in Figure 6.8 and Table 6.4

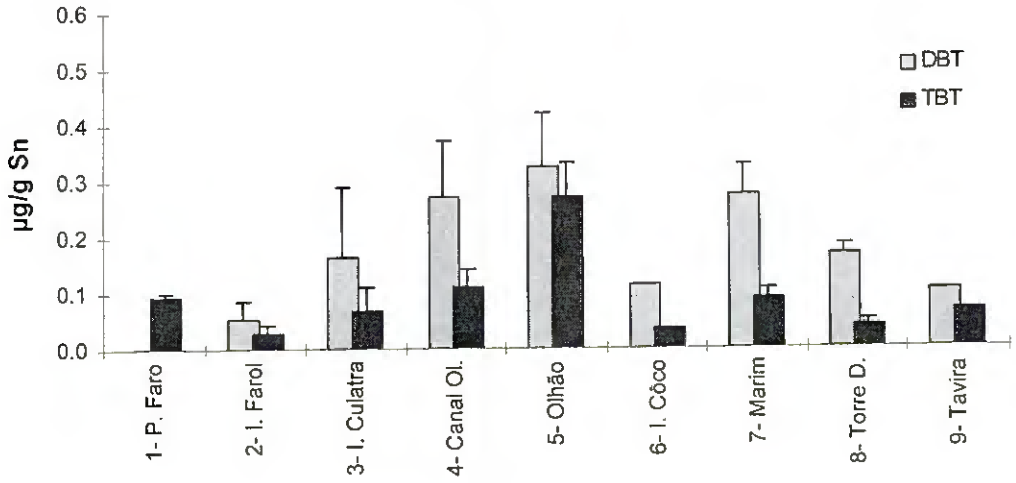

Figure 6.8- *R. decussatus*. TBT and DBT concentrations (µg g^{-1} Sn-d.w.) in the whole soft tissues of clams collected in winter (1992). (Vertical bars are means + st.dev.).

Organotin concentrations in *R. decussatus* sampled in winter 1992, in Ria Formosa, exhibited a marked spatial variation, similar to that observed for the water, with the highest levels at Olhão (0.271 µg g^{-1} Sn and 0.324 µg g^{-1} Sn for TBT and DBT respectively) and lower concentrations at other sites, generally decreasing with distance from Olhão in both easterly and westerly direction.

TBT and DBT concentrations in the whole soft tissues of *R. decussatus* collected in spring 1993, in Ria Formosa are shown in Figure 6.9. and listed in Table 6.4.

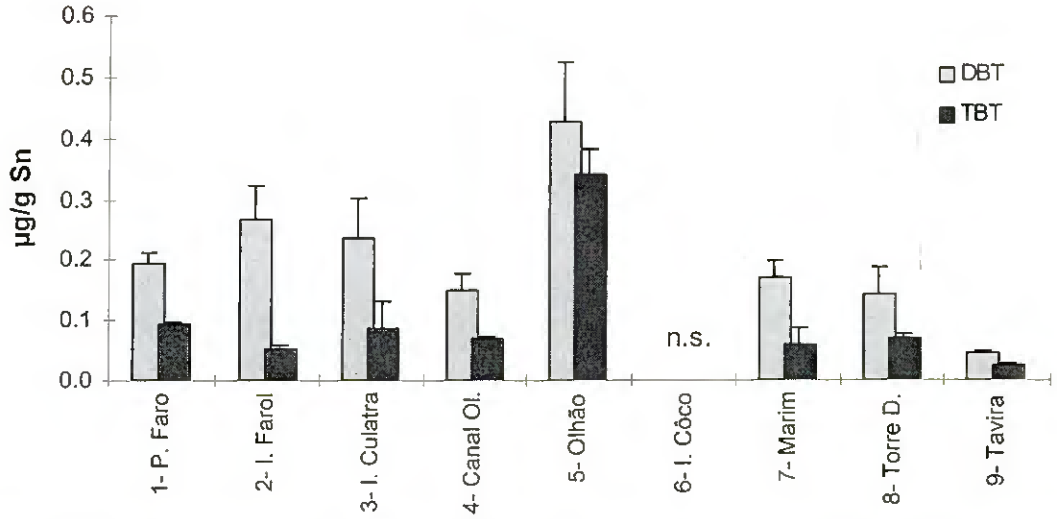

Figure 6.9- *R. decussatus*. TBT and DBT concentrations (µg g^{-1} Sn d.w.) in the whole soft tissues of clams collected in the spring (1993). (Vertical bars are means + st.dev.). (n.s. - not sampled)

Results from spring 93, indicate also a marked spatial pattern in TBT concentrations. As in winter 92, the highest levels of organotins in the whole soft tissues of clams were detected at Olhão (5) (0.338 µg g^{-1} Sn and 0.428 µg g^{-1} Sn for TBT and DBT respectively) (Table 6.4).

TBT and DBT concentrations in the whole soft tissues of *R. decussatus* collected in the summer of 1993, in Ria Formosa are shown in Figure 6.10 and listed in Table 6.4.

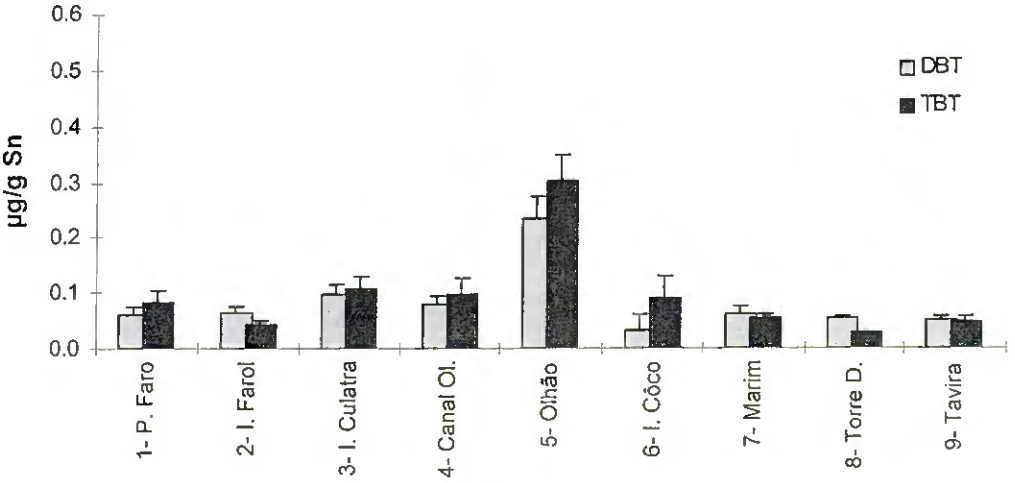

Figure 6.10- *R. decussatus*. TBT and DBT concentrations (µg g^{-1} Sn dw) in the whole soft tissues of clams collected in the summer of 1993. (Vertical bars are means + st.dev.).

In summer, as in the previous seasons, a spatial variation was observed in organotin levels, with a maximum detected at Olhão (5) (0.302 µg g^{-1} Sn and 0.243 µg g^{-1} Sn for TBT and DBT respectively) (Table 6.4).

TBT and DBT concentrations in the whole soft tissues of *R. decussatus* collected in the winter of 1993, in Ria Formosa are shown in Figure 6.11 and in Table 6.4.

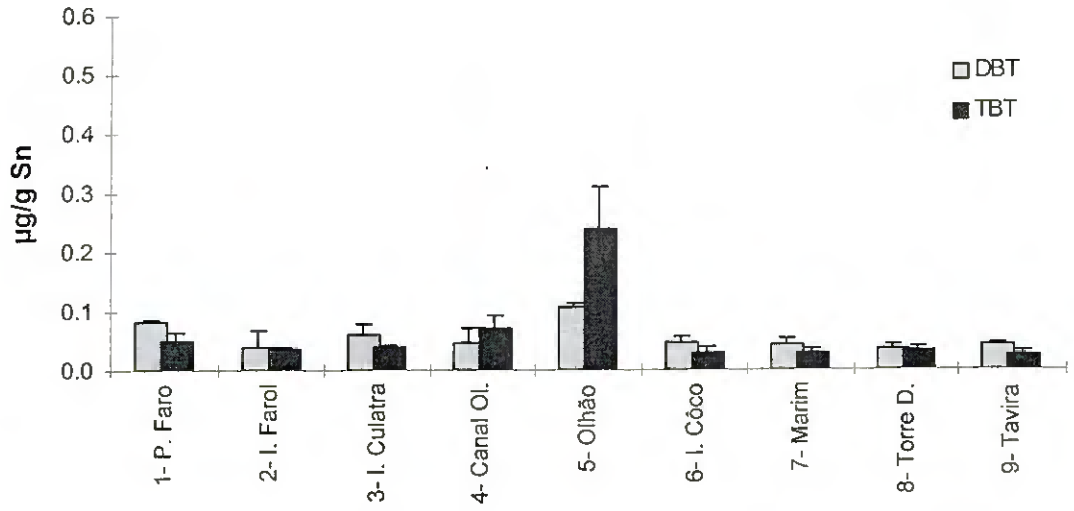

Figure 6.11- *R. decussatus*. TBT and DBT concentrations (µg g^{-1} Sn dw) in the whole soft tissues of clams collected in the winter of 1993. (Vertical bars are means + st.dev.).

Spatial patterns on concentrations of organotins (TBT and DBT) in the whole soft tissues of clams *R. decussatus*, in winter 93, were analogous to those previously reported for other seasons with highest burdens at Olhão (5) (0.239 µg g^{-1} Sn and 0.106 µg g^{-1} Sn for TBT and DBT respectively) (Table 6.4).

As expected, comparing the mean TBT concentrations at each site, over the one year sampling period, the highest mean was observed at Olhão (Fig. 6.12)

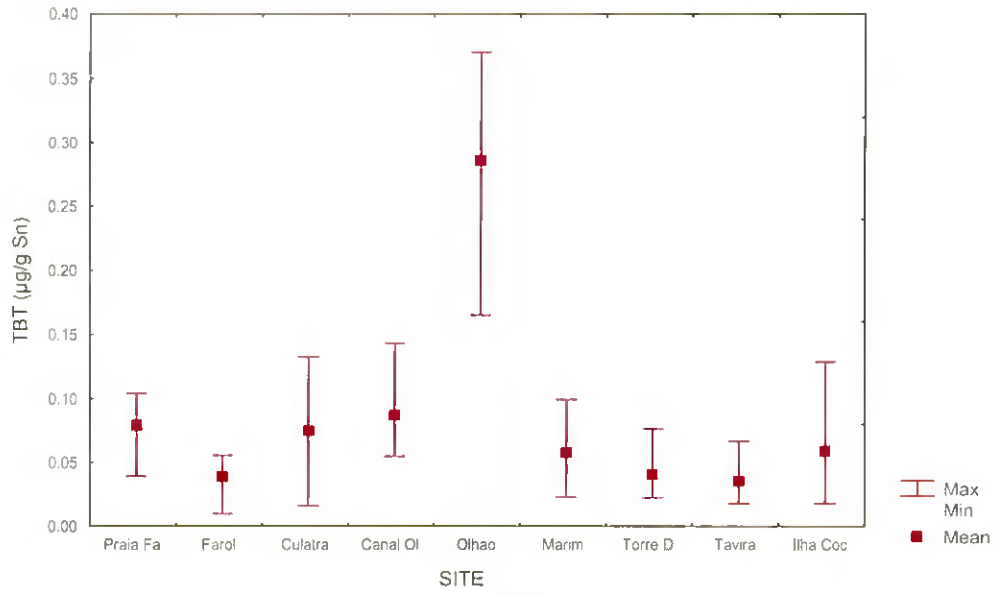

Figure 6.12- Mean, maximum and minimum (n=4) TBT concentrations (ng g^{-1} Sn) determined in *R. decussatus*, collected during a one year period, in Ria Formosa.

Generally, TBT concentrations in the whole soft tissues of clams *R. decussatus*, collected in Ria Formosa during a one year period presented a marked spatial variation, with significantly (p<0.05) higher concentrations at Olhão (5) (Fig. 6.13), as confirmed by a non-parametric statistical test - Kruskall-Wallis Anova (Rice, 1995) - applied to the data.

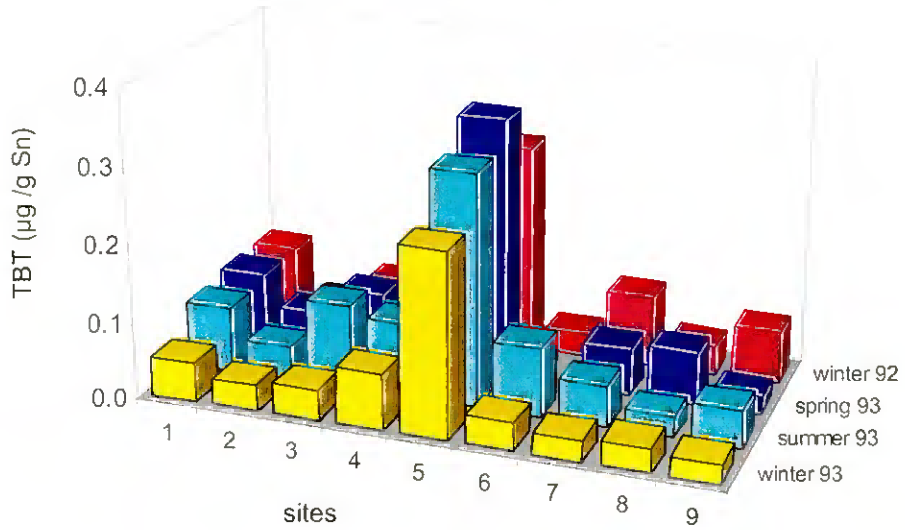

Figure 6.13 - *R. decussatus*. Mean TBT concentrations (µg g^{-1} Sn dw) determined in the whole soft tissues of clams *R. decussatus*, collected during a one year period in Ria Formosa.

Combining data from all samples, no significant differences ($p<0.05$) were observed, in TBT and DBT burdens in the whole soft tissues of *R. decussatus*, among seasons. These results suggest a uniform input of organotin compounds throughout the whole year in the lagoon.

Table 6.4- TBT and DBT concentrations (μg g^{-1} as Sn dw) (mean ± st.dev.) in the whole soft tissues of *R. decussatus* collected in Ria Formosa, in 1992-1993

Site	Winter 92		Spring 93	
	TBT (μg g^{-1} Sn)	DBT (μg g^{-1} Sn)	TBT (μg g^{-1} Sn)	DBT (μg g^{-1} Sn)
1 - Praia Faro	0.093 ± 0.009	<0.005	0.092 ± 0.003	0.195 ± 0.015
2 - Ilha Farol	0.028 ± 0.016	0.053 ± 0.032	0.051 ± 0.008	0.266 ± 0.057
3 - Ilha Culatra	0.066 ± 0.044	0.165 ± 0.122	0.087 ± 0.045	0.236 ± 0.065
4 - Canal Olhão	0.111 ± 0.031	0.272 ± 0.099	0.071 ± 0.004	0.148 ± 0.027
5 - Olhão	0.271 ± 0.060	0.324 ± 0.099	0.338 ± 0.042	0.428 ± 0.096
6 - Ilha Côco	n.s.	n.s.	n.s.	n.s.
7 - Marim	0.091 ± 0.015	0.276 ± 0.052	0.060 ± 0.027	0.169 ± 0.029
8 - Torre D'Aires	0.038 ± 0.013	0.169 ± 0.018	0.068 ± 0.010	0.141 ± 0.046
9 - Tavira (4 Águas)	0.067	0.140	0.024 ± 0.005	0.044 ± 0.004

Site	Summer 93		Winter 93	
	TBT (μg g^{-1} Sn)	DBT (μg g^{-1} Sn)	TBT (μg g^{-1} Sn)	DBT (μg g^{-1} Sn)
1 - Praia Faro	0.083 ± 0.022	0.061 ± 0.012	0.051 ± 0.013	0.082 ± 0.005
2 - Ilha Farol	0.043 ± 0.007	0.063 ± 0.011	0.034 ± 0.004	0.040 ± 0.028
3 - Ilha Culatra	0.109 ± 0.022	0.096 ± 0.018	0.040 ± 0.003	0.061 ± 0.016
4 - Canal Olhão	0.097 ± 0.030	0.078 ± 0.015	0.073 ± 0.020	0.046 ± 0.027
5 - Olhão	0.302 ± 0.046	0.234± 0.038	0.239± 0.070	0.106± 0.008
6 - Ilha Côco	0.091 ± 0.039	0.032 ± 0.028	0.029 ± 0.010	0.048 ± 0.011
7 - Marim	0.055 ± 0.006	0.062 ± 0.012	0.028 ± 0.006	0.043 ± 0.010
8 - Torre D'Aires	0.028 ± 0.001	0.053 ± 0.003	0.032 ± 0.008	0.036 ± 0.005
9 - Tavira (4 Águas)	0.048 ± 0.008	0.049 ± 0.007	0.025 ± 0.008	0.042 ± 0.005

n.s.- not sampled

Mean DBT concentrations in *R. decussatus* ranged from not detected to 0.43 μg g^{-1} Sn. The proportion of extractable butyltin (Σ TBT+DBT) in *R.*

decussatus, which was present as DBT, varied between 0 and 84% (mean= 57 ± 17%).

Results of TBT levels in *R. decussatus* are consistent with data reported for water (Fig. 6.14), confirming the importance of the fishing harbour at Olhão, as a source of TBT contamination in the Ria Formosa. The bioconcentration factor (BCF) (TBT concentration in *R. decussatus*/ TBT concentration in water) obtained for field samples varied between 4.6×10^3 and 9.6×10^4.

By combining all the results for TBT concentrations in these surveys (water, sediments and clams), a positive and significant correlation ($r=+0.506$; $p<0.01$) was obtained between TBT concentrations in water and those in the clams *R. decussatus* whole soft tissues ($[Sn_{water}]$ (ng g^{-1}) = 0.07 $[Sn_{clams}]$ (µg g^{-1})+ 0.05; $r=+0.506$; $p<0.01$)) (Fig. 6.14). However, TBT and DBT burdens in the clams were not significantly correlated ($p<0.05$) with the organotin compounds present in the sediments. These results are consistent with laboratory studies which imply that the major vector for TBT uptake in *R. decussatus* is the water column (Coelho *et al.*, 2002 a,b - Chapter 5).

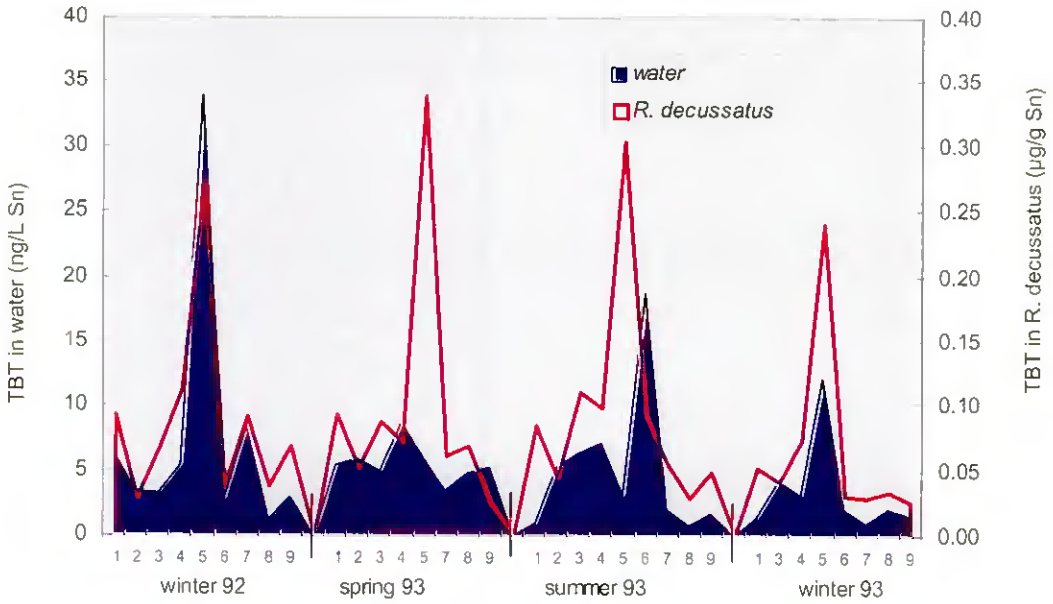

Figure 6.14-TBT concentrations in water (ng L^{-1} Sn) and *R. decussatus* (ng L^{-1} Sn - dw) collected in Ria Formosa, between 1992-1993.

6.4. DISCUSSION

Organotin concentrations measured in the three environmental compartments -water, sediments and clams - in the field survey in the Ria Formosa lagoon showed marked spatial patterns, throughout the year. Generally, higher organotin concentrations were observed at Olhão (site 5), where the most important fishing harbour of the southern coast of Portugal is located. Results indicate that fishing and leisure vessels, mainly moored in the harbour of Olhão (site 5), are the major source of TBT contamination to the Lagoon.

According to Portuguese and European legislation, vessels are nowadays forbidden to use TBT-based antifouling paints, but partial restrictions in Portugal were only started in 1993, and thus were not fully effective at the time of the present survey (1992-93). Later, if restrictions were effective, a significant decrease in TBT concentrations at Olhão would be expected. However, field studies carried out in the same location, more recently, showed little evidence of reduction in TBT burdens, at site (Bebianno *et al.*, 2002; Langston *et al.,* 1997;). TBT contamination in this area of Ria Formosa is unlikely to change for a considerable period of time. Although organotin levels are not excessively high, in view of the importance of the shellfish industry, continued surveillance of TBT contamination should be carried out to ensure risks do not increase.

Since data obtained during the survey did not indicate a significant temporal trend in organotin concentrations, a constant input of TBT throughout the year seems likely. The fact that fishing vessels are probably the major source of contamination in the area may explain a permanent input of TBT, since fishing activity spans the whole year. The influence of TBT contamination from small fishing vessels was also reported for other sites in the Portuguese coast, particularly for Sines harbour (Langston *et al.*, 1997).

TBT concentrations measured in **water** from the Ria Formosa are within the range of those reported for estuarine and mariculture waters at other locations world-wide (Table 6.5)

Table 6.5- Tributyltin concentrations (expressed as ng L^{-1} TBT) in estuaries and mariculture's subsurface waters, determined in this and other studies.

Sampling area	Location	TBT (ng L^{-1}) min	max	Reference
Estuaries / bays	Portugal	✧ 2.5	20	Langston *et al.*, 1997
	Portugal (Tagus)	✧ 3	53	De Bettencourt *et al.*, 1999
	UK	✧ < 3	44	Langston & Burt, 1991
	Chesap.Bay, USA	nd	24	Hall *et al.*, 1987
		5	48	Hall *et al.*, 1988
	San Diego, USA	1	13	Valkirs *et al.*, 1991
	Thailand	<1	3	Larsen, 1997
Maricultures	Portugal	✧ **2.5**	**85**	**This study**
		✧ 2	28	Langston *et al.*, 1997
	France	< 2	17	Gabrielides *et al.*, 1990
	UK	530	730	Waldock & Miller, 1983
	Australia	< 1	40	Batley & Scammell, 1991
	Hong-Kong	< 90	285	Lau, 1991

✧ Converted from concentrations originally expressed as ng L^{-1} Sn (Sn x 2.5 = TBT)
✷ Expressed as concentration of total organotins
nd - not detected

However, TBT levels at several sites in the Ria Formosa exceed the water EQS for TBT (2 ng L^{-1} TBT = 0.8 ng L^{-1} as Sn) adopted in other European countries such as the UK, and the EPA water quality criterion (0.4 ng L^{-1} Sn) (EPA, 2002). Furthermore, the chronic toxicity thresholds of TBT for most bivalve species (Chapter 1- Table 1.7) and particularly for *R. decussatus* and *R. semidecussatus* (Coelho *et al.*, 2001 – Chapter 3; Thain & Waldock, 1986) are of the same order of magnitude of those found in the Ria Formosa. Possible adverse effects on this important bivalve fishery cannot, therefore, be ruled out.

TBT concentrations at which no deleterious effects are observed (NOEC) are in the range of 0.8 - 10 ng L^{-1} TBT (as Sn) for bivalves, generally (Lapota *et al.*, 1993; Salazar & Salazar, 1991; Stephenson, 1991). Early life stages are particularly susceptible. At most of the sampled sites in the present study, TBT water concentrations were above 0.8 ng L^{-1} Sn and thus may induce harmful effects in bivalves, particularly in their early life stages. Results obtained in laboratory experiments with planktonic larvae of *R.*

decussatus have confirmed that TBT concentrations of ≥25 ng L^{-1} TBT (Sn) cause a reduction in growth and development of larvae (Coelho *et al.*, 2001 – Chapter 3). Thus, organotin concentrations detected in Olhão (site 5) (34 ng L^{-1} Sn) are within the range that might influence planktonic larvae in the lagoon, where failure in recruitment could have important economic consequences.

Sediment concentrations (and sediment-water partition coefficient values) for TBT are consistent with those reported for similar habitats elsewhere (Table 6.6 – see Table 1.1 for partition coefficients).

Table 6.6- Tributyltin concentrations (expressed as ng g^{-1} TBT *) in estuaries and mariculture's surface sediments, determined in this and other studies.

Sampling area	Location	TBT (ng g^{-1}) min	max	Reference
Estuaries / bays	Portugal	✧ < 8	53	Quevauviller *et al.*, 1988
		✧ nd	2888	De Bettencourt *et al.*, 1999
		✧ nd	70	Langston *et al.*, 1997
	UK	✧	25	Langston & Burt, 1991
Estuaries / bays	San Diego, USA	2	78	Valkirs *et al.*, 1991
	Chesap. BAy, USA	1	93	Espourteille *et al.*, 1993
	Korea	✧ 55 000	307 000	Hwang *et al.*, 1999
Mariculture	Portugal	✧ 13	85	This study
		✧ nd	90	Langston *et al.*, 1997
	Italy	★ 13	19	Cardellicchio *et al.*, 1992
		★ 14	29	Cardellicchio *et al.*, 1992
	Thailand	4	81	Kan-Atireklap, 1997
	Hong-Kong	52	1100	Lau, 1991

✧ Converted from concentrations originally expressed as Sn (Sn x 2.5 = TBT)
* TBT concentrations in dry weight (dw), except where indicated.
★ Concentrations expressed in wet weight
nd - not detected

In the present study, localised contamination of sediments was observed at Praia de Faro (site 1), in summer, perhaps due to the existence of a high number of boats moored, at this time of the year, in the area.

Despite the localised contamination, generally the TBT burdens in sediments from Ria Formosa represent a moderate to low level contamination.

At most sites, DBT is present in sediments in proportion to TBT, suggesting that some degradation of TBT was occurring in surface sediments.

TBT concentrations measured in ***R. decussatus*** are of the same order of magnitude as those reported for harbours in southern Spain (Table 6.7). Comparable organotin levels have been detected in the oysters *Crassostrea gigas* from the japanese coastal zone and an Australian estuary (Table 6.7) and in mussels *Mytilus galloprovincialis* collected from Portuguese Sado Estuary (Table 6.7). However, levels in *R. decussatus* from the Ria were generally lower than those in clams *Scrobicularia plana* collected from the Portuguese Tagus estuary (Table 6.7).

Table 6.7- Tributyltin concentrations (expressed as ng g^{-1} TBT *) in the whole soft tissues of several species of clams, oysters and mussels, collected in different locations world-wide.

Species	Location/ type	TBT (ng g^{-1})* min.	TBT (ng g^{-1})* max.	Reference
Clams				
Ruditapes decussatus	Portugal / mariculture	✧ **60**	**338**	**This study**
R. decussatus	Spain /harbour	420	710	Morcillo *et al.*, 1997
Scrobicularia plana	Portugal / estuary	✧ nd	730	Langston *et al.*, 1997
	UK / estuary	✧ 88	131	Langston & Burt, 1991
Mya arenaria	Denmark/coastal zone	✧ 628	1 330	Kure & Deplege, 1994
Meretrix spp.	Vietnam / coastal zone	✧ ★3.5	140	Midorikawa *et al.*, 2004
Oysters				
Crassostrea gigas	Japan / coastal zone	50	300	Mizuishi *et al.*, 1989
	Australia / estuary		✧ 439	Batley & Scammell, 1991
C.virginica	USA / coastal zone	7	180	Espourteille *et al.*, 1993
	USA / coastal zone	10	4 030	Uhler *et al.*, 1993
Ostrea angasi	Australia /estuary		✧ < 3	Batley & Scammell, 1991

Species	Location/ type	TBT (ng g^{-1})* min.	max.	Reference
Saccostrea commercialis	Australia / estuary	✧ < 5	879	
Mussels				
Mytilus edulis	Holland / estuary	< 1	530	Ritsema *et al.*, 1991
	Holland / marina	350	2 300	
	USA /bay -estuary	★ 27	390	Valkirs *et al.*, 1991
M. galloprovincialis	Portugal / coastal zone	✧ 40	100	Quevauviller *et al.*, 1988
	Italy / mariculture	★ 10	24	Cardellicchio *et al.*, 1992
		★ 10	13	

* TBT concentrations in dry weight , except where indicated
★ concentrations expressed in wet weight;
✧ Converted from concentrations originally expressed as Sn (Sn x 2.5 = TBT)
nd - not detected

Although organotin levels are not excessively high in most of the Ria Formosa, contamination at low levels is fairly widespread and, as indicated, is of potential significance to early life stages of bivalves.

Generally, TBT concentrations measured in the three environmental compartments (water, sediments and biota) during the 1992-93 survey of the Ria Formosa suggest a low-moderate level of contamination, with the exception of Olhão where the highest TBT concentrations were detected. Probably, the main source of organotins in this area is TBT leached from antifouling paints, used in fishing and leisure vessels from the port of Olhão.

Because of the importance of the lagoon as a shellfishery, its restricted flushing characteristics, and the longevity of TBT contamination in sediments, it would seem important to continue surveillance to ensure that the risks, to this fragile ecosystem, do not increase.

In fact, a recent survey carried out in the same area during 2000 and 2001, has shown that the TBT levels have actually increased and not decreased (Bebianno *et al.*, 2002), revealing that there has been a constant input of organotins in the lagoon. On the other hand, imposex levels screened in gastropods, also for the same area, revealed a 100% imposex in females and, in some sites, female sterilization, thus confirming the organotin contamination in the lagoon. Similarly, a global increase in TBT contamination

and imposex incidence was observed in the remaining Portuguese coastal area from 1997 to 2000 (Barroso & Moreira, 2002). Therefore, in recent years TBT concentrations did not exhibit a significant decrease in the Portuguese coastal area, particularly in the Ria Formosa lagoon, revealing the inefficacy of the 1993 legislation, which banned the use of organotin compounds in small vessels.

Chapter 7

ASSESSMENT OF DEGREE OF IMPOSEX IN ALGARVE

7.1. INTRODUCTION

Prosobranch gastropods exhibit all types of sexuality but in the majority of species the sexes are separated and unchanged throughout the life history of the individual. In the suborder Neogastropoda all species are strictly gonochoristic or dioecious. Sex identification in these species was, until the late 60s, a routine matter, involving simply the observation of the presence or absence of a penis in each specimen. This situation changed around 1970 when penis-bearing females were noted in widespread populations of a few neogastropods species. This type of abnormality was first recorded by Blaber (1970) who described penis-like outgrowths on *Nucella lapillus* females collected in Plymouth Sound (UK). Subsequently, in the USA, Smith (1971) observed comparable masculinisation of *Ilyanassa* (=*Nassarius*) *obsoleta* females although with more advanced features (development of penis, sperm duct (vas deferens) and oviducal convolution), which led to the creation of the term "imposex" to describe this superimposition of male characteristics on to *I. obsoleta* females.

Later, the appearance of snails with this type of masculinisation was linked with pollution from marinas. Studies with transplanted gastropods showed that imposex could be induced when snails were transferred from "clean" sites onto marinas (Smith, 1981a,b). Thus, a number of substances used in marinas came under suspicion and eight chemical products were screened: a chemical toilet disinfectant; a spray detergent; leaded petrol; exhaust emissions and 4 different types of antifouling paints (copper, lead and TBT based paints). The product responsible for this abnormality was identified as a TBT-based antifouling paint (Smith, 1981b). Moreover, laboratory studies with that type of paint demonstrated that imposex could be induced in the laboratory by exposure to tributyltin compounds at concentrations calculated to be in the range of parts per billion ($\mu g\ L^{-1}$) (Smith, 1981c). Field observations on the referred species led to the conclusion that such "pseudohermaphroditism" did not affect *I. obsoleta* reproductive capacity nor the population ecology (Smith, 1981a,b,c), in contrast with other species, as described latter. Simultaneously, imposex studies carried out in Europe

showed that several other species were also affected, particularly *Ocenebra erinacea* around the Atlantic coast of France and *Nucella lapillus* in the UK coastline (Bryan *et al.*, 1986; Féral, 1980a,b)

The imposex syndrome gained a significant ecological dimension following the observation that affected *N. lapillus* populations were declining in abundance in all of the main sites related with boating activity, namely marinas, around the southwest peninsula of England (Bryan *et al.*, 1986). In order to assess imposex, extensive field surveys were carried out and revealed that the degree of imposex increased with proximity to boating centres and was correlated with the concentration of organotins (TBT and DBT) accumulated in gastropod's tissue. The studied populations were characterised by a general scarcity of individuals, no indication of breeding activity, no juveniles and a predominance of old males among survivors (Bryan *et al.*, 1986). A lack of breeding activity would imply a scarcity of larvae, thus impairing recruitment and leading to a lack of juveniles in the population. As a result, a reduction in recruitment caused by a lowered reproductive activity, rather than a high mortality rate, was indicated as probable cause for declining populations. Additionally, laboratory studies suggested that the masculinisation process was being initiated at an ambient TBT concentration of approximately one part per trillion (1 ng L^{-1} Sn), or even less, in the water (Gibbs *et al.*, 1987). At these concentrations, the presence of a penis and a vas deferens does not interfere with the breeding activity of females. Nevertheless, further investigations lead to the conclusion that, at slightly higher TBT concentrations (4 ng L^{-1} Sn), additional morphological modifications which inhibit breeding activity were apparent, and at 7-10 ng L^{-1} Sn no breeding was occurring and populations were probably declining (Gibbs & Bryan, 1986; Gibbs *et al.*, 1987).

Data obtained in field surveys led to the development of indices measuring the level of imposex induced by TBT in *N. lapillus*. One of those indices, the vas deferens sequence (VDS index) is illustrated in Figure 7.1 (Gibbs *et al.*, 1988).

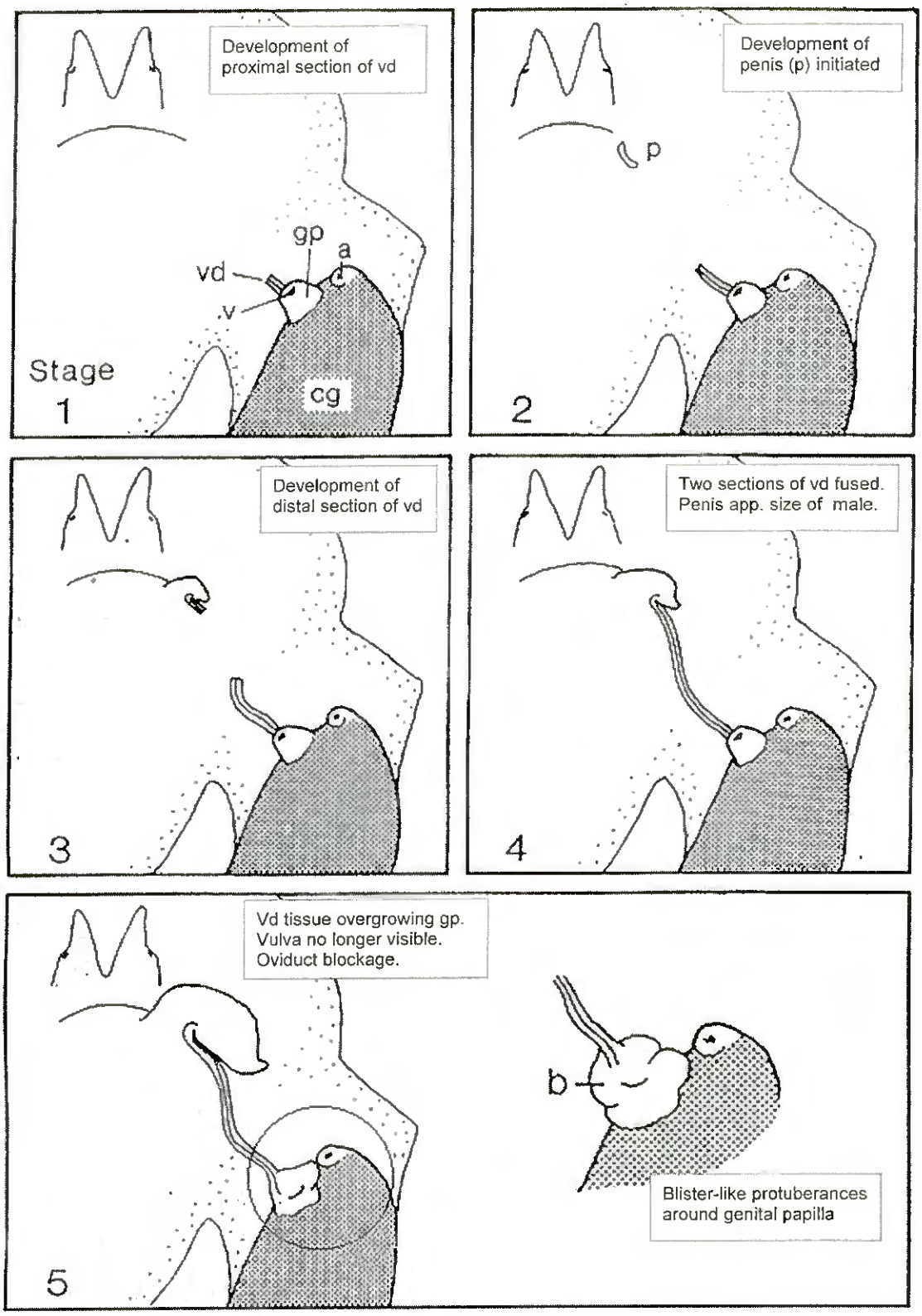

Figure 7.1 – Development of imposex in *Nucella lapillus*, based on the vas deferens sequence (VDS) (After Gibbs *et al.*, 1987).
Abbreviations: *a*- anus; *b*- blister-like protuberance; *cg*- capsule gland; *gp*- genital papilla; *p*- penis; *v*- vulva; *vd* – vas deferens

Furthermore, field surveys on the assessment of imposex in *N. lapillus* revealed that females from highly contaminated sites all exhibited a particular feature, namely a blockage of the oviduct due to an overgrowth of the vas deferens tissue (see Stage 5 in Fig. 7.1). This blockage prevented the normal release of encapsulated eggs, which in turn were accumulated within the oviduct, demonstrating that females were sterile. Moreover, the build-up of aborted capsules induced an injury in females through splitting of the capsule gland, which may have increased mortality rates (Gibbs & Bryan, 1986).

In long term laboratory and field experiments *N. lapillus*, especially growing juveniles, were shown to be extremely sensitive to TBT. A summary of effects of TBT exposure on the reproductive system of female *N. lapillus* is presented in Table 7.1.

Table 7.1- Effects of TBT exposure, on the reproductive system of the female *N. lapillus* during the first 2 years of life, based on field and laboratory data (after Gibbs *et al.*, 1988; 1991).

TBT in water (ng L^{-1} Sn)	Effect on the reproductive system (long term exposure)
< 0.5	Development of penis and vas deferens. Breeding normal.
1-2	Breeding capacity retained by most females, but some sterilised by oviduct blockage. Aborted capsules in capsule gland.
3-5	Virtually all females sterilised. Oogenesis apparently normal

Field data together with rearing of *N. lapillus* from hatching to maturity in the laboratory (> 2 years) showed that imposex was initiated at water TBT concentrations lower than 0.5 ng L^{-1} Sn ; at a concentration of 1-2 ng L^{-1} Sn some females were sterilised and at 3-5 ng L^{-1} Sn all were sterilised and no reproduction occurred (Bryan *et al.*, 1987; Gibbs *et al.*, 1987; 1988).

Laboratory and field studies on *N. lapillus* long-term accumulation of TBT followed by a depuration period demonstrated that imposex is an irreversible syndrome (Bryan *et al.*, 1987).

During the past decades, females of an increasing number of gonochoristic gastropods have been found to exhibit imposex and such abnormal penis-bearing females have been recorded in over 150 gastropod

species in coastal waters world-wide (Bettin *et al.*, 1996; Gibbs & Bryan, 1994; Schulte-Oehlmann *et al.*, 2000), including the coast of Portugal (Barroso *et al.*, 2000; 2002; Santos *et al.*, 2000; 2002), and positively correlated with TBT concentrations. Thus, imposex is a term now widely used to describe the condition of female gastropods that exhibit male characteristics, but should be applied only in those cases where masculinisation has been linked to the presence of TBT, i.e., an external agent causing an unnatural degree of masculinisation (Gibbs & Bryan, 1994).

Although the overall pattern of masculinization under TBT exposure appears similar in all neogastropods, species vary in detail in terms of end-effects (see Gibbs *et al.*, 1991; Gibbs & Bryan, 1994). In some, such as the nassariids *I. obsoleta* and *Nassarius* (=*Hinia*) *reticulatus* the imposition of a penis and sperm duct does not seem to interfere with the female's breeding activity. However, in other groups such as the muricid *N. lapillus*, the masculinisation of females may lead to female sterilisation, resulting in population decline and eventual extinction, as described earlier (Bryan *et al.*, 1986).

More recently, it was demonstrated that females of the neogastropods *Ocenebra erinacea* exhibit advanced imposex in a different manner, presenting a characteristic malformation of the oviduct (Gibbs *et al.*, 1990; 1991). Affected *O. erinacea* females show a longitudinal split of the oviduct wall, causing the bursa copulatrix and capsule gland to open directly into the mantle cavity (Gibbs *et al.*, 1990; Gibbs, 1996) (Figure 7.2). The mantle spilt can be interpreted as a masculinisation of the oviduct, since it mimics the partially open structure of the male prostate (Figure 7.2) (Gibbs *et al.*, 1990). Such malformation is initiated at ambient TBT concentrations of 3 ng L^{-1} Sn, in the water, and leads an unsuccessful sperm transfer during copulation, thus not allowing subsequent encapsulation of the fertilised egg masses (Gibbs, 1996; Gibbs *et al.*, 1990).

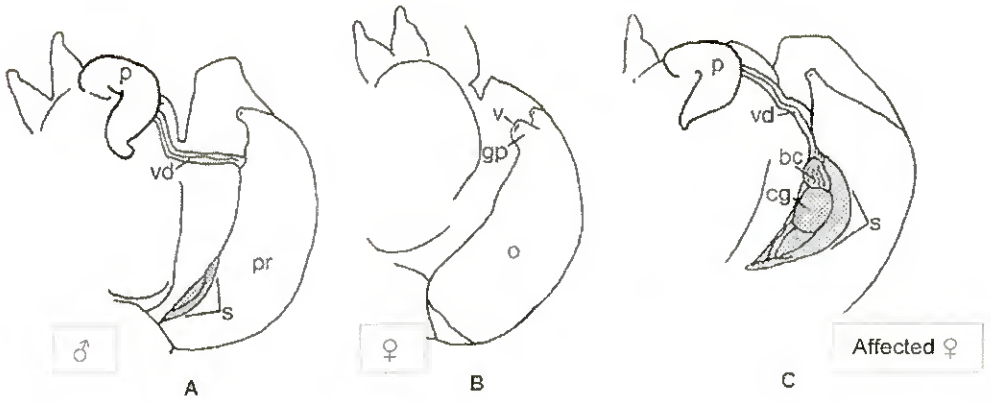

Figure 7.2- Effect of imposex in *Ocenebra erinacea*. A- male; B- normal female; C- non breeding female with advanced imposex (sterility caused by split in the oviduct (shown shaded)
Abbreviations: bc- bursa copulatrix; cg- capsule gland; gp- genital papilla; o- oviduct; p- penis; pr- prostate; s- split; vd- vas deferens (After Gibbs *et al.*, 1990; 1991).

After hatching larvae of *O. erinacea* go through a planktonic phase of up to 5 days (13-16°C) (Gibbs, 1996). The existence of a pelagical larval phase is crucial *O. erinacea* population's abundance, since it allows recruitment of worst-affected populations, with larvae from different locations being carried a considerable distance by water currents. As a consequence, sterility of females will not necessarily result in population extinction, in contrast with species such as *N. lapillus* which lack a pelagic larval phase.

Even with different overall effects, the imposex response has an extraordinary universality and essentially all neogastropods collected at TBT contaminated sites exhibited this syndrome. Despite some variance, overall it is possible to trace certain features of imposex which appear to be applicable to all affected neogastropods: (i) imposex is induced by TBT exposure at or below 0.5 ng L^{-1} Sn; (ii) its degree of development is dose-controlled and (iii) in sterilised species the effect at the population level depends largely on the dispersive capacity of the larvae, *i.e.*, length of the planktonic existence (Gibbs & Bryan, 1994).

Although imposex arises as an universal response, different levels of masculinization can be identified in distinct groups of neogastropods (Gibbs *et al.*, 1997). Four levels were described: zero (no masculinization); level I (penis and vas deferens developed); level II, (oviduct function disrupted); level

III (ovary-testis transformation). Included in the zero level are some neogastropod species, found in TBT contaminated areas, that do not exhibit masculinisation of females. Reported exceptions include *Amphissa columbiana* (Bright & Ellis, 1990), *Cominella glandiformis* (Smith & McVeagh, 1991) and *Columbella rustica* (Gibbs *et al.*, 1997). In fact some authors still think it is a controversial issue (Evans *et al.*, 1995).

The use of imposex as an indicator of TBT pollution, shows it to be widespread in coastal waters throughout the world. In European coastal waters, *N. lapillus* has been extensively used as a bioindicating species because imposex proceeds in a predictable manner (Bryan *et al.*, 1986; Gibbs & Bryan, 1986).

In Portugal, evaluation of imposex was performed mainly in the west coast, using the species *Nassarius reticulatus* e *N. lappilus*. Results have shown that organotin contaminations together with imposex are widespread in estuarine and coastal waters (Peña *et al.*, 1988; Barroso *et al.*, 2002; Santos *et al.*, 2000; 2002; 2004).

The work reported here is an baseline assessment of the impact of TBT pollution on neogastropod species in the coast of Algarve. In addition to the assessment of TBT concentrations in water, sediments and biota (Chapter 6 – Coelho *et al.*, 2002c), the assessment of imposex constitutes a complementary tool to evaluate the degree of TBT contamination in Algarve.

Considering that *N. lapillus* nearly does not occur in the south coast of Portugal, the first task in the present work was to examine availability of neogastropod species to assess the incidence of imposex in the region. Subsequently, the degree of imposex was determined along the coast concurrently with TBT concentrations in gastropods tissues. Most of the sampled sites were located inside the Ria Formosa lagoon system, which occupies a large area of the south coast and where impact of TBT pollution is likely to be greater, as a result of boating activity.

7.2. MATERIALS AND METHODS

7.2.1. Imposex field survey

In order to assess the incidence of imposex in neogastropods, a field survey was carried out in Algarve between Vila Real de Santo António, at the eastern border, and Zavial, near Sagres, at the western end, at 13 different sites (Figure 7.3). Sampling sites were selected to cover the whole coast of Algarve and according to facility of access, existence of suitable substrate for gastropods and presence of boating activity. Collection of samples at these sites was performed in March 1994; April 1995 and April 1996.

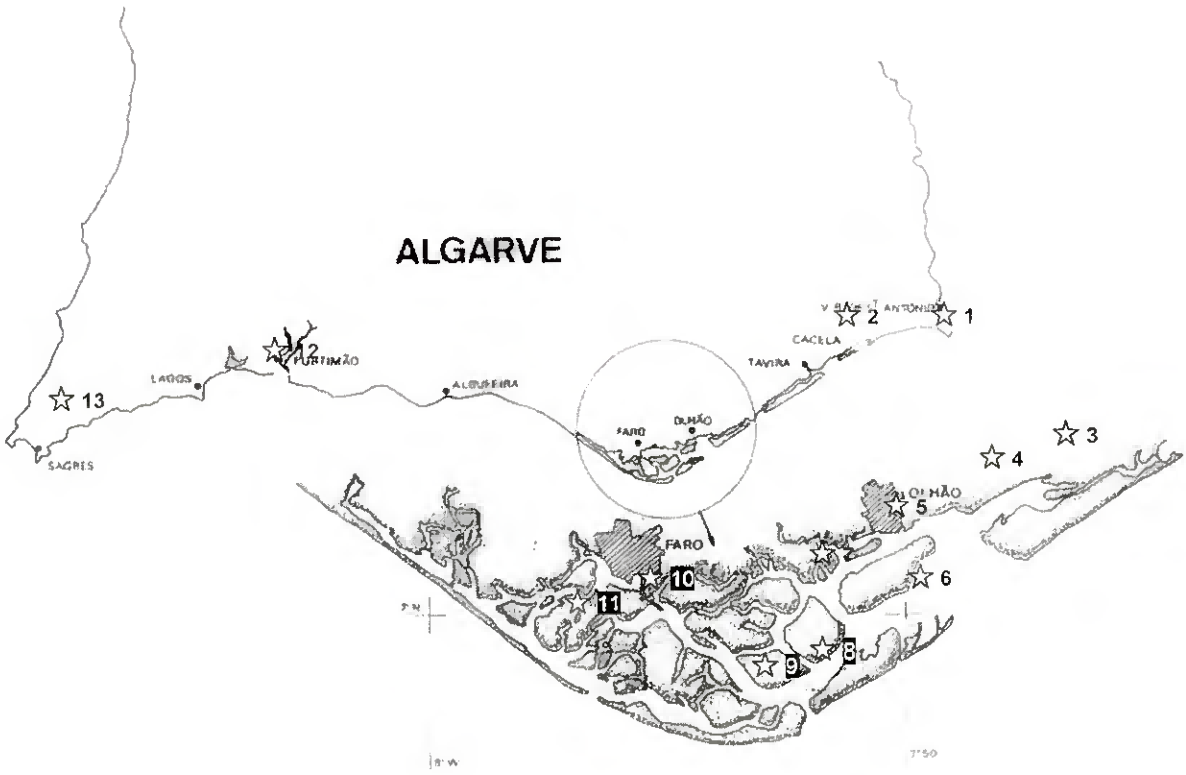

Figure 7.3 – Location of sampling sites (☆) along the coast of Algarve.

Due to the broad spectrum of conditions (substrates, sea-water temperatures, exposed and sheltered) observed at sampling sites, availability of neogastropod species varied with locations. Overall, six neogastropod species were collected and these included *Ocenebra erinacea, Hexaplex trunculus, Murex* (=*Bolinus*) *brandaris, Conus ventricosus, Nassarius reticulatus* and *Nucella lapillus* (Figure 7.4 and Table 7.2).

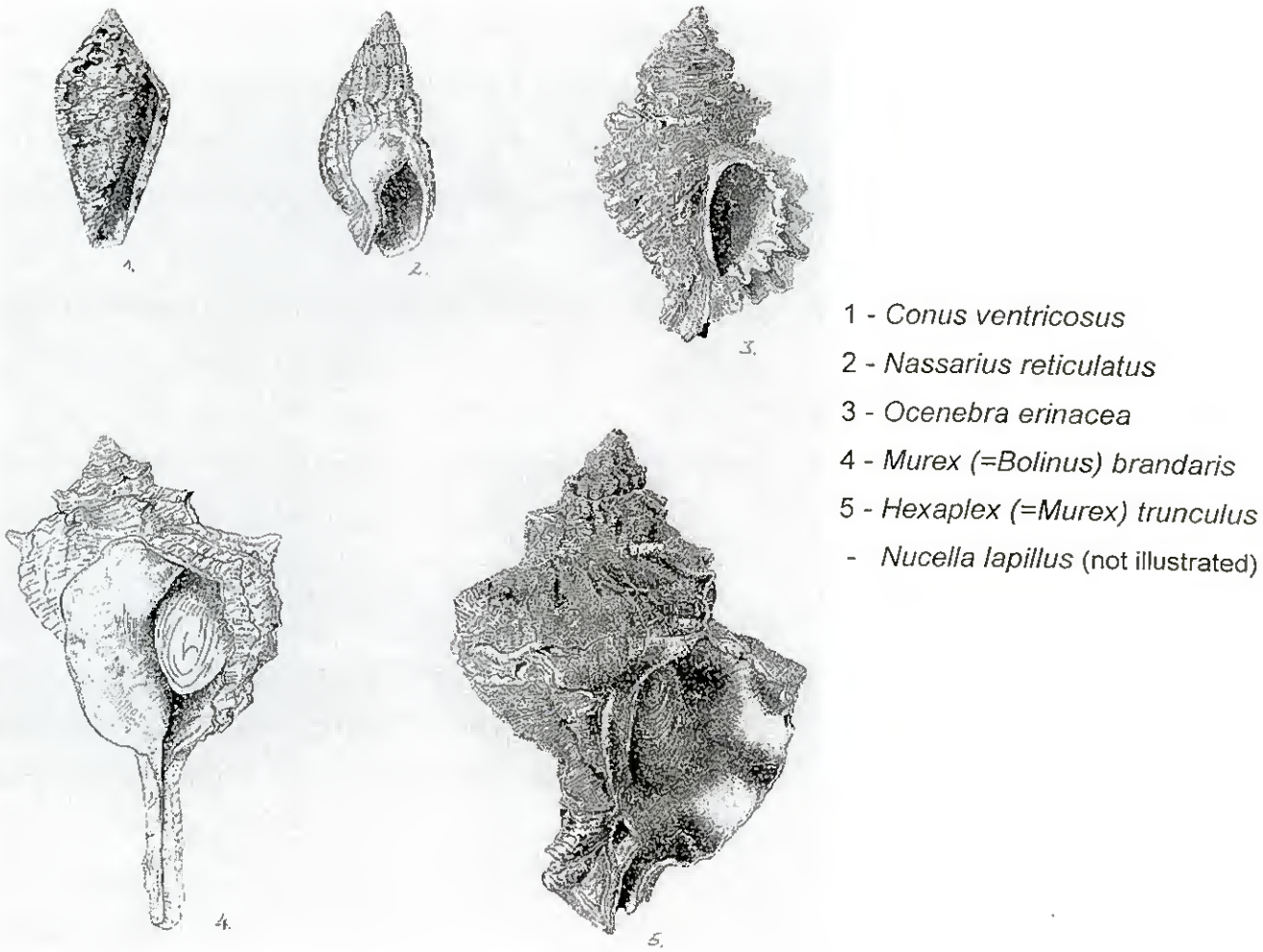

Figure 7.4 – Species of neogastropods collected in the south coast of Portugal, for imposex assessment.

Availability of neogastropod specimens, at sampling sites, was irregular and in a few locations sample numbers were inevitably low. For instance, *C. ventricosus, N. reticulatus* and *N. lapillus* were scarce and collected only in

small numbers. Nevertheless, where possible a sample of 20 adults was collected at each site, at low spring tide, in the intertidal zone, kept in an ice box and transported alive to the laboratory for subsequent observation.

Table 7.2- Neogastropod species collected (x) at each site , in the coast of Algarve, for the assessment of imposex.

Species / Site	*Ocenebra erinacea*	*Hexaplex trunculus*	*Murex brandaris*	*Conus ventricosus*	*Nassarius reticulatus*	*Nucella lapillus*
1- V.R.S. António	x					
2- Cacela	x	x	x	x	x	
3- Cavacos				x		
4- Marim		x			x	
5- Olhão		x				
6- Culatra	x	x	x		x	
7- Canal Olhão		x			x	
8- Salva vidas	x				x	
9- Farol (Bareta)		x	x		x	
10- Cais comercial	x	x			x	
11- Ramalhete				x	x	
12- Portimão	x					
13- Zavial (Sagres)						x

7.2.2. Assessment of imposex

In the laboratory, gastropods were maintained in aquaria with aerated sea-water until subsequent observation, in the same day or within 1 day of collection. The procedure used to assess the degree of imposex was based on methods describe by Bryan *et al.*, (1986) and Gibbs *et al.*, (1987) and summarised below.

Shell length was measured using a calliper, to the nearest 0.1 mm. After removal of the shell, using a bench vice, gastropod's mantle roof was opened using a small scissors or scalpel, to expose the mantle cavity.

Observations were performed using a binocular microscope and, if possible, were carried out under water.

Animals were sexed from the presence of the capsule gland, genital papilla, sperm ingesting gland, and accessory organs (Gibbs *et al.*, 1987; Gibbs, pers.comm.).

After observation and assessment of imposex, as described below, gastropod's opercula was removed, males and females were separated and tissues kept in glass vials for subsequent organotin extraction and analysis, using the method described in Chapter 2.

Imposex was quantified using two indices, both described by Gibbs *et al.* (1987): (i) the relative penis size index (RPSI), which relates the size of female penis to that of the male and (ii) the vas deferens sequence (VDS) index, which indicates the steps of the vas deferens formation. These indices have been devised for *N. lapillus* and used successfully in other species within the suborder Neogastropoda for over a decade (Gibbs & Bryan, 1996).

7.2.3. Relative Penis Size Index (RPSI)

RPSI expresses the extent of penis development in the female and it is considered a convenient indicator of TBT exposure – it compares the bulk (i.e. volume, weight) of female penis with the bulk of that of the male, for the same population.

In both males and affected females the length of the penis, from its tip to its junction with the body wall (Fig. 7.1-7.2), was measured using millimetre-graduated graph paper, to the nearest 0.1 mm. The RPS index, for individuals of the same population, was then calculated according to the equation:

$$RPSI = \frac{(\text{mean length of female penis})^3}{(\text{mean length of male penis})^3} \times 100$$

A female without any sign of a penial outgrowth was registered as zero and this value was included in the calculation for mean length.

7.2.4. Vas Deferens Sequence Index (VDSI)

In females, besides the penis length, the presence and developmental stage of a vas deferens (sperm duct) was also recorded, since, in some species, it may interfere with the reproductive competency of females. The recognition of different stages in vas deferens development therefore provides a more sensitive method of categorising the intensity or expression of imposex (Gibbs *et al.*, 1987).

An index, the so called vas deferens sequence index (VDSI) (Gibbs *et al.*, 1987) was initially defined to *N. lapillus*, and adapted to sampled species in the present field survey. A VDS index with 5 categories was used for most species including *O. erinacea, H. trunculus, B. brandaris, C. ventricosus* (Gibbs, pers.comm.). In the case of *N. reticulatus*, slightly different categories were adopted, as described bellow.

The VDSI describes the development of the vas deferens, in females, and for species other than *N. reticulatus*, the VDSI was categorised from Stage 0 (normal female) to Stage 4 (full developed vas deferens). In the case of *O. erinacea* an additional Stage 5 was included to account for females with a split oviduct wall, as described above (Fig.7.2) (Gibbs, 1996). For this species, the **VDS index** can be described as follows:

Stage 0 - "normal" female with no male character being visibly imposed;
Stage 1 - No visible vas deferens; Development of a penis initiated with the formation of a small "button", behind the right tentacle
Stage 2 - No visible vas deferens. Development of a measurable penis behind the right tentacle.
Stage 3 - Development of vas deferens starting from the base of the penis; measurable penis formed.
Stage 4 - Complete vas deferens formed from the base of the penis to the vulva; well developed penis approaching a size similar to the male.
Stage 5 -(*Ocenebra erinacea*) - Complete vas deferens; longitudinal split of the oviduct wall, causing the bursa copulatrix and capsule gland to open directly into the mantle cavity; well developed penis.

For the species *Nassarius reticulatus* another VDSI was devised, with less categories as follows (Gibbs, pers.comm.):

Stage 0 - no visible vas deferens
Stage 1 - 'early' stage involving a short vas deferens
Stage 2 - 'late' stage characterised by the presence of a complete vas deferens, from the base of the penis to the vulva.

Based on all the observed females in each sample, mean VDSI values were obtained for each population, as well as mean RPLI values.

Evidence has demonstrated that in populations highly impacted by TBT, the sex ratio typically becomes biased in favour of males, as a consequence of increased female mortality (Gibbs & Bryan, 1986). Thus, this parameter was also included in the present imposex assessment.

Overall, results obtained for the assessment of imposex will be expressed as sex ratio (n males: n females), percentage of affected females; mean penis length of males and females; mean RPLI and VDSI for each species collected at each location.

7.2.5. Intercomparison exercises

The participation, in 1998, in the QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring in Europe) laboratory performance study (Davies *et al.*, 1999) provided information regarding the quality assurance of methods employed in the present study for imposex assessment. Satisfactory results were obtained for the following parameters, measured in *Nucella lapillus*: sex identification, penis length, VDSI and RPSI.

In addition, an intercomparison exercise of the measurement techniques employed in this study was also undertaken, with two independent observers, at the University of Algarve to "calibrate" assessment of the imposex stages (VDSI, RPLI) in the species H. *trunculus* and *O. erinacea*.

The same exercise for determining RPS and VDS indices, for two populations of *N. lapillus*, was also carried out at the Plymouth Marine Laboratory. Results of this exercise demonstrated good agreement in results obtained by independent observers (Langston *et al.*, 1997).

7.3. Results and Discussion

In the present study, the degree of imposex was determined using the two indices RPLI and VDSI and complementary information. In this way, results obtained in terms of sex ratio (n ♂:n ♀); percentage of affected females (n affected females:n total females), penis length (for ♀ and ♂) and mean RPLI and VDSI for each species, at each site, are presented in Table 7.3. In addition, visible signs of sterility, such as a split bursa in *O. erinacea* females, were also registered.

Table 7.3 - Results from the imposex field survey in southern Portugal: % females ;% penis-bearing females, sex ratio; imposex index (RPSI and VDSI) and eventual signs of sterility.

Site	Species	date	sex ratio m:f	% female	% females penis-bearing	mean penis length male	mean penis length female	index of imposex RPSI	index of imposex VDSI	signs of sterility
	1994									
4 -Moinho-Marim	*Nassarius reticulatus*	28/03/94	3:1	25	100	8.33	0.4	0.011	1	
5 -Olhao	*Hexaplex trunculus*	28/03/94	6:0	0	-	10.33	-	-	-	
9 –Farol (Bareta)	*Nassarius reticulatus*	27/03/94	4:15	79	80	10.38	0.04	0	0.3	
6 -Culatra	*Hexaplex trunculus*	28/03/94	8:8	50	100	9.26	3.40	4.9	4	
	Nassarius reticulatus	28/03/94	11:9	45	78	8.73	0.04	0.0	0.1	
10-Cais comercial	*Hexaplex trunculus*	27/03/94	3:3	50	100	9.83	5.33	16.0	0	
	Nassarius reticulatus	27/03/94	5:2	29	100	6.40	0.75	0.2	0.5	
11 -Ramalhete	*Conus ventricosus*	30/03/94	10:3	23	100	4.79	3.23	30.8	3.3	
	Nassarius reticulatus	27/03/94	1:2	67	100	8.50	1.20	0.3	0	
12 -Portimao	*Ocenebra erinacea*	29/03/94	9:10	53	100	3.40	1.86	16.4	3.6	
13 -Zavial	*Nucella lapillus*	29/03/94	4:16	80	0	3.50	0	0	0	
	1995									
1 -Vila Real	*Ocenebra erinacea*	17.04.95	12:9	43	100	3.08	0.20	0.0	4	
2 -Cacela	*Ocenebra erinacea*	17.04.95	0:1	100	100	-	0.70	-	4	
	Conus ventricosus	17.04.95	1:0	0	-	5.5	-	-	-	
	Nassarius reticulatus	19.04.95	0:1	100	100	-	0.40	-	0	
4 -Moinho-Marim	*Hexaplex trunculus*	17.04.95	6:6	50	100	8.90	5.53	24.0	4	
	Nassarius reticulatus	18.04.95	1:4	80	100	9.50	1.50	0.4	1.5	
7 -Canal Olhao	*Hexaplex trunculus*	18.04.95	2:7	78	100	8.85	3.56	6.5	4	
	Nassarius reticulatus	18.04.95	3:20	87	95	10.83	0.42	0.0	0.9	
6 -Culatra	*Hexaplex trunculus*	18.04.95	9:1	10	100	10.17	3	2.6	4	
	Murex brandaris	19.04.95	3:3	50	100	7.83	2	1.7	4	
6 -Culatra	*Ocenebra erinacea*	18.04.95	0:1	100	100	-	1.80	-	4	
	Nassarius reticulatus	18.04.95	10:19	66	47	9.40	0.00	0	0.2	
8 –Salva-vidas	*Ocenebra erinacea*	19.04.95	3:21	88	100	3.27	1.19	4.8	3.9	1 ♀ split oviduct

Site	Species	date	sex ratio m:f	% female	% females penis-bearing	mean penis length male	mean penis length female	index of imposex RPSI	index of imposex VDSI	signs of sterility
8 –Salva-vidas	*Nassarius reticulatus*	19.04.95	4:15	79	100	8.00	0.21	0	0.8	
9 –Farol (Bareta)	*Hexaplex trunculus*	19.04.95	2:2	50	100	10	1.5	0.34	2	
	Murex brandaris	19.04.95	2:2	50	100	8	3	5.3	4	
	Nassarius reticulatus	19.04.95	2:8	80	63	8.00	0.15	0	0.3	
10-Cais comercial	*Hexaplex trunculus*	18.04.95	4:4	50	100	9.88	5.83	20.5	4	
	Nassarius reticulatus	19.04.95	7:13	65	100	9.36	1.66	0.6	1.2	
11 -Ramalhete	*Conus ventricosus*	20.04.95	1:1	50	100	4.5	9	12.5	-	
12 -Portimao	*Ocenebra erinacea*	20.04.95	4:18	82	100	2.68	1.37	13.5	4	
	1996									
2 -Cacela	*Hexaplex trunculus*	17.04.96	4:3	43	100	8.88	2.43	2.1	4	
	Murex brandaris	16.04.96	2:1	33	100	6	2.75	9.6	4	
	Nassarius reticulatus	16.04.96	9:16	64	69	6.89	0.20	0	0.1	
	Conus ventricosus	16.04.96	13:6	32	100	4.33	1.38	3.3	4	
3- Cavacos	*Conus ventricosus*	19.03.96	9:7	44	100	5.64	1.39	1.5	4	
4 -Moinho-Marim	*Hexaplex trunculus*	18.04.96	0:2	100	100	-	3.3	-	4	
	Nassarius reticulatus	18.04.96	0:1	100	100	-	3.5	-	1	
6 -Culatra	*Hexaplex trunculus*	18.04.96	0:9	100	100	-	2.76	-	4	
	Ocenebra erinacea	18.04.96	0:1	100	100	-	1.00	-	4	
	Nassarius reticulatus	18.04.96	14:10	42	50	9.14	0.00	0	0.2	
8 –Salva-vidas	*Ocenebra erinacea*	17.04.96	2:21	91	100	2.90	1.50	14.0	4.1	1 ♀ split oviduct
	Nassarius reticulatus	17.04.96	3:21	88	90	8.33	0.29	0	0.5	
9 –Farol (Bareta)	*Nassarius reticulatus*	17.04.96	2:2	50	50	7.25	0.00	0	0.5	
10-Cais comercial	*Hexaplex trunculus*	17.04.96	1:4	80	100	9.5	5	14.6	4	
	Ocenebra erinacea	17.04.96	6:14	70	100	3.83	2.19	18.5	4	
10-Cais comercial	*Nassarius reticulatus*	17.04.96	6:3	33	100	8.08	0.67	0.1	1	
11 -Ramalhete	*Nassarius reticulatus*	18.04.96	16:6	27	100	9.53	1.30	0.3	1	
12 -Portimao	*Ocenebra erinacea*	18.04.96	12:11	48	100	2.68	1.42	14.8	4	

Concentrations of organotins (TBT and DBT) measured in the neogastropods tissues are shown in Table 7.4.

Extraction of organotins was carried out in all specimens collected in Algarve. However, due to problems during transport of hexane extracts from Portugal to Plymouth (UK), where further analysis were performed, 33 samples were lost and results presented bellow correspond to the remaining 13 samples.

Table 7.4 - Organotin (TBT and DBT) concentrations (µg g^{-1} Sn dw) in the whole soft tissues of male and female neogastropods collected in Algarve.

Site	species	Date	tissues organotin (µg g^{-1} Sn dw)			
			male		female	
			TBT	DBT	TBT	DBT
2- Cacela	*C. ventricosus*	16.04.96	0.057	0.039	0.128	0.012
	N. reticulatus	16.04.96	0.156	0.050		
	H. trunculus	17.04.96	0.052	0.000	0.033	0.008
	M. brandaris	16.04.96	0.180	0.005	0.094	0.035
6- Culatra	*H. trunculus*	18.04.96			0.056	0.040
	N. reticulatus	18.04.96	0.047	0.045		
	O. erinacea	18.04.96			0.166	0.017
8- Salva-vidas	*N. reticulatus*	17.04.96			0.041	0.062
	O. erinacea	17.04.96	0.556	0.085	0.095	0.055
9- Farol	*N. reticulatus*	17.04.96	0.259	0.225		
	H. trunculus	19.04.95	0.037	0.042	0.040	0.030
10-Cais Com.	*O. erinacea*	17.04.96			0.100	0.124
11- Ramalhete	*N. reticulatus*	18.04.96	0.057	0.044	0.045	0.045
12- Portimão	*O. erinacea*	18.04.96	0.129	0.075	0.275	0.097

Imposex was observed at all the sampled sites in Algarve, except in Zavial (13), where all females *Nucella lapillus* were unaffected by imposex (Table 7.3), probably reflecting a low TBT contamination in the area. According to studies performed with this species (Gibbs *et al.*, 1988) absence of imposex in *N. lapillus* females is probably a result of extremely low ambient TBT concentrations in water, since TBT concentrations as low as 0.5 ng L^{-1} Sn were shown to initiate imposex. Zavial is an exposed beach, near Sagres, with infrequent boating activity and consequently with low TBT inputs, in contrast with all the other sampled locations were boating activity is more

intense. Females of *N. reticulatus* and *N. lapillus* collected in exposed beaches, at the western part of Algarve, were also unaffected by imposex (Santos *et al.*, 2000; Barroso *et al.*, 2002). However, a low level of imposex was detected in 3-4% of females, in Ingrina, a beach next to Zavial (site 13) (Santos *et al.*, 2000; 2002). The proximity of the fishing port of Sagres may help to explain the presence of imposex in these females.

At all the other sampled sites in the Algarve, imposex was registered in 100% of the collected females, at least in one species. At the 12 sites, and from the 48 samples obtained (1 sample = 1 species at one site), 46 included both males and females, while in the remaining 2 samples only males were collected (*H. trunculus*, 5-Olhão; 1994; *Conus ventricosus*, 2-Cacela; 1995) (Table 7.3). In approximately 80% of the samples, all the collected females - from all species - were affected with imposex. In the remaining 20%, the proportion of affected females varied from 47-95% (Table 7.3). All unaffected females belonged to the species *N. reticulatus*. At some sites, including Cacela (2), Culatra (6) and Farol (9), *N. reticulatus* were collected along with other neogastropod species. Since all the specimens were probably exposed to similar environmental conditions, a comparison on the imposex incidence among species was possible (Figure 7.5).

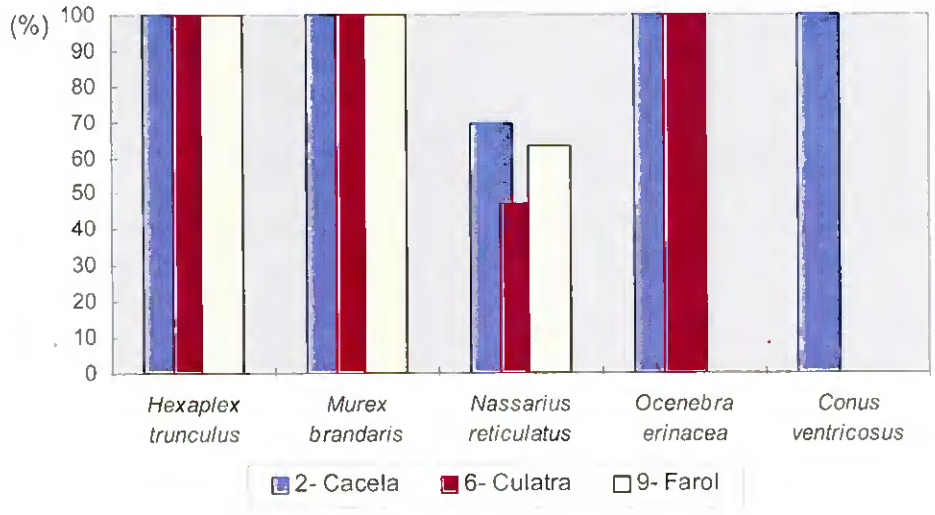

Figure 7.5- Proportion (%) of females affected by imposex, from different neogastropod species collected at Cacela (2), Culatra (6) and Farol (9), in the field survey.

Results illustrated in Figure 7.5 show that, in samples collected at Cacela (2), Culatra (6) and Farol (9), most species (*O. erinacea, H. trunculus, B. brandaris, C. ventricosus*) exhibited 100% of females affected by imposex. However, in the case of *N. reticulatus*, some unaffected females were also observed at the same sites, suggesting lower sensitivity of this species to TBT contamination. Other studies also demonstrated that this species was less sensitive than *Nucella lapillus*, although it was pointed as a useful alternative particularly in highly contaminated areas where the most sensitive species may already be in extinction or even extinct (Barroso *et al.*, 2002; Bryan *et al.*, 1993b; Gibbs *et al*, 1997).

From all the selected species of the Algarve coast, only *O. erinacea* showed signs of possible sterilisation. Two females collected at the Salva-vidas station (8) presented a split oviduct (Table 7.3 & Figure 7.2), which precludes a normal breeding function and probably leads to sterilisation (Gibbs *et al.*, 1990; Gibbs, 1996). Nevertheless, abundant egg capsules and juveniles were observed at the same site, confirming the existence of recruitment. Salva-vidas (8) is located near the main artificial inlet of the Ria Formosa - barra do Farol – (Figure 7.3) and consequently has a considerable boat traffic intensity. Concentrations of organotins in *O. erinacea* tissues, particularly in males, were notably high at this site (Table 7.4), confirming the existence of some TBT contamination in the area.

Other neogastropods observed, particularly *H. trunculus* , *N. reticulatus* and *B. brandaris,* showed an incidence of 100% of affected females, at all sites. At other locations world wide, imposex was also described for these species (see Gibbs and Bryan, 1994, Axiak *et al.*, 1995; Terlizzi *et al.*, 1998; Pellizzato *et al.*, 2004). Masculinisation of females, in these species, included the presence of a penis and a vas deferens, but no other characteristic malformations or indications of sterilisation were observed. Moreover, growth of the sperm duct did not appear to obstruct the vulva since it grew parallel to the oviduct.

At a few sites within the Ria Formosa another neogastropod species, *Conus ventricosus,* was also collected. Imposex has not been described for this species and although all females exhibited masculinisation (penis and

vas deferens), the general scarcity of specimens did not allow a detailed observation and comparison of males and females, neither conclusive observations on the possible interference of imposex with breeding activity in *C. ventricosus*. It is worth noting, however, that according to older fisherman, *C. ventricosus* were quite abundant in Ria Formosa 30 years ago and appear to have declined dramatically in abundance. The fact that all *C. ventricosus* found in Ria Formosa were adult individuals (no juveniles were observed) with a predominance of males (68%), may indicate a possible interference with reproduction and even mortality of females, in a similar way to the *N. lapillus* populations (Bryan *et al.*, 1986). However, further studies with *C. ventricosus* should be carried out to confirm this hypothesis.

A large variation was obtained on the neogastropods sex ratio, but no comparisons can be made due to the difference in the total number of specimens in each sample.

Although imposex was widespread, the intensity of imposex - described by the RPLI - in female gastropods collected along the Algarve coast, showed some variations among sites (Table 7.3). For *N. reticulatus*, higher RPLI values were obtained at (10) Cais Comercial (0.06-0.56), (4) Moinho de Marim (0.39) and (11) Ramalhete (0.25-0.28). Concerning *H. trunculus*, higher values were also observed at (4) Moinho de Marim (24.03) and (10) Cais Comercial (14.68-20.52). *Ocenebra erinacea* also presented higher RPLI values at (10) Cais Comercial (18.54), (12) Portimão (13.50-16.37) and the (8) Salva-vidas station (13.97). In the case of *C. ventricosus*, higher values were observed at (11) Ramalhete (30.75). These results indicate that along the coast, (10) Cais Comercial, near Faro, and (4) Moinho Marim, next to Olhão, appear to be the most contaminated sites, followed by, (11) Ramalhete and (12) Portimão. In fact, Cais Comercial is the only port within Ria Formosa used by large vessels, which were entitled to use TBT up to 2003. Similarly, in Portimão, some TBT pollution was probably related with the presence of large vessels that call the commercial harbour of the city. But, if at Cais Comercial some TBT contamination could be expected, this should not be the case for other sites in Ria Formosa such as Moinho de Marim and

Ramalhete where boat traffic is dominated by small vessels, prohibited from using TBT paints since 1993.

The use of imposex as an indicator of TBT pollution shows it to be widespread throughout the Algarve, particularly in the Ria Formosa. Virtually all mature females of the most sensitive species were highly masculinised and some signs of sterilisation were observed in two *O. erinacea* females. At present, there appears to be no threat to the neogastropod's population. However, a slight increase in TBT contamination, particularly inside Ria Formosa, could bring serious consequences for these populations.

Overall, *Nassarius reticulatus, Hexaplex trunculus,* and *Ocenebra erinacea* showed a wide distribution in Algarve. Considering both the wide distribution of species and its high sensitivity, results from this work suggest that *H. trunculus* and *O. erinacea* present a good potential as bioindicators for TBT contamination in this region.

Further studies should be performed with the species *Conus ventricosus*, in order to identify the possible causes of a decline in the Ria Formosa populations. Laboratory studies with species observed in the Algarve coast could also be useful to identify concentrations that initiate imposex, in different neogastropods. In addition, research on the sterilisation of females from various species should also be performed.

Results of imposex in gastropods will not be related to TBT concentrations measured in water in the TBT field survey (Coelho *et al.*, 2002c - Chapter 6) since the two surveys were not concomitant in time. However, taken together, data from the two surveys (TBT levels and imposex) suggest a wide TBT contamination in Algarve.

In the present work, identification of gonochoristic neogastropod snails exhibiting imposex was shown to be a valuable complementary tool for the assessment of TBT contamination in Algarve. Furthermore, imposex is recognised as an extremely sensitive ecotoxicological bioassay and its continuous use should allow potential areas of risk to be identified and monitored in the future.

Chapter 8

GENERAL DISCUSSION

Aquatic toxicology has been defined as the study of the effects of chemicals on aquatic organisms with special emphasis on adverse or harmful effects (Rand *et al.*, 1995). The present work intended to assess the possible harmful effects of TBT leached from antifouling paints, in clams *R. decussatus*, one of the most commercially important species in southern Portugal. Three aspects of ecotoxicology were examined, namely: (1) the possible toxic effects of TBT to early life stages of *R. decussatus*; (2) the preferential route of TBT uptake in this species and (3) an assessment of TBT contamination in the field.

8.1. Toxicity of TBT to early life stages of clams

Molluscs, and in particular the early life stages of bivalves, are among the most sensitive *taxa* to TBT. Exposure of embryos and larvae to levels up to 100 ng L^{-1} TBT was linked with harmful effects, in different bivalve species such as mussels, oysters and clams (Table 1.8). But, how toxic is TBT to the larvae and juveniles of *R. decussatus* ?

Toxicity studies performed in this study with *R. decussatus* larvae have confirmed the high toxicity of TBT to this species of clams. TBT levels as low as 25 ng L^{-1} Sn ($\approx 10^{-10}$ M TBT) in water were shown to inhibit *R. decussatus* larval growth and development. For bivalves, the larval period of their life cycle is crucial since it determines the successful dispersal of the species, culminating in the metamorphosis and settlement as a benthic organism (Dame, 1996). Therefore, recruitment is dictated from the ability of larvae to successfully complete their development. Low concentrations of TBT in the water column may, in the long run, pose serious risks to recruitment and hence to the *R. decussatus* abundance in coastal waters, as observed with oyster populations both in Arcachon bay (Alzieu, 1991) and in the Tejo estuary (de Bettencourt *et al.*, 1999) when TBT was initially introduced in antifouling paints.

Results obtained in this study confirm indications of reductions in growth and development shown for other bivalve embryos and larvae,

including mussels *M. edulis*, oysters, *C. gigas* and *Pinctada fucata*, and clams *S. plana* (His *et al.*, 1983; Inoue *et al.*, 2004; Lapota *et al.*, 1993; Ruiz, 1995a; Stenalt *et al.*, 1997).

In the field survey performed in Ria Formosa (Coelho *et al.*, 2002c - Chapter 6), levels up to 34 ng L^{-1} Sn were observed in water and are common in low-moderately contaminated environments (Estuaries, bays and maricultures - see Table 1.3). Results from this work with *R. decussatus* larvae (Coelho *et al.*, 2001 - Chapter 3) indicate that concentrations within this range (≥ 25 ng L^{-1} Sn) could pose a threat to bivalve larvae, including *R. decussatus*, which inhabit coastal waters, particularly sheltered lagoons as Ria Formosa. In order to prevent damaging effects on *R. decussatus* populations, which support a very important commercial and social activity in Ria Formosa, a permanent surveillance on TBT concentrations should be carried out in the area.

During the course of the experiments with *R. decussatus* larvae, it was not possible to derive the 'No Observed Effect Concentration' (NOEC), since the lowest tested concentration (25 ng L^{-1} Sn) induced deleterious effects on larvae development. Deleterious effects on growth of *M. edulis* post-larvae were observed at extremely low TBT concentrations (2.3 ng L^{-1} Sn) (Stenalt *et al.*, 1998). Probably, exposure to TBT concentrations lower than 25 ng L^{-1} Sn could induce harmful effects on larval development and the NOEC value should lie within the interval of 0-25ng L^{-1}. However, further research should be performed to test this hypothesis.

Microlayer enhancement of TBT concentrations (GESAMP, 1995) can be an additional threat to planktonic larvae. In the case of TBT, microlayer enrichment can be one order of magnitude over the sub-surface waters (Cleary & Stebbing, 1987; Hall *et al.*, 1987). Clam embryos and larvae which may temporary inhabit the surface microlayer, may be seriously affected by exposure to high levels of TBT found in this surface layer. It would be interesting to study this subject under laboratory and field conditions in the future.

Comparing with other stages of the life cycle, namely juveniles and adults, larvae were shown to be the most sensitive stage to dissolved TBT, as suggested for other bivalve species (Stenalt *et al.*, 1997). Nevertheless, additional investigations are required on the effects of contaminated water and sediments on settling larvae and juveniles. In the later, growth and burying activity, were shown to be adversely affected by exposure to TBT concentrations in water (0.05-0.5 µg L^{-1} Sn and 0.5-4 µg L^{-1} Sn, respectively) and also in sediments (0.4 µg/g Sn dw) for the species *S. plana* (Ruiz, 1993) Settling pediveligers of *S. plana* exposed to TBT concentrations of ≥ 70 ng L^{-1} Sn in water, suffered significant mortalities (Ruiz, 1995a). In order to protect settling larvae and juvenile clams, the establishment of Environmental Quality Standards is required not only for the water media but also for the sediments. Still, further studies should be performed, particularly on the toxicity of TBT contaminated sediments for *R. decussatus* settling larvae and post-larvae.

There are indications that TBT may also affect developing eggs. At concentrations in the range of 10^{-9} - 10^{-4} M, TBT was shown to be a potent cytotoxic compound to the eggs of sea urchin, blocking egg cleavage and suggesting an inhibition of fundamental cellular processes such as protein synthesis and DNA synthesis (Girard *et al.*, 1997). Studies on the toxicity of TBT at the cellular level should also be performed in bivalves to better understand the mechanisms of toxicity of this compound.

8.2. Routes of TBT Uptake in R. decussatus

The accumulation of chemicals in organisms is of major importance in environmental hazard assessment. Internal concentrations in the body may increase, by accumulation, to a level that induces toxic effects. The extent to which compounds accumulate and the preferential routes by which they are taken up, are of great concern in the study of a toxic compound such as TBT.

Clams, *R. decussatus* were shown to accumulate TBT in their soft tissues. However, bioaccumulation presented a significant variation with the different routes of uptake studied (water, sediments and diet). Experimental work with *R. decussatus* led to the conclusion that water acts as major uptake

pathway, although a minor fraction of TBT is also incorporated via benthic sediments and phytoplankton. It is suggested that feeding habit is of obvious major significance in terms of determining which TBT-phase poses most risk to bivalves. Higher accumulation from the water route is probably linked to the suspension feeding habits of this species while, in contrast, deposit feeding clams as *S. plana*, exposed to TBT in similar conditions, exhibited preferential uptake from sediments (Langston & Burt, 1991).

In extrapolating laboratory results as with the ones obtained in this work, to a field situation, extreme caution has to be taken to account for the multitude of factors that interact in the field. In the case of *R. decussatus*, results obtained in this study appear to be environmentally relevant since realistic TBT concentrations were used in the experiments. However, different ecological conditions, such as the density of phytoplankton populations in the water column, may influence the degree of deleterious effects that will arise from TBT contamination in the field.

Another aspect that should be taken into consideration is the interaction between TBT and other pollutants that occur simultaneously in the environment and its influence on TBT accumulation in clams. A synergistic interaction may occur (Batley, 1992; Fernandez-Alba *et al.*, 2002) and toxicity and accumulation in tissues could be even higher than predicted from laboratory experiments, thus posing higher risk to bivalves. However, more evidence is required to better understand these possible interactions.

It is probable that, for *R. decussatus*, the risk posed by TBT in water is higher than TBT bound to sediments or phytoplankton. Therefore, together with the new European Regulation, further restrictions should be established for TBT in water. If bioaccumulation is to be prevented in *R. decussatus*, a maximum allowable concentration - or equivalent measures such as Environmental Quality Standards (EQS) or water quality criteria - should be set for the water media. Moreover, results from experiments with *R. decussatus* larvae, confirm that water appears to be the appropriate media upon which to apply regulations to protect *R. decussatus* early-life stages. In addition, studies with phytoplankton suggested that low TBT concentrations

(0.01-0.6 nM ~ 3-200 ng L^{-1} TBT), usually found in low-moderately contaminated areas, may alter the typical species succession of phytoplankton communities (Petersen & Gustavson, 1998), confirming the deleterious effects of dissolved TBT on coastal environments.

8.3. Adverse effects and intake of TBT by humans

In recent years, research has indicated adverse changes in the reproductive health and fecundity of different animal species and humans. Laboratory studies have pointed out that some chemicals in the environment, both natural and synthetic, have the potential to disrupt the endocrine system, and could be partly responsible for the observed changes. Organotins are among the synthetic chemicals identified as possible endocrine disrupters (Harrison *et al.*, 1997; Oetken *et al.*, 2004). In addition, a critical immunotoxic effect was detected in humans with the suppression of natural killer cell activity by tributyltin (Ghoneum *et al.*,1990; Whalen *et al.*, 1999; Whalen *et al.*, 2002). Furthermore, the detection of organotins in human blood samples raised the level of concern regarding human exposure to organotins (Kannan *et al.*, 1999).

Since organotins accumulate in the marine food chain, TBT and its breakdown products have been detected in different marine organisms such as fish, squid, shellfish, and in top predators as whales, dolphins, seals and fish-eating birds (see review by Belfroid *et al.* (2000) and WWF (1999)). Taking into account that *R. decussatus* is an edible species, bioaccumulation of organotins should be prevented to avoid risks for humans as a result of the ingestion of TBT contaminated shellfish. Besides clams, gastropods, mainly *Hexaplex trunculus* and *Murex brandaris*, are also edible shellfish and should also be considered.

For human consumption, a tolerable daily intake (TDI), quantifying the maximum dose of TBT considered safe, when taken in man, has yet to be established in the European Union. However, some TDI values for TBT have been proposed, in different countries, and range from 0.25 µg / kg body weight / day (ww) (Penninks, 1993) up to 3.2 µg kg^{-1} body weight (ww) (Schweinfurth & Gunzel, 1987). The value of 0.25 µg / kg body weight / day derived by

Penninks (1993) is generally accepted and it means a tolerable daily intake (TDI) of 15µg TBT per person per day for a 60 kg person (Belfroid *et al.*, 2000; WWF, 1999;).

R. decussatus collected in the field survey (Ria Formosa) exhibited TBT tissue concentrations up to 0.34 $\mu g g^{-1}$ Sn (dw). Clams were collected at culture sites, thus similar concentrations could be found in marketable clams. Assuming a dry weight percentage of 10%, TBT concentrations in *R. decussatus* from the Ria would be 0.085 $\mu g g^{-1}$ TBT (ww). Considering an average body weight of 60 kg, and the TDI (15µg TBT per person per day) for human consumption, approximately 180 g of clam flesh would have to be ingested daily to exceed the referred TDI values for TBT. Such a daily consumption is possible taking into account that the average seafood consumption in Portugal is very high (160 g per day per person)(FAO, 1998). A similar approach was presented by Belfroid *et al.* (2000). Based on the referred TDI, and the average seafood consumption in different countries, these authors calculated the Tolerable Average Residue Levels (TARL) for TBT, in seafood. For Portugal, the calculated TARL was 93 ng TBT / g seafood product (ww) for an average person of 60 Kg. The maximum TBT concentrations detected in clams *R. decussatus* (85 ng /g ww) are close to the TARL, raising some concern over commercially exploited shellfish, especially bivalves. Average residue levels are used to establish whether a population may be at risk, through dietary intake. In the case of Portugal, the residue levels of TBT in clams may be close or exceeding TARL. Thus, specific consumption recommendations should be published and maximum residue levels (MRL) for TBT, and other organotin compounds, in seafood products should be set to minimize human risks.

8.4. Field Survey

TBT concentrations measured in three environmental compartments (water, sediments and clams) in Ria Formosa are within the range of those reported for low-moderately contaminated estuaries and mariculture areas world-wide.

There was a considerable spatial and seasonal variation in TBT levels determined in samples from the Ria Formosa lagoon, although no evident seasonal pattern was observed (Table 1.2).

As far as the water media is concerned, higher TBT levels were detected in the Olhão area, where a major fishing port and a small leisure harbour are located. Following a similar trend, higher levels of this compound were also detected in tissues of *R. decussatus* near Olhão. These results suggest that fishing and leisure vessels, mainly from the port of Olhão, were the most important source of TBT contamination in Ria Formosa, at the time of sampling (1992-93). Thus, TBT leached from antifouling paints was probably responsible for the greater input of this compound in the area, although some contamination may also result from the slow release of TBT from sediments to the water column, as registered at some sites in the UK (Langston *et al.*, 2000; Langston *et al.,* 1994). Data from TBT concentrations obtained in Olhão, in more recent surveys, showed little evidence of reduction in TBT burdens, at site (Bebianno *et al.*, 2002; Langston *et al.,* 1997). Hence, periodical monitoring is crucial and should be carried out to observe the validity of restrictions in Portugal and possible decline in TBT levels in the environment.

A study on the progression of a parasitic disease in TBT-exposed oysters suggested that chronic TBT exposure could increase the progression of *Perkinsus marinus* infection in *C. virginica* as well as disease related mortality (Anderson *et al.,* 1996; 1998). At times, *Ruditapes decussatus* from Ria Formosa were shown to be infected by this parasite and thus, the potential synergistic effect of TBT and parasitic diseases in this species should be investigated.

As stated earlier, an Environmental Quality Standard should be set for water, although criteria to derive this standard for TBT is still not well established. According to experts (OSPAR, 1994), available ecotoxicological data on TBT should be considered and following the use of a so called 'application factor' an extrapolated concentration, or range of concentrations, should be set as assessment criteria for TBT. Thus, considering available

toxicity data and 'application factors', the following provisional ranges have been suggested for TBT (EPA, 2002; OSPAR, 1994):

water (µg/L)	sediment (µg/g dw)*	mussel tissue (µg/g dw)
0.0001-0.001	0.0001-0.001	0.05-0.5

* sediments with 1% organic carbon

Although these values were considered provisional and should only be used as guidelines, most of the water and sediment TBT concentrations observed in Ria Formosa exceeded the higher value of the described ranges. This is a further indication of the urgent need to enforce the new legislation that bans the use of TBT and, eventually, establish quality standards in Portuguese coastal waters.

8.5. Imposex

Additional evidence of TBT pollution in the Algarve region was obtained from the survey performed to assess the degree of imposex in neogastropod females from this region.

Six neogastropod species were studied including *O. erinacea, H. trunculus, B. brandaris, C. ventricosus, N. reticulatus* and *N. lapillus*. Results indicate that TBT pollution is widespread throughout the Algarve, since virtually all mature females collected were masculinised. An exception was observed for females of *N. lapillus* obtained in the area of Sagres, which were not affected by imposex.

Imposex assessment was carried out mainly inside Ria Formosa, from Cacela to Praia de Faro. Data obtained in this area points to a higher TBT contamination at the Cais Comercial, in Faro and Moínho de Marim, near Olhão. Cais comecial is the only commercial port within the Ria Formosa called by large vessels - potential sources of TBT. Boat traffic near Moínho de Marim is dominated by small leisure and fishing vessels, presumably not allowed to use TBT-based paints.

Results from imposex assessment in Ria Formosa are in agreement with those obtained in the field survey (Coelho *et al.*, 2002c - Chapter 6), where higher TBT burdens were detected in water and clam samples from the Olhão area - sampling was not carried out in Faro. However, imposex cannot be directly related with TBT levels obtained in water samples from the field survey, because the two studies were not carried out simultaneously.

The fact that, in neogastropods, TBT interferes with the endocrine system raises a great concern over the potential effects of this compound on other organisms (Oetken *et al.*, 2004), including humans. There is evidence of a significant interaction of TBT with the androgen metabolism of marine molluscs (Morcillo & Porte, 2000; Morcillo *et al.*, 1998), but no investigation was carried out on higher organisms. No direct evidence exists to indicate a causal link between TBT exposure and adverse reproductive and developmental effects on mammals or humans. However, TBT may potentially disrupt the endocrine system (Harrison *et al.*, 1997) and clearly further studies are needed to assess the risk of TBT exposure.

8.6. Alternatives to the use of TBT

The difficulty in finding an 'ideal' biocide for antifouling paints, arises from the fact that such a compound should only be toxic to target species (including algal spores and barnacles) and in addition be rapidly converted into substances which are biologically harmless. Restrictions on the use of TBT in marine antifouling paints have lead to an increase in the investigation and use of alternative antifoulants.

In the 90's, research on biocidal antifoulings was focused on non-metallic organic compounds that, theoretically, would not persist in the environment due to rapid degradation in sea water and thus would have little or no impact on non-target organisms (Callow, 1990). However, as demonstrated in the case of TBT, new compounds are usually authorised until their deleterious effects to the environment is recognised.

Since the restrictions on the use of TBT that copper compounds have been utilised again, as the principal biocide. However, a number of booster

biocides have been used in conjunction with copper to improve its antifouling characteristics (Konstantinou & Albanis, 2004). An example of a new booster biocide is the triazine herbicide 'Irgarol 1051' (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-s-triazine), which is now used in over 80 antifouling paints. Despite its efficacy, 'Irgarol 1051' was found to be extremely toxic to zoospores of *Enteromorpha intestinalis* at a concentration of 100 ng L^{-1} 'Irgarol 1051', in contrast with a *no effect concentration* of 200 ng L^{-1} 'Irgarol 1051' quoted by the manufacturer (Scarlett *et al.*, 1997; van Wezel & van Vlaardingen, 2004). A comparative environmental assessment of biocides, used in antifouling paints, was published by Voulvoulis *et al.* (2002). This study focused on nine booster biocides including chlorothalonil, dichlofluanid, diuron, Irgarol 1051, zinc pyrithione, zineb, kathon 5287, TCMTB and TCMS pyridine, recently approved as active ingredients in antifouling products in the UK. As a conclusion, three biocides (dichlofluanid, TCMTB and TCMS pyridine) demonstrated similar environmental characteristics to TBT; four other biocides (chlorothalonil, Irgarol, Kanthon and diuron) widely used nowadays, were found to present insufficient data on its toxicity and persistence in the environment. Only the use of zinc pyrithione and zineb was pointed out as less hazardous for the aquatic environment. If research on the antifouling efficacy of these two biocides proves that they are as effective as TBT, then a replacement of organotin products by them may be beneficial to the environment.

Another compound, thriphenyltin (TPT), widely used as agricultural fungicide, has also been proposed as an alternative biocide to TBT in antifouling paints. However its high toxicity has been demonstrated (Jarvinen *et al.*, 1988; Semlitsch *et al.*, 1995) and thus, its utility as an alternative to TBT is now doubtful. The addition of ammonium salts to a vinyl copolymer (Mellouki *et al.*, 1989), and the use of antibiotics in the paints (Clark, 1987) were also suggested as alternatives to prevent fouling.

More 'environmentally friendly' compounds, mainly extracts from marine organisms that maintain foul-free surfaces, are also being tested as potential natural antifoulants (Rittschof, 2000). Essentially, marine natural antifoulants are extracts from marine organisms of different *phyla* including

Cnidaria, Porifera, Bryozoa, Chordata and macroalgae Thallophyta and Angiospermae (Clare, 1996). Lately, there has been a significant increase in investigations on these natural chemical defences against biofouling, but in only a few studies have the natural antifoulants been isolated and chemically characterised (see review by Clare, 1996).

Uncertainty on the future of TBT containing paints combined with the long and costly procedures for gaining approval from governmental authorities for the use of new biocides is leading to new forms of fouling control. Research is now being taken on the use of non-biocidal antifouling coatings that, instead of releasing a biocide, act by altering the substrate texture, utilising the concept of low energy surfaces associated to a weak adhesion of organisms. The main types of coatings include fluoropolymers (Bultman & Griffith, 1984; 1994), silicone elastomers (Rittschoff, 2000; Callow *et al.*, 1986) and the 'thethering' of drag-reducing molecules such as polyox (polyoxyethylene) to surfaces (Gucinski *et al.*, 1984). However, the use of these substrate textures has some drawbacks: some accumulation of fouling organisms still occurs, although their attachment is weak; thus they do not prevent biofouling and require regular cleaning of the vessels hulls; in addition, the current generation of these coatings is not sufficiently robust for most applications including shipping (Clare, 1996). Furthermore, both ultrasonic waves (14kHz) and low frequency sound waves (30 Hz) were shown to inhibit barnacle settlement and may have some application to fouling control, in certain circumstances (Branscomb & Rittschoff, 1984; Suzuki & Konno, 1970).

In summary, although a large number of alternatives have been evaluated, none of these methods can still compete with organotin antifouling paints and clearly there is an urgent need to develop all the potential 'non-toxic' antifoulants as alternatives to the widely used organotin paints.

Some authors argue that until an effective alternative to TBT has been developed and approved, a TBT ban will pose serious environmental problems on a global scale and the environmental benefits from restricting TBT are yet to be assessed. According to these authors, a ban on TBT antifouling paints will the increase ships fouling leading to (1) an increase in

fuel consumption, and consequently to higher carbon dioxide and sulphur dioxide emission; (2) a more frequent docking of ships, rising the need for additional docking capacity and increasing the volume of waste; (3) a shift towards less environmentally friendly modes of transport such as land and air and (4) a transference of TBT contamination to countries with less restrictive legislation, usually less able to deal with such a problem (Abel, 2000; Champ, 2000; Strandenes, 2000).

8.7. Final remarks

Results from this work constitute a further step to confirm that TBT is among the most toxic chemicals introduced in the marine environment. Its potential for negative impact on the benthic community is evident. In the particular case of *R. decussatus* cultured in Ria Formosa and other important mariculture sites (including Ria de Alvor and Sado estuary), a continued input of organotins will probably cause serious damage to shellfish species, particularly to its early life-stages.

Until recently, most legislation worldwide focused on small vessels, based on the belief that the effects of TBT were restricted to inshore waters and caused mainly by input from leisure vessels. However, TBT has continued to be used in larger vessels and (1) the perception of open sea effects correlated with shipping lanes (Ten Hallers-Tjabbes *et al.*, 1994; 2002), (2) the recognition of deleterious effects caused by large vessels in coastal environments (Santos *et al.*, 2002; Langston *et al.*, 1997) together with (3) the detection of organotin contamination in organisms of deep-sea and remote ecosystems (Negri *et al.*, 2004; Takahashi *et al.*, 1997), raised further concerns on this subject.

Taking all this into consideration, international attention has finally focus on the 'TBT problem' and **Chapter 17 (17.32) of Agenda 21** adopted by the United Nations Conference on Environment and Development, in 1992, calls upon States to take measures to reduce pollution caused by organotin compounds used in anti-fouling systems. On the 5th October 2001, the Marine Environmental Protection Committee (MEPC) of the International Maritime Organization (IMO) adopted a new "International Convention on the

Control of Harmful Anti-fouling Systems on Ships" (**AFS-Convention**) that will ban TBT used on antifouling paints, on a global scale, by 2008. Even though it was adopted, it will only enter into force 12 months after 25 States (representing 25% of the world's merchant shipping tonnage) have ratified it. Up to September 2004, the Convention was not ratified. This type of concerted action is probably the only solution for such a toxic compound as TBT.

Based on the AFS Convention, the European Union adopted, on the 5th May 2003, a Regulation on the prohibition of organotin compounds on ships (Regulation (EC) 782/2003 of the 14th April 2003), as an attempt to "reduce or eliminate the adverse effects on the marine environment and human health caused by organotin compounds". According to this regulation, from July 2003 organotin compounds shall not be applied on ships operating under the authority of a Member State and 1st January 2008 will be the last date for having TBT-based antifouling paint on any ship sailing to or from ports of the EU Member States, regardless of the flag they fly.

Although the sale and use of TBT-based antifouling paints is now banned in Portugal, significant TBT concentrations in the environment are likely to occur in the near future (Barroso & Moreira, 2002; Santos *et al.*, 2002), as a result of leaching from TBT painted vessels that do not operate under the authority of a EU Member State or as a consequence of slow release from the sediment reservoir into the water column. Thus, as stated earlier, in order to protect the coastal environment the enforcement of the new European Regulation and the establishment of Environmental Quality Standards, in Portugal, should be considered.

According to the World Health Organisation (WHO, 1990), recommendations for protecting human and environmental health against the damaging effects of TBT include (1) the establishment of restrictions on the use of TBT compounds; (2) evaluation of organotins inputs to the environment from sources other than antifouling paints; (3) improvement of methods for the safe application removal and disposal of organotins. Furthermore, future research needs comprise improvement of detection and analysis of organotins and better knowledge of toxicity, namely immunological

effects, mechanisms of toxicity, endocrine effects and mammalian toxicity including studies on potential carcinogenic effects. Attention should also be given to the search for other sensitive bioindicator species, including freshwater organisms and finally more information is required on butyltin residues in fish and shellfish for human consumption.

It is important to emphasise that once a compound, as TBT, is found to be environmentally dangerous and is banned, it is inevitably replaced by others, which may cause its own, probably less well-known, problems. Thus, the fate and effects of chemicals introduced, in the future, as alternatives to TBT into the marine environment should be kept under close and continued control.

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