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UNIVERSITY OF SOUTHAMPTON

ORGANOTIN AND OSMOREGULATION: QUANTIFYING THE EFFECTS OF ENVIRONMENTAL CONCENTRATIONS OF SEDIMENT-ASSOCIATED TRI-N-BUTYLTIN AND TRIPHENYLTIN ON OSMOREGULATORY PROCESSES IN THE EUROPEAN FLOUNDER PLATICHTHYS FLESUS (L).

by

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A Thesis submitted to the University of Southampton
for the Degree of Doctor of Philosophy

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

SCHOOL OF OCEAN & EARTH SCIENCE

Doctor of Philosophy

ORGANOTIN AND OSMOREGULATION: QUANTIFYING THE EFFECTS OF ENVIRONMENTAL CONCENTRATIONS OF SEDIMENT-ASSOCIATED TRI-N-BUTYLTIN AND TRIPHENYLTIN ON OSMOREGULATORY PROCESSES IN THE EUROPEAN FLOUNDER PLATICHTHYS FLESUS (L).

by Mark G. J. Hartl

The disruption of physiological and morphological aspects of osmoregulatory processes in freshwater-adapted 0-group European flounders, Platichthys flesus (L.), caused by exposure to environmental concentrations (150 ng triorganotin g⁻¹ dry weight sediment) of sediment-associated tri-*n*-butyltin chloride (TBTCl) and triphenyltin chloride (TPhTCl), was examined and quantified. Radiotracers were used to measure hydromineral fluxes, the water balance and passive sodium efflux of chronically (35 days) exposed fish. The water permeabilities of exposed flounders varied during the course of the experiment and were significantly lower than the corresponding controls, that did not change significantly with time. It was found that the maximum change in water permeability of TBTCl- and TPhTCl-exposed fish occurred after 14 days and 21 days, respectively; thereafter there was an increase towards control values, suggesting adaptation to compensate for the effects of the organotin exposure. Drinking rates increased significantly in both organotin groups but urine production rates did not change. The effects of organotin exposure on the passive sodium efflux and Na⁺/K⁺-ATPase activity showed an inverted relationship in the TBT group, where the Na⁺/K⁺-ATPase activity was reduced and the passive sodium efflux was increased. TPhT had no inhibitory effect on Na⁺/K⁺-ATPase activity and the passive sodium efflux increased only gradually. The overall effect of these changes in these components of hydromineral regulation was to reduce the mean blood osmolalities of the organotin groups compared to the control values.

The effects of chronic exposure to sediment-associated triorganotin compounds during sea water adaptation was examined and quantified by measuring the active sodium efflux, Na⁺/K⁺-ATPase activity and structural changes to the gill epithelium usually encountered in euryhaline fish during adaptation to sea water. Following the transfer to sea water, the Na⁺/K⁺-ATPase activity and the active sodium efflux were decreased in the TBT group but increased significantly in both the TPhT and control groups. Similarly, the morphological changes to the gill epithelium, involving chloride cell distribution, associated with sea water adaptation, took place in the control group and only partially in the TPhT group but were significantly inhibited or delayed in the TBT group. The exposure to organotin caused the mean blood osmolalities in fish of the TBT and TPhT to rise beyond the expected values that were observed in the control group.

The results presented in this study lead to the conclusion that tri-*n*-butyltin chloride and triphenyltin chloride in sediments are capable of significantly disrupting both the physiological as well as morphological components of osmoregulatory functions of an estuarine fish, at concentrations currently found in local sediments.

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*To my parents Pat & Herold Hartl,
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List of publications from this project

- Hartl, M. G. J., Hutchinson, S. & Hawkins, L. E. (in press). Organotin and osmoregulation: quantifying the effects of sediment-associated TBT and TPhT on freshwater-adapted European flounders (*Platichthys flesus* L.). *J. exp. Mar. Biol. Ecol.*
- Hartl, M. G. J., Hutchinson, S., Hawkins, L. E. & Eledjam, M. (2000). The biometry of gills of 0-group European flounder, *Pleuronectes flesus* L. *J. Fish Biol.* 57/4: 1037-1046.
- Hartl, M. G. J., Hutchinson, S. & Hawkins, L. E. (2000). Reduced blood osmolality in freshwater-adapted European 0-group flounder, *Platichthys flesus* (L.), exposed to environmental levels of sediment-associated tributyl- and triphenyltin. *Mar. Pollut. Bull.* 40/9: 792-794.

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- Hartl, M. G. J., Hutchinson, S. & Hawkins, L. E. (2000). Effects of sediment-associated organotin compounds on the osmoregulation of 0-group European flounders, *Platichthys flesus* (L.). In *Fish Toxicology - Symposium Proceedings International Congress on the Biology of Fish* (eds. C. J. Kennedy & D. MacKinlay) Am. Fish. Soc., Aberdeen, Scotland, UK, 23-27 July, 2000: 67-82.
<http://www-heb.pac.dfo-mpo.gc.ca/congress/Papers/toxicologypdf/hartl.pdf>.
- Hartl, M. G. J., Hutchinson, S. & Hawkins, L. E. (1999). Organotins and osmoregulation: Quantifying the effects on the European flounder *Platichthys flesus*. In *Proceedings of the International Marine Pollution Symposium* (ed. P. Povinec) IAEA, Vienna, Monaco. pp 487-488.

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Chapter 1 – General Introduction

The European flounder *Platichthys flesus* (L.), family Pleuronectidae, is a common euryhaline flatfish found around the coasts of Europe, from the White Sea to the Black Sea. It is a catadromous species, feeding and growing in estuaries during the summer and returning to the sea in winter, where mature age groups spawn (Wheeler, 1969). According to FAO statistics (FAO, 1998), the economic significance of flounders is ranked behind that of other species of flatfish. However, their hardiness, euryhalinity and the fact that flounders are readily available, makes them ideal subjects for osmoregulatory physiology experiments.

In order for flounders to survive in estuarine habitats and to exploit this highly productive environment, a number of physiological adaptations have evolved, that have been extensively studied on a variety of developmental stages, ranging from eggs, larvae and 0-group (Hutchinson, 1984; Hutchinson & Hawkins, 1990; 1993) to mature adults (Henschel, 1936; Motais, 1961a, b; Mactz & Evans, 1972; Smith *et al.*, 1975). These studies found flounders to be hyperosmoregulators in fresh water, with blood osmolarities ranging from 240 to 304 mOsmol l⁻¹ and hyposmoregulators in sea water, displaying blood osmolarities between 297 and 364 mOsmol l⁻¹.

The study of the physiological mechanisms for ionic- and osmoregulation has a long tradition, beginning with Smith (1930; 1932) and Keys (1931) and has since been widely investigated and is summarised below.

In the case of marine osmoconformers, such as many invertebrates, seawater acts as the extracellular medium without the blood acting as a buffer. Osmolality is controlled by the swelling of the coelomatic cavity or cells with specialised vacuoles. In contrast, the blood of freshwater-adapted fish is hyperosmotic with respect to the external medium, causing an increase in osmotic water influx and a loss of electrolytes along the respective concentration gradients. In order to maintain constant intracellular conditions enabling efficient cellular function, a number of regulatory mechanisms have been developed, including the regulation of body surface permeability for water, the maintenance of an electrochemical gradient across the gill membrane in order to conserve ions and the control of drinking and urine production. The osmotic water flux across the body surface occurs mainly across the thin gill membranes (Evans, 1969b). The water permeability of gills can

be regulated by adjusting the fatty acid composition in the gill membranes (Motais *et al.*, 1969a; Morris *et al.* 1987). This physio-morphological response is accompanied by an endocrine-controlled substantial reduction of drinking rates (freshwater-adapted fish drink occasionally) and the production of large amounts of diluted urine (Evans, 1969b, Motais *et al.*, 1969b). In addition to these osmoregulatory mechanisms, measures are undertaken to replenish lost ions by an active ionic influx across the gills (Fig. 1.1) facilitated by the activity of ATPases in specialised cells, the chloride cells, of the secondary or lamellar gill membrane.

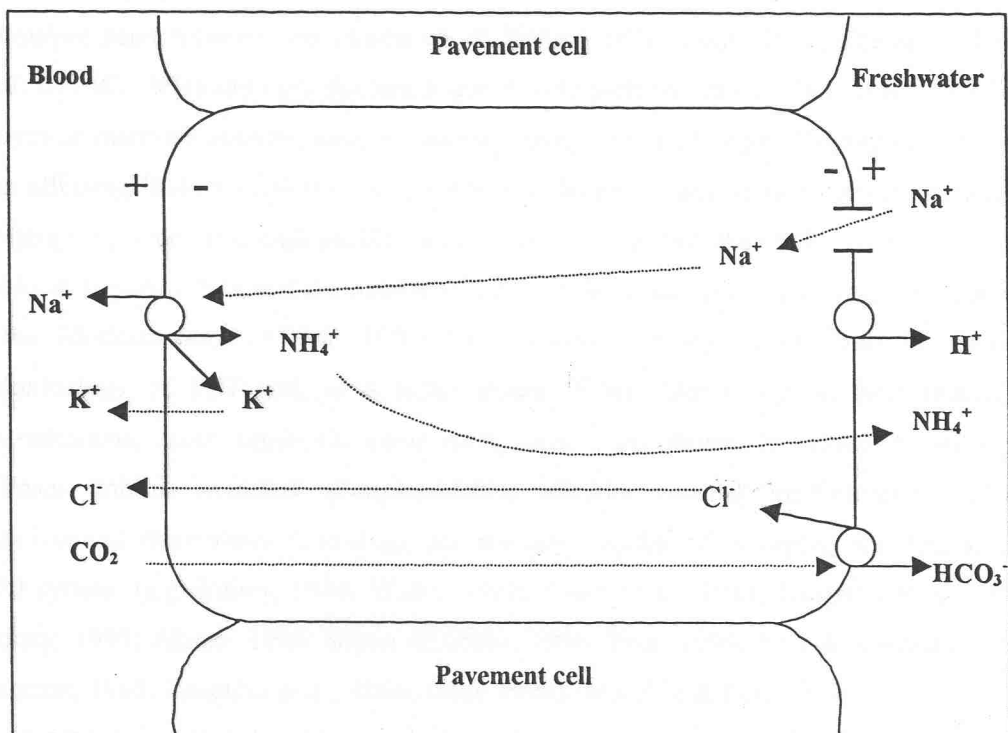


Fig. 1.1. Schematic representation of active ionic uptake in freshwater-adapted euryhaline fish. Circles represent membrane-bound ATPases (from Evans, 1999)

In sea water the situation is reversed: the fish is hypoosmotic in respect to the environment and therefore faces constant osmotic water loss. The water balance is maintained by the frequent drinking of sea water and a reduction of urine production (Evans, 1984). Excess ions are actively excreted by chloride cells in the primary gill membrane (Fig. 5.1).

A wealth of literature on osmo- and ionoregulatory mechanisms is available and has been summarised in a number of reviews (see Potts, 1968; Maetz, 1970; 1971; Motais & Garcia Romeau, 1972; Evans, 1984; Payan & Girard, 1984; Perry, 1997).

The high productivity in most estuarine environments presents ideal nursery grounds for juvenile flounders (Summers, 1974; 1979; Gibson, 1997). However, many estuaries also attract a high level of human activity, of both commercial and recreational nature. Therefore, estuaries are often also the most heavily polluted marine environments, due to communal and industrial effluent discharge and pesticide input from agricultural runoff and antifouling paints applied to boats and mariculture equipment. In particular, organotin compounds of the trialkyl- and triaryltin type, notably tri-*n*-butyltin (TBT) and triphenyltin (TPhT), owing to their high toxicity, have found widespread use as the active ingredient in antifouling paint formulations (Anderson & Dalley, 1986; Kuch, 1986; Champ & Pugh, 1987; CEFIC, 1994) and have become a world-wide problem, especially in areas with high degrees of maritime activity, such as marinas, large ports and major shipping lanes. Apart from affecting fouling organisms, organotin compounds are also toxic to a wide variety of non-target species. The high-profile cases of imposex in the dogwhelk, *Nucella lapillus* (Gibbs & Bryan, 1991), and the virtual collapse of the oyster (*Crassostrea gigas*) industry in the Mediterranean (Alzieu, 1986) have generated many studies into the aquatic ecotoxicology of TBT and, to a lesser extent, TPhT. Depending on their respective concentrations, these organotin compounds have been shown to inhibit or stimulate ATPases, inhibit oxidative phosphorylation, thereby causing malformations in the mitochondrial membranes (swelling), and are also capable of disrupting the cytochrome P450 system. (e.g. Bailey, 1986; Walsh, 1986; Thain *et al.*, 1987; Langston *et al.*, 1990; Cooney, 1995; Alzieu, 1996; Bryan & Gibbs, 1996; Fent, 1996; Hall & Bushong, 1996; Langston, 1996; Laughlin *et al.*, 1996; IMO, 1998a; Morcillo & Porte, 2000)

The recognition of the deleterious effects of organotin compounds in the late 1980s, led to the partial ban of organotin containing antifouling paints on vessels smaller than 25 m and on mariculture equipment and to the development of environmental quality standards (EQS) for organotin in most industrialised countries (Anderson & Dalley, 1986; Kuch, 1986; Champ & Pugh, 1987; CEFIC, 1994; Bosselmann, 1996; Cartwright & Lewis, 1996; Waite *et al.*, 1996). Following legislation¹ restricting the use of organotin-based antifouling paints, water concentrations of tri-*n*-butyltin (TBT) and triphenyltin (TPhT) have

¹ During the 41st session of the Marine Environmental Protection Committee (MEPC) of the International Maritime Organisation (IMO), the proposal for the total ban on the use of organotin compounds in antifouling paint formulations, to take effect in 2003, was endorsed (IMO, 1998b; IMO, 1998c). This decision has sparked a fierce debate concerning the benefits and risks of a total ban in light of the lack of suitable alternatives and polarised the scientific community. A wide range of publications documenting this debate is available, (e.g. Champ & Wade, 1996; Champ, 1999; Evans, 1999; Evans *et al.*, 2000; Ten Hallers-Tjabbes, 2000).

dropped dramatically, albeit with hotspots remaining in areas of intense shipping activity and regions with high agricultural run-off (Waldock *et al.*, 1988; Waite *et al.*, 1991; Stewart, 1996; Waite *et al.*, 1996; Ten Hallers-Tjabbes, 1997). However, there is an increasing amount of evidence to show that, owing to their high octanol/water coefficients, organotin compounds readily adsorb to particles suspended in the water column (Harris & Cleary, 1987; Batley, 1996; Unger *et al.*, 1996; Weidenhaupt *et al.*, 1997; Arnold *et al.*, 1998; Bueno *et al.*, 1998). This tends to enhance their partitioning into the sediment, where they can persist for many months, years or even decades. Estuarine sediments play an important role in the biogeochemical cycles of many inorganic and organic pollutants (Adelman *et al.*, 1990; Craig & Miller, 1997; Dahllöf *et al.*, 1999) and therefore much attention has been given to the interaction of benthic organisms with estuarine sediments and the bioavailability of sediment-associated inorganic and organic pollutants (McCain *et al.*, 1978; Luoma, 1983; Bryan, 1985; Knezovich *et al.*, 1987; Landrum *et al.*, 1987; Brezonik *et al.*, 1991; Langston & Burt, 1991; Bryan & Langston, 1992; Absil, 1993; Fent & Looser, 1995; Fent & Looser, 1998; Looser *et al.*, 1998).

Estuarine sediments have been shown to act as reservoirs and sources (Fig. 3.1) for the secondary introduction of TBT and TPhT to the environment (Waldock *et al.*, 1990; Langston & Burt, 1991; Watanabe *et al.*, 1995; Harris *et al.*, 1996). Despite this wealth of data, there are few studies on the effects on benthic organisms exposed to environmental concentrations of organotin compounds in sediments; the most recent of these being the studies by Krone *et al.* (1996) and Rouleau *et al.* (1998) who examined organotin concentrations in stomach contents of benthic fish and identified diet as a route of exposure and Werner *et al.* (1998) found an inhibitory effect of organotin-contaminated sediments on stress protein expression in the amphipod *Ampelisca abdita*.

The persistence of sediment-associated organotin compounds and the fact that various fish species showed no avoidance reaction to environmental levels of organotin (Hall *et al.*, 1984), with only very high concentrations triggering a response (Pinkney *et al.*, 1985), indicates the vulnerability of benthic species, such as flounders, that rely on the intimate contact with sediments for protection and food. Furthermore, the chemical structures of TBT and TPhT (Fig. 1.2) and their high lipophilic properties increase the bioavailability of these compounds to benthic organisms (Fent & Looser, 1995; Fent & Looser, 1998; Looser *et al.*, 1998).

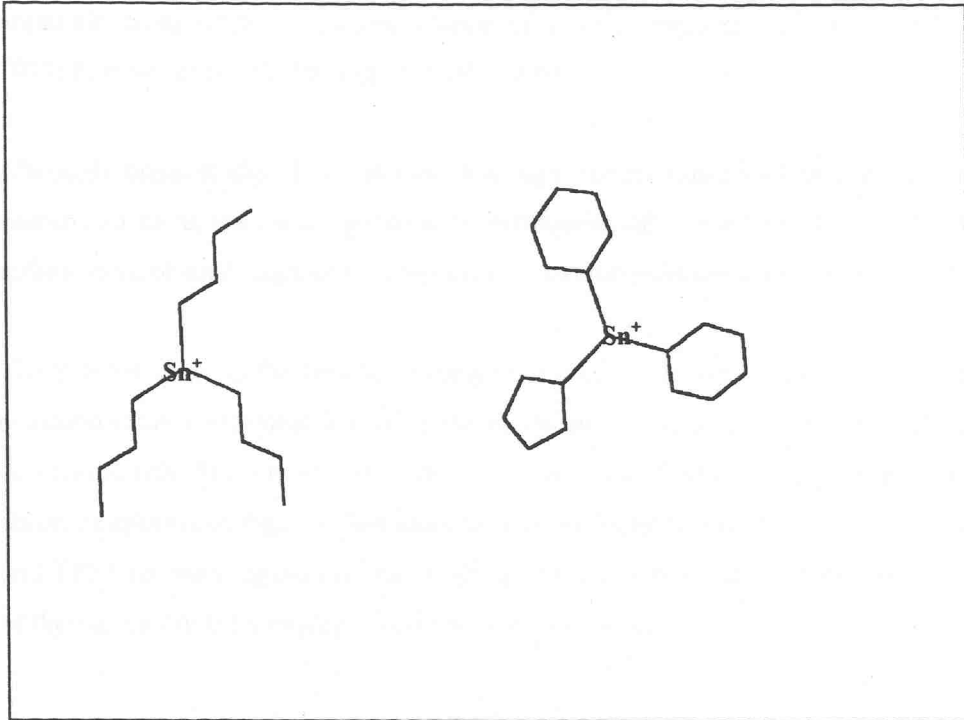


Figure 1. 2. Structural diagrams of TBT and TPhT as present in natural water.

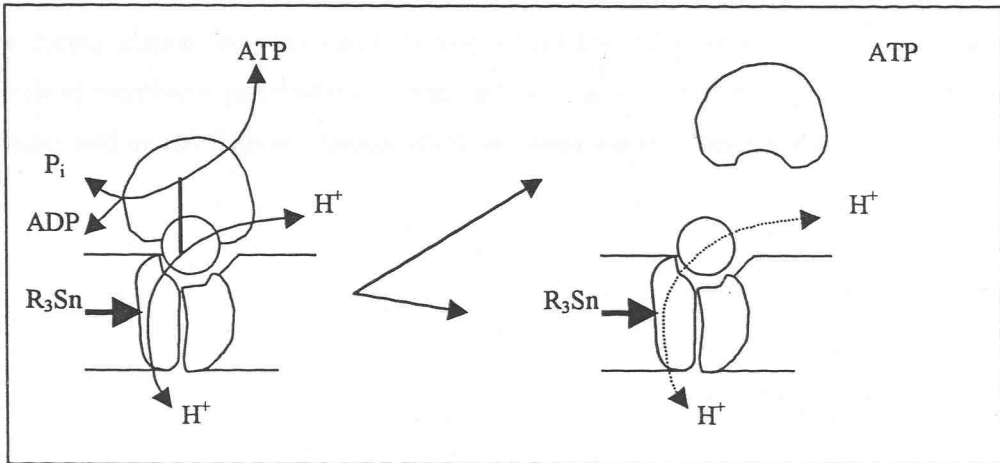


Figure. 1.3. Schematic representation of the proposed effect of triorganotins on the membrane-bound Na^+/K^+ -ATPase. Organotins cause an uncoupling of the ATPase and a H^+ -porter, leading to the cessation of ATP hydrolysis (after Selwyn, 1976).

The gills with their thin membranes and large surface areas present a prime target for aquatic-borne pollutants and several studies have investigated the physiological and morphological effects organotin exposure has on these organs (Josephson *et al.*, 1989; Kannan & Lee, 1996; Schwaiger *et al.*, 1996; Grinwis *et al.*, 1998; Lignot *et al.*, 1998). In particular, the work by Aldridge (1976) on the *in vitro* effects of TBT on Na^+/K^+ -ATPase (Fig. 1.3) , a key enzyme in ionic regulation, spawned several studies on the effects of

organotin compounds on osmoregulation of aquatic organisms (Chliamovitch & Kuhn, 1977; Pinkney *et al.*, 1989b; Lignot *et al.*, 1998).

Although these studies have shown that high concentrations of organotins in aqueous suspension disrupted osmoregulation in euryhaline fish, no information on the effects of sediment-associated organotin compounds on osmoregulation is available.

The present study is the first to investigate the effect on osmoregulatory mechanisms of environmental concentrations of sediment-bound organotin compounds on a benthic euryhaline fish. The aim of this study was to detect and quantify any significant effects of chronic exposure of 0-group flounders to environmental levels of sediment-associated TBT and TPhT on osmoregulation, that might affect the viability of such juvenile fish in terms of their ability to fully exploit a euryhaline environment.

In order to address this aim, experiments were performed on 0-group flounders (*P. flesus*) exposed to sediment-associated TBT and TPhT, either under freshwater-adapted conditions or during adaptation to sea water. Various parameters relevant to osmo- and ionoregulation, such as membrane permeability, water balance, blood osmolality, enzyme activities, ionic fluxes and morphological changes of the gill membranes, were examined.

Chapter 2 - Materials & Methods

2.1. The European flounder *Platichthys flesus* (L.)

The European flounder *Platichthys flesus* (L.), family Pleuronectidae, is a common euryhaline flatfish found around the coasts of Europe, from the White Sea to the Black Sea. It is a catadromous species, feeding and growing in estuaries during the summer and returning to the sea in winter, where mature age groups spawn (Wheeler, 1969). The European flounder has been widely used as a model euryhaline species in eco-physiological studies, because it is easily obtained, hardy in the laboratory and fully euryhaline (Motais *et al.*, 1969a, b; Potts & Eddy, 1973; Potts *et al.*, 1973; Hutchinson, 1984; Hutchinson & Hawkins, 1990). Flounders are also commonly chosen as bio-indicators in eco-toxicology surveys, because of their abundance and ubiquitous distribution in coastal waters and estuaries (Vethaak & Reinalt, 1992; Fent & Stegeman, 1993; Eggens *et al.*, 1995a, b; Besselink *et al.*, 1996; Beyer *et al.*, 1996; Hylland *et al.*, 1996; Besselink *et al.*, 1998; Kirby *et al.*, 1999; Rotchell *et al.*, 1999).

2.2. Fish collection and husbandry

0-group flounders were collected in the River Itchen estuary just below Woodmill, Southampton, U.K. During low tide a large sand bank on the east side of the river is uncovered, forming several shallow pools between itself and Woodmill bridge, trapping 0-group flounders, where they are relatively easy to catch using hand-held fishing nets. Fish size ranged, depending on the time of year, from 10 mm to 75 mm.

Several hundred flounders were collected at low tide ($S < 2$) from April to October and a stock population kept in fresh water in a 3.500 litre glass-fibre fish-farming tank at the Southampton Oceanography Centre (SOC). The hatchery is shielded from direct sunlight and rain by a roof, but exposed to natural temperature fluctuations and light/dark cycles. Prior to experiments, fish were sampled from the stock population and acclimated for at least 2 weeks in tapwater ($S = 0 - 2$; 15°C).

2.3. Biometric methods

2.3.1. Weights

Fish were placed in a plastic kitchen strainer, blotted dry with tissue paper and transferred to a pre-weighed glass beaker filled with water of the same temperature and salinity as the acclimation medium and weighed on a 'Sartorius' electronic analytical balance, after which the weight increase to the third decimal place was noted.

2.3.2. Lengths

The length of each fish was measured at the beginning and in weekly intervals during the experiments, in order to monitor growth. While in the kitchen strainer the length of the fish was measured to the nearest first decimal place with a pair of vernier callipers. Care was taken that each fish was lying flat and straight. Measurements were made as total length, from the tip of the lower jaw to the end of the tail fin.

2.4. Anaesthesia and euthanasia

Fish were anaesthetised using MS-222 (Tricaine; methanesulfonate salt, 0.1 mg l⁻¹) prior to intramuscular injection of ⁵¹Cr-EDTA for the determination of urine production rates.

Schedule 1 killings were performed by an overdose of MS-222 (5 mg l⁻¹) following the destruction of the brain in accordance with the Animal (Scientific Procedures) Act of 1986 (HMSO, 1986).

2.5. Culture of brine shrimp

Artemia salina L. cysts (Ocean Star International, Inc.) were hatched out in a vigorously aerated 20 litre glass flask containing sea water (S = 32 - 35, 20°C). On hatching, after approximately 48 hours, the aeration was turned off and the flasks partially darkened for 3 to 4 hours. The positive photo-taxis of the nauplii was then used to separate the empty cysts from the nauplii, the former sinking to the bottom, the latter aggregating near the surface. The nauplii were siphoned off, caught in a 125 µm sieve and transferred to the fish tanks.

Artemia for larger fish (> 15 mm) were produced in a similar way as food for small fish, except that on hatching the aeration was turned down to a minimum and cultures of *Isochrysis* sp. were added. Over several weeks the growing *Artemia* were collected at intervals as described above. During the holding period, the fish were fed *ad libitum*,

depending on size, with either nauplii or adult *Artemia*. Fish were also fed during the experiments but starved 24 hours prior to measurements.

2.6. Experimental tanks

The choice of tanks was dictated by the need to minimise adsorption of TBT to the walls of the container, so polyethylene buckets were chosen as experimental vessels, as the material of choice, polycarbonate, was not available in a suitable form. The next best material was polyethylene (Carter *et al.*, 1989).

2.7. Husbandry conditions during experiments

The fish were kept on silver sand (fine-grained horticultural silica sand) in 25litre polyethylene buckets in aerated water, depending on the experiment (steady state fresh water or fresh water-sea water transfer experiments), either tap water (S = 2; ; ionic composition (mmole l⁻¹): Na: 0.2; Cl: 0.11; Ca: 0.62; NH₄: 0.027; Mg: 0.21; K: 0.03; pH 6.5) or full strength sea water, at a constant temperature of 15°C (+/- 1°C; controlled temperature room) and subjected to a 12 hour light/dark regime. The buckets were covered loosely by lids. This ensured dimmed light conditions, as well as gas exchange with the environment.

2.8. Sediment preparation

2.8.1. Experimental sediments

Rogers *et al.* (1998) identified those estuaries in southern England containing *P. flesus*; comparison with Langston *et al.* (1994) showed that all were contaminated with organotin compounds. Other than organotin compounds, sediments in the River Itchen are known to contain high levels of trace metals, in particular mercury and arsenic (Langston, 1980; 1982; Ramshaw, 1990; Hill *et al.*, 1993). In order to prevent masking any effects of organotin compounds by background levels of other contaminants, commonly found in estuarine sediments (Stronkhorst *et al.*, 1999) and to have a reliable control, artificial sediments were prepared. These were prepared from a mixture of clean silver sand and deep-sea mud. The deep sea mud was collected from Porcupine Abyssal Plain, North Atlantic (4,800 m). Organotin levels in mud from this deep-sea site were below the detection limit of 1 ng g⁻¹. The mud provided the organotin compounds with the necessary substrate to remain adsorbed to the sediment, protecting it from debutylising factors such as

light and oxygen. This ensured a nearly constant organotin concentration during the experiments (see Chapter 3).

The dry weight of sediment samples was measured by weighing and drying a sediment sub-sample at 50°C to a constant weight. The factor obtained was used to estimate the total dry weight of the sediment in the bucket in order to calculate the amount of TBTCI and TPhTCI to be added. The sediment water content estimated by this method was needed during organotin analysis (see below).

The bulk sediment for all experiments was weighed on a 'Mettler' P1200 electro-mechanical balance. Dry weight was determined using a 'Sartorius' electronic analytical balance as described above.

2.8.2. Organotin concentration in experimental sediments

Itchen River sediments² were repeatedly surveyed by Langston *et al.* (1994). In the 'upper Itchen', they found sediment TBT concentrations of 222 ng.g⁻¹ (89 ng Sn.g⁻¹). In 1997, a little downstream at Woodmill, TBT levels averaged 130 ng.g⁻¹ (52 ng Sn.g⁻¹; Hartl, unpublished data); at St. Denys, a site just down-stream from Woodmill, Langston *et al.* (1994) reported TBT concentrations of 305 ng.g⁻¹ (122 ng Sn.g⁻¹). From these data it is evident that significant degradation has not taken place in River Itchen sediments (Langston, personal communication). On the basis of these data an experimental organotin concentration of 150 ng.g⁻¹ sediment was chosen as a representation of present environmental concentrations for the area. For an account of sediment-water interactions of organotins see chapter 3.

2.8.3. Application of organotin to the experimental sediments

TBTCI and TPhTCI in glacial acetic acid were adsorbed onto approximately 20 g of dried deep-sea mud (Porcupine Abyssal Plain; organotin concentration < 1 ng.g⁻¹) and then mixed into a slurry of clean fine-grained silica sand (horticultural 'Silver Sand'; grain size < 1 mm) in the bucket in order to produce an organotin concentration of 150 ng.g⁻¹. The sediment preparation for the control groups consisted of silver sand and approximately the same amount of mud, but without organotin. Sediment samples from both experimental and control buckets were taken immediately after application (t_0) and frozen at -20°C until further analysis. It has been demonstrated that the degradation rate of tripropyltin, added to

² All sediment organotin concentrations are expressed as dry weight.

the frozen samples as an internal standard, was negligible and therefore freezing at the above temperature a suitable form of storage (M. Waldock, personal communication). Preliminary experiments in both fresh and sea water were performed over a six week period. Repeated sediment samples were taken to establish the rate of organotin degradation in the sediment preparations.

2.8.4. Total organic carbon and sediment particle size distribution

The adsorption of organotin compounds to sediment particles depends on the organic content and also on the sediment particle size. In order to characterise the sediment, total organic matter and particle size distribution were determined.

Total organic matter (TOC) was measured as ash-free dry weight (AFDW). A sediment sample was dried at 50°C to a constant weight, combusted in a muffle furnace (500°C; 4 h), left to cool in a desiccator and re-weighed (Dean, 1974).

The combusted sediment sample was weighed and then shaken through a series of 'Fritsch' shaking sieves (63, 90, 125, 180, 250, 355, 500, 710 and 1400 µm). The resulting grain size fractions were weighed and expressed as a percentage of the total sample weight.

2.9. Organotin Analysis

Sediments were analysed at the Centre for Environment, Fisheries and Aquacultural Science (CEFAS), Burnham on Crouch, after hydride generation, by gas chromatography with flame photometric detection (GC-FPD), following the procedure by Waldock *et al.* (1989). Briefly, methanol was used to extract organotin compounds from a sediment slurry. These were then back-extracted into hexane and derivatised with sodium borohydride, before analysis by GC-FPD. The internal standard was tripropyltin chloride. For details see Appendix I.

2.10. Sodium efflux

Flounders (15 fish, 5 per bucket for each treatment and control group) acclimated to fresh water (2 weeks prior to experiments) were exposed to sediment containing 150 ng.g⁻¹ TBTCI or TPhTCI for 6 weeks in order to demonstrate the effect of organotin compounds on the ability of flounders to control Na⁺-fluxes under hypo- and hyper-osmotic conditions. Passive Na⁺-efflux was estimated using the method described by Shaw (1959) and modified for flounders by Hutchinson (1984). A control group was maintained simultaneously in exactly the same conditions, but without the addition of organotin. Measurements were carried out at t₀ (before organotin exposure) and then subsequently at weekly intervals for both groups. Flounders acclimated to fresh water were weighed, their

lengths measured and then loaded for five hours in a fresh water loading medium containing $^{22}\text{Na}^+$ at an activity of $106 \text{ kBq}\cdot\text{ml}^{-1}$ ($2.9 \text{ }\mu\text{Ci}\cdot\text{ml}^{-1}$). The fish were rinsed to remove any $^{22}\text{Na}^+$ from the body surface, placed in a small crystallising dish filled with water of the same temperature and salinity as in the acclimating medium and whole body counts performed using a 'Panax' NaI well γ -scintillation counter (see 2.12). Measurements of fish activity were made in triplicate and corrected for background counts. The fish were then transferred to a large volume of unloading medium, with the same temperature and salinity as the loading medium, and left for two hours, after which the fish were recounted. It is assumed that constant circulation of the large volume (approximately 5 litres) of unloading medium prevented any significant backflux of $^{22}\text{Na}^+$ into the fish. The efflux was calculated from the half time of exchange of $^{22}\text{Na}^+$ and from the total body Na^+ content. The exchange is described by the equation for an exponential loss:

$$A_0 = A_t \cdot e^{-kt_{\text{unload}}} \quad (\text{Eq 1})$$

The rate constant k is calculated as

$$k = \frac{1}{t_{\text{unload}}} \cdot \ln \frac{A_0}{A_t}, \quad (\text{Eq 2})$$

where t_{unload} is the unloading time [h], A_0 are the counts at the end of the loading period and A_t are the counts at time t_{unload} . Using the rate constant (k), the half time of exchange ($T_{1/2}$) can be determined as

$$T_{1/2} = \frac{\ln 2}{k} \quad \text{and} \quad (\text{Eq 3})$$

Na^+ -efflux is thus calculated using

$$\text{Na}^+\text{-efflux} = 1000 \cdot \frac{\ln 2 \cdot [\text{Na}^+]}{T_{1/2} \cdot A} \text{ nmoles mm}^{-2} \text{ h}^{-1}, \quad (\text{Eq 4})$$

where $T_{1/2}$ is the half time of exchange [h] and $[\text{Na}^+]$ is the total sodium concentration of a fresh water acclimated flounder ($142 \text{ }\mu\text{moles g}^{-1}$; Hutchinson, 1984) and A is the gill area [$\text{mm}^2 \text{ g}^{-1}$].

In order to determine the effect of environmental concentrations of sediment-associated organotin compounds on the active Na^+ -efflux during adaptation to sea water, freshwater-adapted flounders were exposed to either TBTCI or TPhTCI for 15 days, the time it takes for flounders to become fully adapted to sea water (Harding *et al*, 1997). The fish were

transferred to full strength sea water on day 3. The Na^+ -efflux was monitored throughout the experiment on days 0, 3, 6, 9 and 15. The loading and unloading media were fresh water on days 0 and 3 and sea water on days 6, 9 and 15. Loading and unloading were performed in the same way as described above.

Various authors have used radioisotopes, ^{22}Na (Hutchinson, 1984; Carroll *et al.*, 1995; Hansen *et al.*, 1999) and ^{24}Na (Motais *et al.*, 1966; Bornancin & De Renzis, 1972; Potts *et al.*, 1973), to measure the active efflux of sodium in euryhaline fish, either by measuring the rate of appearance of the tracer in the unloading medium or by performing whole body counts and measuring the amount of tracer remaining in the fish. There are several advantages in using ^{22}Na rather than ^{24}Na in Na^+ -flux experiments. (1) ^{22}Na is a γ -emitter, permitting whole body counts and thus the measurement of tracer remaining in the fish at a certain time. ^{24}Na , on the other hand, is a β^- -emitter allowing the appearance of tracer to be measured in the unloading medium only. The measurement of any tracer remaining in the fish would require its destruction (maceration), which would clearly not have served the purpose of this study. (2) ^{22}Na has a half-life of 2.6 years, whereas ^{24}Na has a half-life of 14.9 hours, that means the decay rate during a several hour experiment has to be taken into account with the use of ^{24}Na but is negligible when using ^{22}Na . (3) The long half-life of ^{22}Na enables the fish to be loaded in an appropriate medium rather than administering the tracer by injection, that would be likely to cause additional stress, especially to the small fish used in the present study. For both isotopes, care must be taken to avoid the potential back flux of tracer into the fish and also the possibility of isotope adherence to the skin. Both problems can be mitigated by either regularly changing the unloading medium or by using large volumes of constantly circulating unloading medium and by rinsing the animals before placing them into the unloading medium. Whichever tracer is used, the efflux is chiefly a gill efflux, because the contribution of the skin and kidney is negligible (Motais & Maetz, 1965), thus allowing the flux rate to be normalised to gill area.

2.11. Water balance

Flounders acclimated to fresh water were exposed to sediment containing $150 \text{ ng}\cdot\text{g}^{-1}$ TBTCI or TPhTCI for 6 weeks. The drinking and urine production rates were measured in order to demonstrate the effect of organotin compounds on the water balance of 0-group flounders. A control group was run parallel under exactly the same conditions, but lacking

organotin exposure. Measurements were carried out at t_0 (before organotin exposure) and then subsequently in weekly intervals for both groups.

2.11.1. Drinking rates

Drinking rates were estimated using ^{51}Cr -ethylendiamine tetra-acetic acid (^{51}Cr -EDTA; 'Nycomed Amersham plc') (Hutchinson & Hawkins, 1990). The animals were placed in an appropriate loading medium (fresh water) of the same temperature as in the experimental tanks (15°C) and exposed to the tracer in the loading medium for two hours. The activity in the medium was 774 Bq ml^{-1} ($0.02 \mu\text{Ci ml}^{-1}$). After loading, the animals were placed in clean, non-radioactive water, in order to remove any excess tracer from both buccal cavity and pharynx. The activity in the fish was measured by performing whole body counts of the fish in a 'Panax' NaI well γ -scintillation counter (see 2.12). Measurements of fish activity were made in triplicate and corrected for background counts. The activity of $100 \mu\text{l}$ of loading medium was also measured and the drinking rates estimated using the following equation:

$$D = \frac{C_f \cdot 100}{C_m \cdot t_{load} \cdot ww} \text{ \% body weight h}^{-1} \quad (\text{Eq 5})$$

where D is the drinking rate as a percentage of body weight, C_f are the fish counts, C_m are the counts in the loading medium, t_{load} is the loading time [h] and ww is the fish wet weight [g]. Owing to the size of the molecule, it is assumed that ^{51}Cr -EDTA is not immediately absorbed by the gut and thus is not metabolised during the short-term experiment. Furthermore, it is assumed that all the activity measured stems from imbibed ^{51}Cr -EDTA (Bolt *et al.*, 1980). This method was tested further by Hutchinson (1984), who found no significant difference between the activity of the whole animal and that of the excised gut, thus demonstrating that the adsorption of ^{51}Cr -EDTA to the epidermis is negligible.

2.11.2. Urine production

The urine production rate was estimated from the clearance rate of injected ^{51}Cr -EDTA (Babiker & Rankin, 1975; Hutchinson & Hawkins, 1990). The activity of the tracer was 0.37 MBq ml^{-1} ($10 \mu\text{Ci ml}^{-1}$) and was increased by freeze drying to 3.7 MBq ml^{-1} ($100 \mu\text{Ci ml}^{-1}$) (Hutchinson, 1984). Freshwater-adapted animals were injected intramuscularly in the dorsal caudal region with $2 \mu\text{l}$ of ^{51}Cr -EDTA, with an activity of approximately $3.7 \text{ kBq} \cdot \mu\text{l}^{-1}$ ($0.1 \mu\text{Ci} \cdot \mu\text{l}^{-1}$). The fish were then transferred to a large volume of clean, non-radioactive and aerated water (fresh water) of the same temperature (15°C) as the acclimation medium, to allow recovery and the removal of any surface radioactive material

not injected into the body. The animals were left for three hours after which it was assumed that the $^{51}\text{Cr-EDTA}$ was evenly distributed throughout the animal (Hutchinson, 1984). Following the recovery period, the animals were placed in a 'Panax' NaI well γ -scintillation counter (2.12) and whole body counts performed (A_0). The animals were counted again four hours later (A_t). A semi-logarithmic plot of $\log_{10}(A_0 - A_t)$ against time gives a straight line (Hutchinson, 1984). The half-time of exchange ($T_{1/2}$) can be calculated in the same way as for Na^+ -efflux (2.10). From the estimation of the activity of $^{51}\text{Cr-EDTA}$ in the urine (A_U) and blood (A_B), so that the ratio A_U/A_B is obtained, it is possible to calculate the presumptive urine production rate:

$$U_v = \frac{V_b \cdot \ln 2 \cdot V_w}{\left(\frac{A_U}{A_B}\right) \cdot T_{1/2} \cdot 100} \text{ \% body weight h}^{-1}, \quad (\text{Eq 6})$$

where U_v is the volume of urine (% wet weight h^{-1}); V_b is the volume of blood and lymph calculated as the $^{51}\text{Cr-EDTA}$ space as a percentage of the total fluid volume; V_w is the water content as a percentage of the wet weight and $T_{1/2}$ is the half-time of exchange. For the values used in the calculation of urine flow see Table I.

Absolute values for urine flow can be calculated using,

$$U_{tot} = \frac{U_v}{100 \cdot U_s} [\text{ml} \cdot \text{g}^{-1} \cdot \text{h}^{-1}], \quad (\text{Eq 7})$$

where U_{tot} is the total urine flow in $[\text{ml g}^{-1} \text{h}^{-1}]$, U_v is the urine flow [% wet weight h^{-1}] and U_s is the specific gravity of urine.

Table 2. 1. Values used in the calculation of urine flow (Hutchinson, 1984)

| | |
|--------------------------------|-------|
| V_w | 80.49 |
| V_b | 8.51 |
| $\left(\frac{A_U}{A_B}\right)$ | 0.95 |
| U_s | 1.002 |

2.12. 'Panax' NaI well γ -scintillation counter

The Activity of the fish after loading was measured with a PANAX NaI well γ -scintillation counter. In order to account for instrument drift the energy was set at 2000 volts for ^{22}Na and for ^{51}Cr (Fig. 2.1)

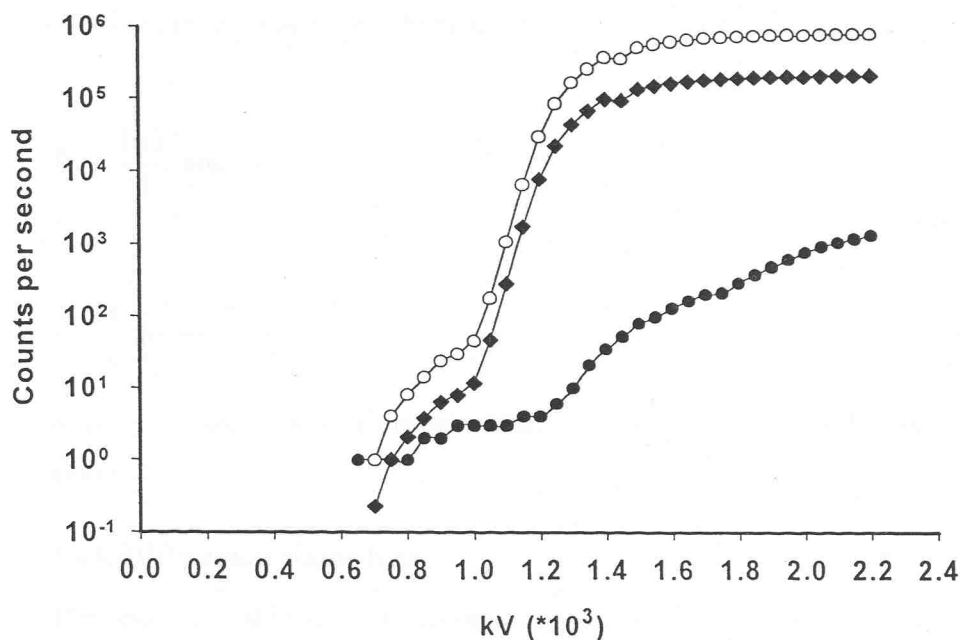


Figure 2. 1. . Scan of 10 kBq ^{22}Na (O), ^{51}Cr (◆) and BKG levels (●) across the energy range of the 'Panax' NaI well γ -scintillation counter.

2.13. Water permeability

Flounders acclimated to fresh water were exposed to sediment containing 150 ng.g^{-1} TBTCI or TPhTCI for 6 weeks in order to demonstrate the effect of organotin compounds on water permeability. Measurements were carried out at t_0 (before organotin exposure) and then subsequently in weekly intervals for both groups. The water permeability of 0-group flounders was determined by measuring the efflux of tritiated water (THO) as the half-time of exchange ($T_{1/2}$) as described by Lockwood *et al.* (1973) and adapted for flounders by Hutchinson (1984). The exchange is described by the equation for an exponential loss (Eq 1). The fish were loaded in fresh water (15°C) containing THO with

an activity of 1 MBq ml⁻¹ (27 μCi ml⁻¹) for ten times the expected half-time of exchange (loading time 5 hours, Hutchinson (1984)). The fish were then removed from the loading medium, rinsed and placed in an unloading medium of the same temperature and salinity as the loading medium. The vessels containing loading and unloading media were kept sealed at all times in order to avoid evaporation, except briefly for sampling water. Water samples (250 μl) were drawn at t_1 (exactly 5 mins) and t_∞ (5 h), added to 5 ml of scintillation cocktail ('Optiphase HiSafe 3') and counted in a 'Wallac' 1409/11 Liquid scintillation counter. The counter is automatically calibrated against an external ¹³⁷Cs source and a pre-set tritium program was used. From these values $T_{1/2}$ can be calculated using,

$$T_{1/2} = \frac{\ln 2}{k} \text{ and} \quad (\text{Eq 8})$$

$$k = \frac{1}{t} \cdot \ln \left(\frac{A_\infty}{A_\infty - A_t} \right), \quad (\text{Eq 9})$$

where A_∞ is the activity of the unloading medium at t_∞ (5 h) and A_t is the activity at t_1 (5 mins).

2.14. Diffusional water flux

The percentage of total water exchanged per unit time (R) can be calculated from the following expression (Hutchinson & Hawkins, 1990):

$$R = \frac{100 \ln 2}{T_{1/2}} \text{ \% weight unit time}^{-1} \quad (\text{Eq 10})$$

The unidirectional flux was estimated as

$$F_{uni} = \frac{R \cdot Q}{100} \text{ \% weight unit time}^{-1}, \quad (\text{Eq 11})$$

where Q is the percentage water content.

2.15. Na⁺-K⁺-ATPase activity

Freshwater-adapted flounders were exposed to either TBTCI or TPhTCI for 15 days in order to determine the effect of environmental concentrations of sediment-associated organotin compounds on the activity of Na⁺-K⁺-dependent adenosine triphosphatase (Na⁺-K⁺-ATPase) during adaptation to sea water. The fish were transferred to full strength sea water on day 3 and the Na⁺-K⁺-ATPase activity was monitored throughout the experiment on days 0, 3, 6, 9 and 15.

The Na⁺-K⁺-ATPase activity was determined using a modification of the method by Mayer-Gostan & Lemaire (1991). This assay is based on the rate of orthophosphate (P_i) accumulation in the reaction medium as the product of the enzymatic hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) + P_i. The P_i concentration [P_i] is an indirect measure for ATPase activity that is expressed as μmol P_i μg protein⁻¹ min⁻¹.

2.15.1. Preparation of gill tissue

All eight gill arches were excised, wrapped in 'Cling' film, dipped in liquid nitrogen and stored at -70°C until required. The frozen gill tissue was then thawed on ice and placed in ice-cold homogenising buffer at pH 7.4. The gill membranes were separated from the underlying cartilage by homogenisation using an 'Ultra Turrax' homogeniser and the homogenate centrifuged at 2,212 g for 10 mins in a 'MSE Coolspin' centrifuge. The pellet was discarded, the supernatant drawn off and centrifuged again at 2,212 g for 10 mins. The supernatant was drawn off and this time discarded. The resulting pellet was rinsed twice in ice-cold isosmotic sucrose solution and re-suspended in ice-cold buffered reaction medium (pH 7.4; for details see Appendix II).

2.15.2. Phosphate determination

A series of orthophosphate (P_i) standards was prepared from a sodium dihydrogen orthophosphate (NaH₂PO₄) solution (0, 20, 40, 60, 80 and 100 μmol). 100 μl of each solution and 150 μl of the colour reagent (Appendix II) were pipetted in quintuplet into separate microplate wells and mixed by repeatedly drawing and expelling the solution. The absorbance was measured at 620 nm using a 'Labsystems Multiskan RC' microplate reader. The readings were corrected for the reagent blank (0), plotted against the known P_i and the resulting calibration regression was used to calculate the P_i in the membrane preparations (Fig. 2.2).

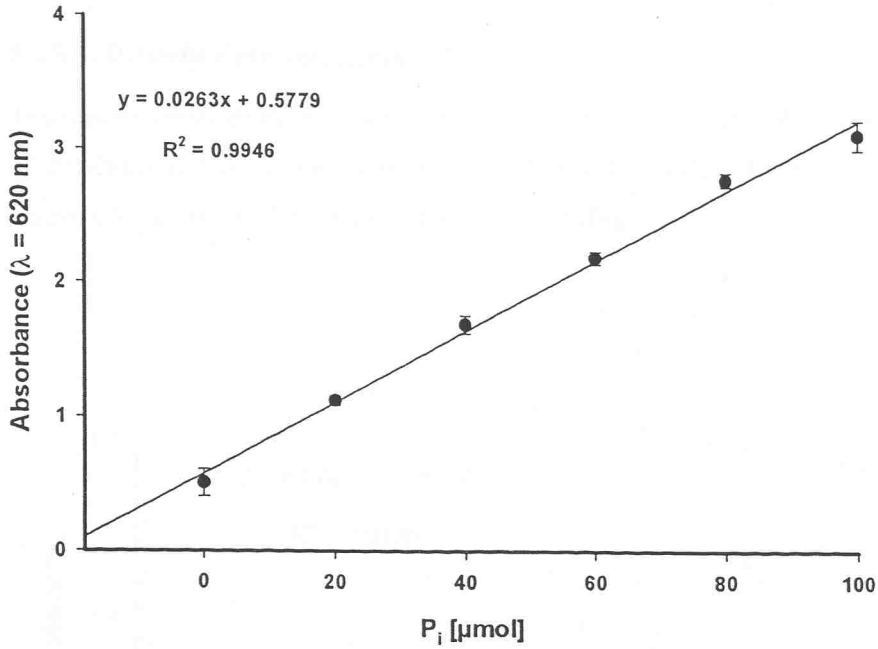


Figure 2. 2. Calibration regression used for the calculation of P_i ; $n = 5 \pm \text{S. D.}$

100 μl of the buffered membrane preparations were pipetted into two series of microplate wells. Ouabain was added to half of the wells in order to inhibit the ATPase and determine the P_i at the start of the assay. The enzyme reaction was activated in the remaining wells by adding 50 μl of ATP. The microplate was incubated for 10 min at 37°C on a shaker table and the reaction terminated by the addition of 150 μl of colour reagent.

The absorbance in both groups was measured at 620 nm and P_i is calculated as follows:

$$f = (P_{620} - B_{620}) - (O_{620} - B_{620}), \quad (\text{Eq 12})$$

where f is the absorbance at 620 nm (P_{620}) and Ouabain reading (O_{620}) corrected for the reagent blank (B_{620}).

P_i is calculated as

$$P_i = \frac{f}{0.0263} - 0.5779 \mu\text{mol} \quad (\text{Eq 13})$$

2.15.3. Protein determination

The protein concentration in the reaction medium was determined according to the method of Bradford (1976). A series of protein standards was produced using bovine albumin fraction V (0, 10, 20, 30, 40 and 50 $\mu\text{g } 0.1 \text{ ml}^{-1}$) (Fig. 2.3).

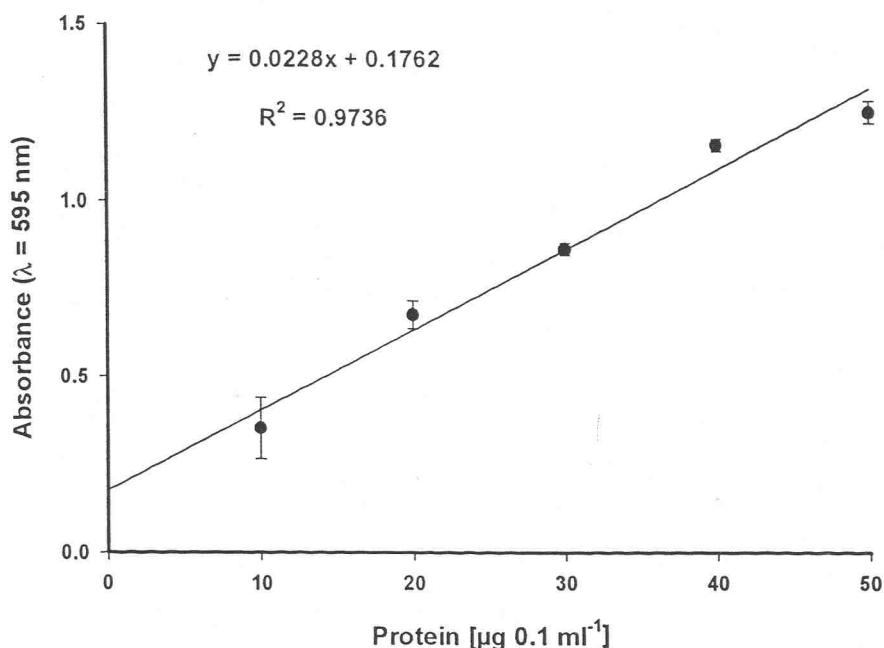


Figure 2. 3. Calibration regression used for the calculation of the protein concentration; $n = 5 \pm \text{S. D.}$

In a 1 ml cuvette 100 μl of standard solution were added to 900 μl of colour reagent and the absorbance measured at 595 nm in a 'Cecil Series 2' spectrophotometer. The protein concentration was then calculated using the following equation:

$$\text{protein} = \frac{A_{595}}{0.0228} - 0.1762 \mu\text{g } 0.1 \text{ ml}^{-1} \quad (\text{Eq 14})$$

where A_{595} is the absorbance at 595 nm. The readings were corrected for the reagent blank by frequently measuring a reagent blank and setting the instrument to zero. The enzyme activity was then calculated using:

2.16. Blood osmolality

The osmolality of the blood was determined using a modification of the method of Ramsay and Brown (1955). Blood was sampled from a caudal blood vessel with an acid cleaned drawn out pasteur pipette. These primary blood samples were stored under liquid paraffin in a siliconised ('Repelcote') solid watch glass until further use. Sub-samples were sandwiched between liquid paraffin in a piece of drawn out silica glass tubing, so that the sample was twice as long as wide. The tube was sealed with wax and inserted into a larger tube of soda glass that was also filled with liquid paraffin. This tube was also sealed with wax and then mounted onto a brass holder, again using sealing wax (Fig. 2.4).

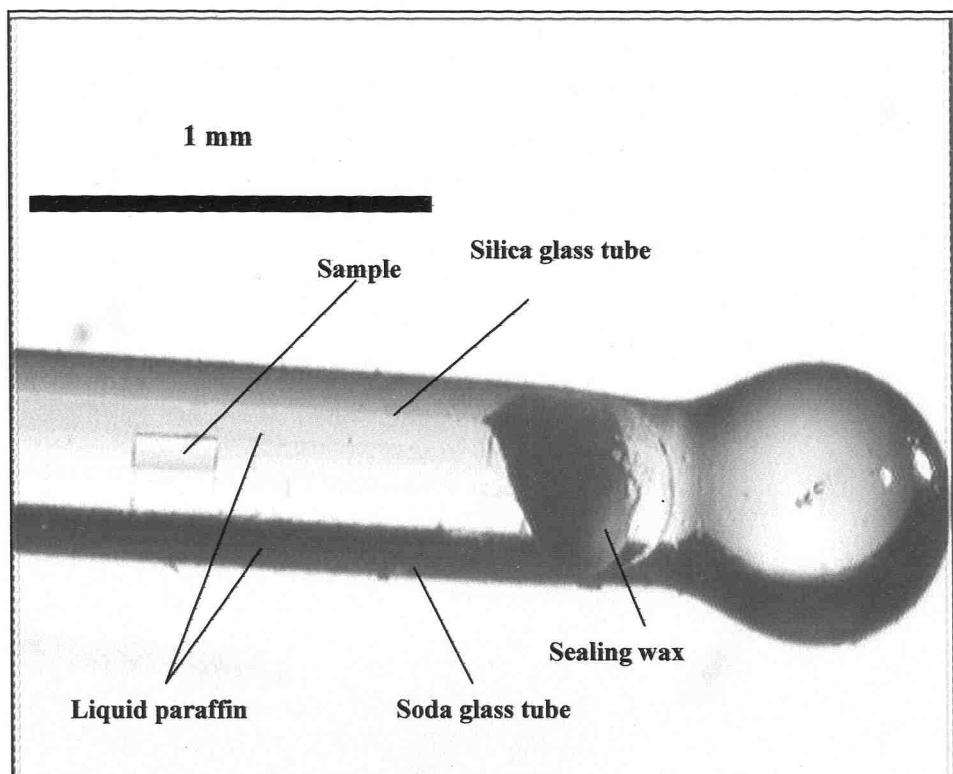


Figure 2. 4. Sample preparation for measuring the freezing point depression of blood used for the determination of blood osmolality.

The samples were frozen with an aerosol spray and placed into a 30 % alcohol bath cooled to below the freezing point of the sample. The sample was observed using a modified microscope. The freezing point of the sample was defined as the point at which the last observable crystal melted. This temperature was measured using a 'Beckmann'

thermometer calibrated against a NaCl standard: $400 \text{ mOsmol kg}^{-1} = 0.7432 + 1.67^{\circ}\text{C} = 2.4132 = 0^{\circ}\text{C}$.

The osmolality was then calculated using:

$$FD = 2.4132 - Temp \quad (\text{Eq 16})$$

and

$$Os = \frac{FD \cdot 1000}{1.8580} \text{ mOsmol kg}^{-1}, \quad (\text{Eq 17})$$

where FD is the freezing point depression and Os is the osmolality [mOsmol kg^{-1}].

2.17. Chloride cell distribution during adaptation to sea water

A variety of histochemical and immunocytochemical methods for identifying and localising chloride cells have been applied: dimethylaminosterylmethylpyridinium-iodine (DASPMI) (Van der Heijden *et al.*, 1997); 2-(4-dimethylaminostyryl)-1-ethylpyridinium iodide (DASPEI) (Bereiter-Hahn, 1976; Wendelaar Bonga *et al.*, 1990; Hiroi *et al.*, 1999; Lin *et al.*, 1999; Sakamoto *et al.*, 2000); avidin-biotin-peroxidase complex (ABC) (Uchida *et al.*, 1996, 1997; Dang *et al.*, 2000); Champy-Maillet (Madsen & Korsgaard, 1989; Madsen, 1990; Madsen *et al.*, 1994), radiochemical (Shirai & Utida, 1970; Hiroi *et al.*, 1998) and several other immunocytochemical methods (Hirai *et al.*, 1999; Masini *et al.*, 2000)

Champy-Maillet's method (Maillet, 1959; Garcia-Romeu & Masoni, 1970) was chosen for this study, because it specifically stains chloride cells and is easy to apply. The other cells constituting the epithelial tissue are stained slightly grey, so that the chloride cells appear as well-defined black dots on a light grey background (Figs. 2.5, 5.10 – 13). This has the advantage that the chloride cells are clearly visible in relation to the surrounding tissue, so that the exact position within the gill epithelium can be determined, which is not always possible with the aforementioned staining techniques. However, this method does not allow the observation of the sequential development of individual chloride cells during sea water adaptation, as is possible with vital probes such as DASPEI (Hiroi *et al.*, 1999). This has the disadvantage that the different stages of chloride cell development during sea water adaptation were observed on tissue samples from different fish. In addition, the possibility of artefacts occurring during fixation with Champy-Maillet's fixative can not be completely ruled out, because of the failure of the method to distinguish between mature chloride cells and accessory cells.

The first two gill arches from the upper gill system were removed and immediately placed in Champy-Maillet's fixative for 18-24 hours, after which the tissue turned a brownish-black. The gill arches were then rinsed for approximately 4 hours in distilled water and then transferred to 70% ethanol and left over night. The silver-blackened tissue was dehydrated through increasing concentrations of ethanol, cleared with toluene and embedded in paraffin wax. 5 µm sections were produced using a 'Jung' microtome. These were de-waxed with xylene, mounted in 'DEPEX' and viewed with an 'Olympus' BH-2 light microscope.

Following treatment with Champy-Maillet's fixative, chloride cells appear as black bulges in the gill epithelium (Fig. 2.5).

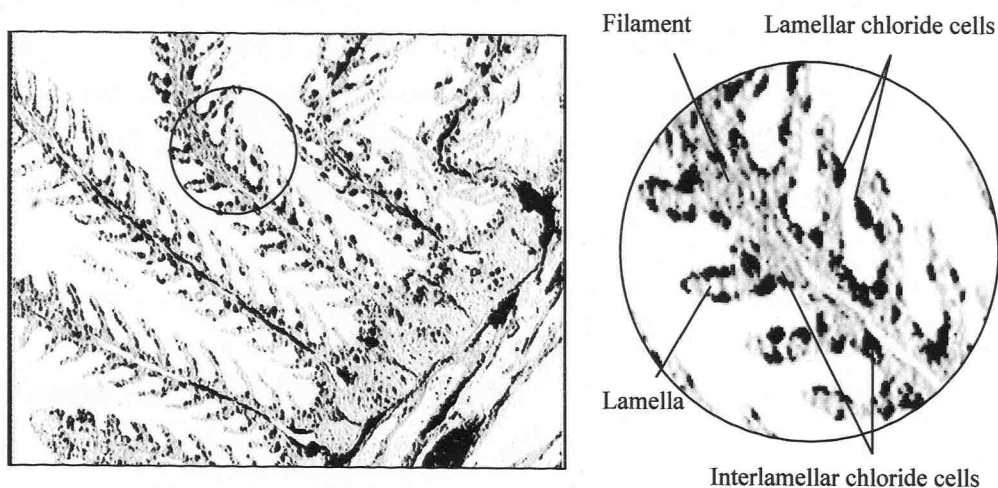


Figure 2. 1. Light micrograph of a 5 µm longitudinal section through a gill arch treated with Champy-Maillet's fixative (left). The encircled area is enlarged on the right, showing a detail of a filament with lamellae branching either side. The black bulges in the gill epithelium are specifically stained chloride cells.

The number of both lamellar (CCLam) and interlamellar (CCilam) chloride cells were counted and divided by the number of lamellae and interlamellar spaces, respectively. The ratio CCLam/CCilam was used to determine the degree of chloride cell redistribution during sea water adaptation. At least 10 sections were chosen at random from each fish. The measurement of chloride cell size was performed by tracing the outline of stained chloride cells using the digital image analysis software package 'SigmaScan Pro' 4.0. Images were grabbed with an 'Olympus' BH-2 light microscope linked by a 'Panasonic' F10 CCD video camera to a desktop PC using 'Matrox Rainbow Runner'. The system was calibrated with the grid of a 'Neubauer' haemocytometer.

2.18. Gill morphometrics

The biometry of fresh and preserved material was determined by adapting the weighted method described by Hughes & Morgan (1973), making use of the digital image analysis software package 'SigmaScan Pro' 4.0 (see 2.17). The gill pouches in longitudinally symmetrical fish are approximately the same size and it is sufficient to make the primary measurements on the gill arches of one pouch and multiply the result by two. In post-metamorphic flounders the 'upper' and 'lower' gill pouches were asymmetric and measurements had to be made on all eight gill arches.

Flounders ($n = 17$; wet weight: 0.008 - 2.860 g) were killed by an MS222 overdose (5 mg l^{-1}) and immediately processed. All eight gill arches from each specimen were removed and preserved in cold Bouin's fixative, following the protocol described by Hughes (1984); who also noted that there is unavoidable shrinkage during fixation with Bouin's.

In the present study the shrinkage was quantified by extracting five individual filaments of different initial sizes which were placed in water on a cavity slide and filament length, interlamellar space and unilateral lamellar area were measured using the methodology described below. The water was replaced by Bouin's solution, and the slide covered and refrigerated. Further measurements were made after 24 h, 72 h; 1 week, 2 weeks and 6 weeks. All measurements were corrected for shrinkage (correction factors for filament length: 1.093, interlamellar space: 1.077 and unilateral lamellar area: 1.165; see also Table 6.5).

The length of each filament of both hemibranchs of each gill arch was measured from base to tip, taking the curvature of some filaments caused by fixation into account. A histogram was produced from these data representing the hemibranch in question (Fig. 2.6).

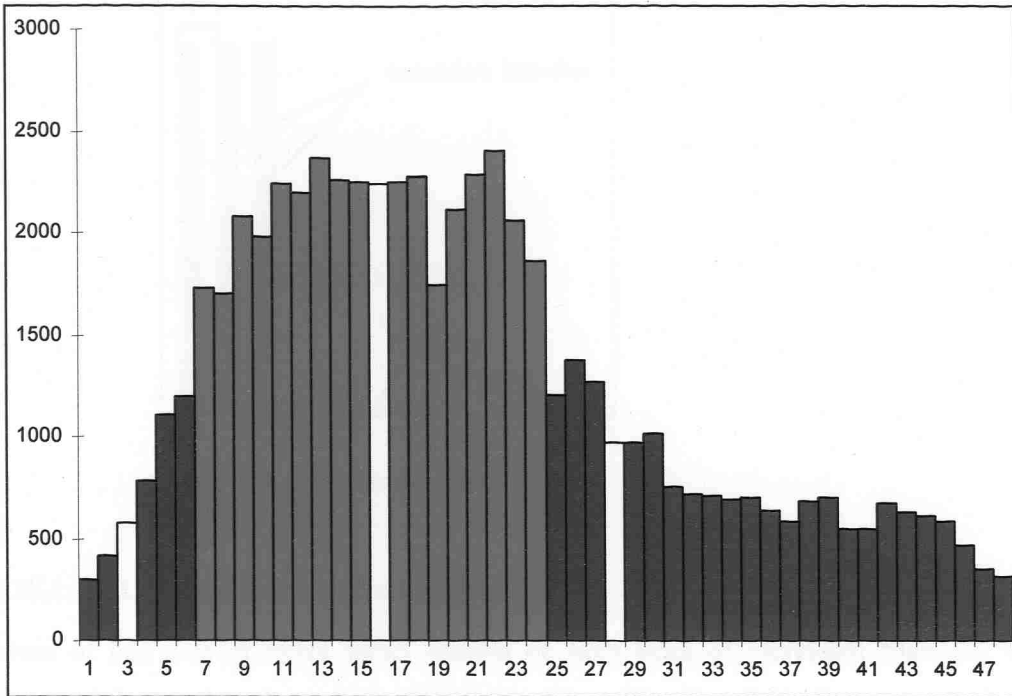


Figure 2. 6. Schematic diagram of one hemibranch of the first gill arch of the upper gill system of an O-group *P. flesus* (weight: 2.88 g; length: 71 mm; total gill area: 99.13 mm²). The 48 filaments were divided into 3 distinctly visible length groups (red, green, blue). One medium sized filament (white) of each group was then chosen on which all subsequent measurements were performed.

Using this diagram (Fig. 2.6) the filaments were divided into 3 length groups. A medium-sized filament of each section was selected and every interlamellar space (d') on both sides of individual filaments was measured. This was achieved by separating the filament from the gill arch and placing it with one side facing upwards into a cavity slide completely filled with water and sealed with a cover slip. Large filaments were turned over with a dissecting needle but with very short filaments it was often enough to move the cover slip along the plane of the slide; the resulting movement of the water in the cavity then rolled the filament onto the opposite side. By measuring the total length of all filaments of a selected group of a given hemibranch and calculating the average d' , the total number of lamellae in that particular group was estimated.

$$n = \frac{fil}{d'} \cdot 2, \tag{Eq 18}$$

where fil is the total length of the filaments in question and d' is the average interlamellar spacing (Fig. 2.7). This result was multiplied by two in order to account for both sides of the filament.

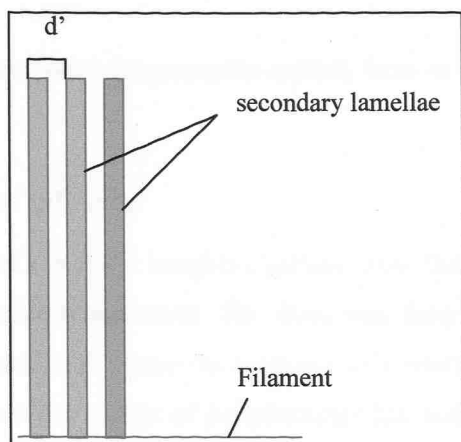


Figure 2. 7. Schematic illustration indicating d' (Hughes, 1966).

2.18.1. Total lamella area of a weighted group

Areas of one side of every intact lamella on both sides of individual filaments were measured, by tracing the outline of individual lamellae (Fig. 2.8). These measurements were then averaged and used to calculate the total surface area of the particular group.

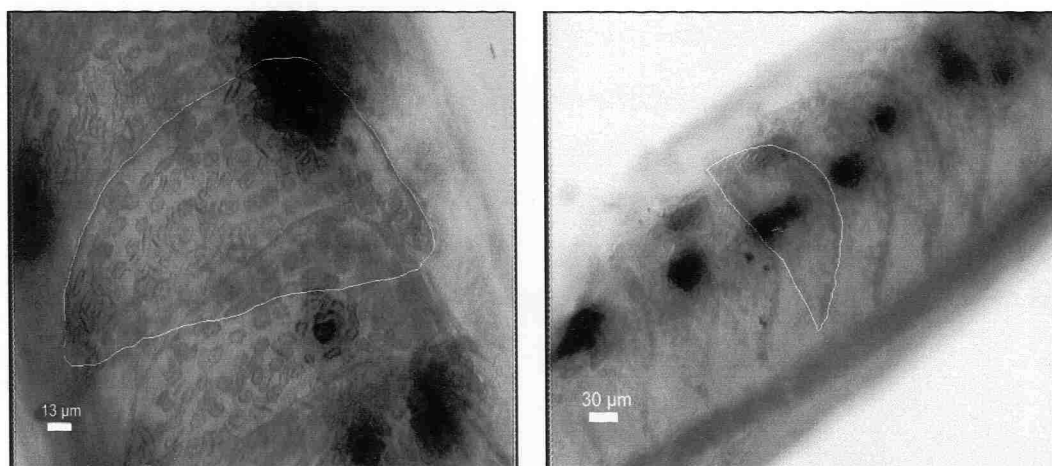


Figure 2. 8. Two examples of digitised microscopic images used for the measurement of unilateral lamellar area. The lamellae were outlined by hand (white line) and the enclosed areas integrated by a PC running 'Sigma Scan Pro' 4.0.

Total area of a given group was calculated using

$$A = \frac{n \cdot a \cdot 2}{10^6} \text{ mm}^2, \quad (\text{Eq 19})$$

where A is the total area of a given group [mm^2], n is the number of lamellae on the filaments (Eq. 18) and a is the average lamellar area for that group. The result is multiplied by 2 in order to account for the bilateral surface area of the lamellae. Lamellae at the tip of

large filaments were occasionally curled, bent or otherwise deformed and therefore not measured.

2.18.2. Total gill area

The results of the three weighted groups were then added together to give the total area estimate of each hemibranch. The same was done for the 15 remaining hemibranchs and their results added to obtain the total gill area estimate. The total gill area for each fish was correlated with the weight of the particular fish and used to develop an allometric equation of the type $y = m \cdot x^c$. Length-area correlation was preferred rather than the traditional weight-area correlation for all the experiments that depend on accurate gill area estimates, because the length of the fish did not fluctuate during the experiments as was the case for weight.

2.19. Statistical analysis

Organotin degradation was analysed with a Student's *t*-test. The osmolality data were examined with a one-way analysis of variance (ANOVA), followed by a Student-Newman-Keuls pair-wise multiple comparison test

Apart from net water balance data, that first required a square root transformation, all data sets were normally distributed. Comparisons between TBTCl, TPhTCl and control groups were analysed by a repeated measurement one-way analysis of variance (RM-ANOVA), followed by a Student-Newman-Keuls multiple comparison procedure. Maximum increases or decreases within each treatment group were compared to the respective initial values (t_0) using a paired *t*-test (Fry, 1993).

Chapter 3 – Experimental sediment preparations

3.1. Introduction

As explained in Chapter 2.8, great care was taken to produce an experimental substrate to which tri-*n*-butyltin chloride (TBTCI) and triphenyltin chloride (TPhTCl) would adsorb and maintain a reasonably stable sub-lethal concentration throughout the experiments. It was also important to create controlled conditions acceptable to flounders. In this chapter, the results of this initial phase of the project are presented. Triorganotin compounds may exist in water as cationic and/or as various neutral species that may exhibit quite different partitioning behaviour between water and non-aqueous phases (Arnold *et al.*, 1997).

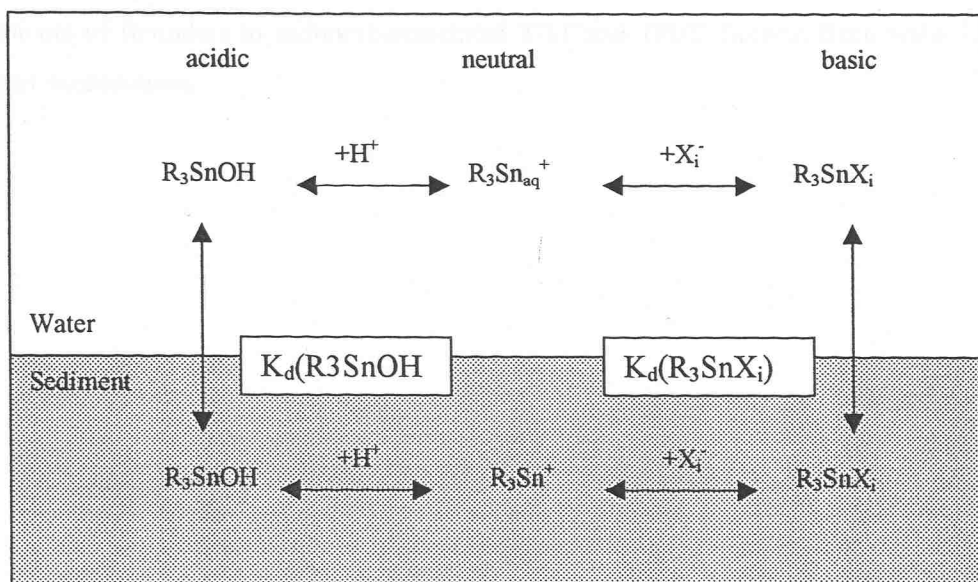


Figure 3. 1. Schematic representation of the partitioning behaviour of triorganotins in the sediment-water interface (after Arnold *et al.*, 1997)

Their adsorptive and partitioning behaviour is dependent on many factors such as salinity, pH, particle concentration and the organic content of these particles (Laughlin *et al.*, 1986; Harris & Cleary, 1987; Fent & Looser, 1995; Batley, 1996; Harris *et al.*, 1996; Arnold *et al.*, 1997). Despite the dynamic ionic conditions of many estuaries, the behaviour of triorganotin compounds seems to be dominated by their hydrophobic moiety (Harris & Cleary, 1987). The chemical characteristics of TBTCI and TPhTCl, in particular their hydrophobicity, favour their attachment to suspended particulate organic matter (POM), that can lead to an accelerated incorporation into the sediment (Tolosa *et al.*, 1992; Batley, 1996).

TBTCl and TPhTCl suspended in the water column have relatively short half-lives of 4 to 19 d (Seligman *et al.*, 1996) and 18 d (Soderquist & Crosby, 1980), respectively. However, once they have been incorporated into the sediment, they can persist, especially under anoxic conditions and shielded from photo- and often also biodegradation, for months, years, possibly even decades (Thain *et al.*, 1987; Adelman *et al.*, 1990; Waldock *et al.*, 1990; Watanabe *et al.*, 1995). There is also evidence of abiotic degradation of TBT, caused by the catalysis by clay minerals in sediments with a low organic content (Stang *et al.*, 1992; Weidenhaupt *et al.*, 1997).

The purpose of this chapter was to assess the quality of the sediment preparations in terms of the stability of TBT and TPhT concentrations and their suitability for chronic exposure experiments of flounders to sediment-associated TBT and TPhT, both in fresh water and sea water preparations.

3.2. Results

3.2.1. Sediment characteristics

The particle size range was measured with a series of shaking sieves and characterised the sediment as 67 % medium-fine sand, 30 % fine sand and 3 % coarse silt (Fig. 3.1). The experimental sediments contained 0.4 % TOC. The pH was 6.5 in fresh water and 8.4 in sea water preparations.

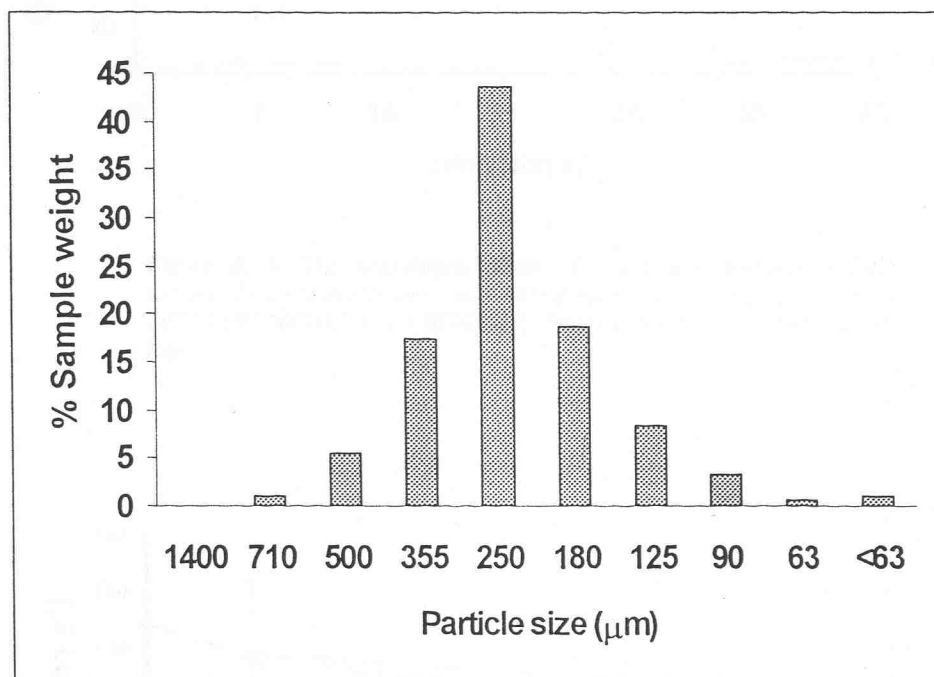


Figure 3. 2. Representative sediment sample showing the particle size distribution in the experimental sediment preparation.

3.2.2. Organotin concentrations and degradation products

Organotin-spiked sediments were sampled at weekly intervals throughout preliminary experiments. Speciation analysis was carried out as described above (see 2.9). The maximum TBTCI degradation after 6 weeks (42 days) of incubation was 19 % in fresh water and 15 % in sea water preparations, falling from initially 150 ng.g⁻¹ to 121 ng.g⁻¹ and 147 ng.g⁻¹ to 125 ng.g⁻¹, respectively (Figs. 3.2 & 3.3). Extrapolation of these values indicated a half-life of 95 d for fresh water and 110 d in sea water preparations. DBTCI₂ concentrations peaked at 8.9 ng.g⁻¹ in fresh water and 6.4 ng.g⁻¹ in sea water preparations. MBTCI₃ concentrations slowly increased peaking at 8.3 ng.g⁻¹ in fresh water, but were below the detection limit of 1 ng.g⁻¹ in sea water.

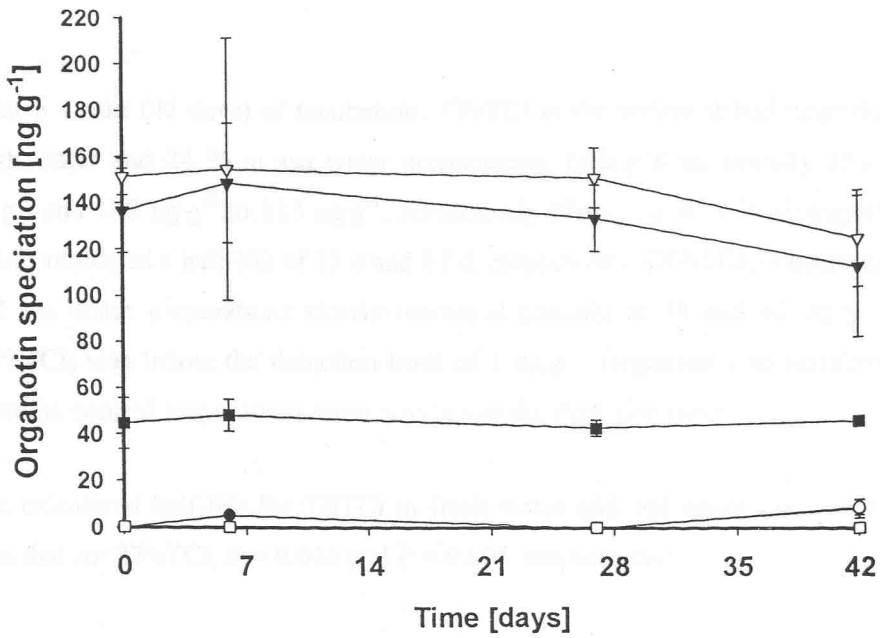


Figure 3. 3. The degradation pattern for sediment-associated organotin compounds in the experimental sediments prepared in fresh water ($n = 3 \pm \text{S.D.}$; TBTCI [▼]; DBTCI₂ [□]; MBTCI₃ [●]; TPhTCl [▽]; DPhTCl₂ [■]; MPhTCl₃ [□]).

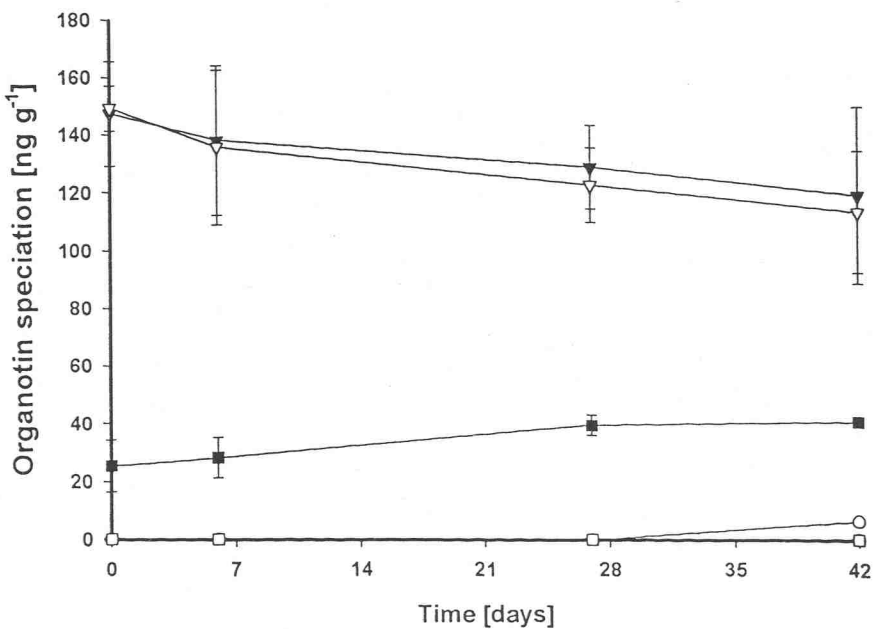


Figure 3. 4. The degradation pattern for sediment-associated organotin compounds in the experimental sediments prepared in sea water ($n = 3 \pm \text{S.D.}$; TBTCI [▼]; DBTCI₂ [□]; MBTCI₃ [●]; TPhTCl [▽]; DPhTCl₂ [■]; MPhTCl₃ [□]).

After 6 weeks (42 days) of incubation, TPhTCI in the sediment had degraded by 29 % in fresh water and 24 % in sea water preparations, falling from initially 151 ng·g⁻¹ to 115 ng·g⁻¹ and 149 ng·g⁻¹ to 113 ng·g⁻¹, respectively (Figs. 3.2 & 3.3). Extrapolation of these values indicated a half-life of 75 d and 87 d, respectively. DPhTCI₂ concentrations in fresh- and sea water preparations slowly increased peaking at 48 and 40 ng·g⁻¹, respectively. MPhTCI₃ was below the detection limit of 1 ng·g⁻¹. Organotin concentrations in samples from the control preparations were also below the detection limit.

The calculated half-life for TBTCI in fresh water and sea water was significantly higher than that for TPhTCI, $P = 0.003$ and $P = 0.005$, respectively.

3.3. Discussion

Estuarine waters are usually characterised by high loads of mineral particles, as well as high concentrations of dissolved organic material (DOM) and particulate organic matter (POM), derived from continental runoff and decomposing organic matter. Fluctuating salinity and changing pH through tidal action, as well as the particle concentration and their organic content have an effect on the adsorption behaviour and bioavailability of organotin compounds (Clavell *et al.*, 1986; Fent & Looser, 1995; Batley, 1996; Meador *et al.*, 1997). Desorption and re-introduction of sediment-associated triorganotin compounds to water have been extensively studied, because of the possible implications of organotin compounds leaching from dredged sediments. In enclosure experiments in Pearl Harbour, Hawaii, using an *in situ* dome, Stang & Seligman (1987) measured daily TBT adsorption rates of $0.23 \text{ ng Sn cm}^{-2}$ ($0.58 \text{ ng TBT cm}^{-2}$), but no TBT was observed to desorb from the undisturbed bottom sediments. Unger *et al.* (1988) examined adsorption and desorption behaviour of TBT from vigorously mixed natural sediments from Chesapeake Bay. They observed that the adsorption process was reversible, indicating that TBT contaminated sediments could act as secondary sources for dissolved TBT. Similar conclusions were made by Watanabe *et al.* (1997), who examined adsorption behaviour of TBT on sediments from the Toba estuary, Japan.

In order to interpret the results of ecotoxicological experiments, it is necessary to know the nature and concentrations of the pollutants involved. It is therefore quite common to spike sediments in order to achieve a specific nominal concentration (Meador *et al.*, 1997; Arnold *et al.*, 1998; Stronkhorst *et al.*, 1999); these authors reported recovery rates ranging from 50 to 110 %. This is consistent with the recovery ratios for TBTCI and TPhTCI in the sediment preparations used in this study, that were well over 90 %. Ideally, natural sediments are the material of choice. The scarcity of readily available non-contaminated sediment for this study made it necessary to produce an artificial substrate, that would ensure a stable organotin concentration and also be acceptable to flounders. The fine silver sand used would alone not have offered organotin compounds the required conditions for effective adsorption and bioavailability (Batley, 1996). Bueno *et al.* (1998) reported the adsorption behaviour of TBTCI to 'pure' quartz sand to be highly pH-dependent and found adsorption and desorption to be in equilibrium at pH 6 – 8. The fine deep-sea mud added to the silver sand preparations in the present study provided the organotin compounds with an increased surface area for adsorption and adjusted the TOC content of the sediment to

0.4 %. This falls within the range of sediment TOC concentrations (0.3 - 1 %) found by Meador *et al.* (1997) to yield the highest sediment-water partitioning coefficient (K_d) values. They spiked natural sediments containing a range of TOC concentrations with TBT and noted an inverse relationship between K_d values and TOC in sediments exceeding TOC levels of 1 %. This relationship has also been reported by Unger *et al.* (1988), who attributed the reduced K_d values to increased organotin degradation caused by biological activity. Similar observations were reported by Seligman *et al.* (1996), who spiked coastal sediment samples (TOC 2 - 22 %) from different US ports and harbours, containing a range of initial TBT concentrations ($< 2 - 241 \text{ ng.g}^{-1}$) with 300 ng.g^{-1} TBT. The observed half-lives ranged from $< 2 - 15 \text{ d}$, where the lowest TOC concentrations coincided with the longest half-lives, probably because of reduced biodegradation from a lack of microbial activity. This may also be true for the sediments used in this project. The low TOC concentrations (0.4 %) may explain the long half-life of 95 d and 110 d for TBTCI and 75 d and 87 d for TPhTCI, in fresh water and sea water, respectively. The significantly longer half live of TBTCI in both fresh water and sea water and the fact that DPhTCI₂ concentrations were higher than DBTCI₂ concentrations are consistent with octanol-water partitioning coefficient (K_{OW}) and K_d values given for TBTCI and TPhTCI (Fent & Hunn, 1991; Tolosa *et al.*, 1992) and indicate different adsorption and partitioning behaviour for TPhT and TBT.

In laboratory experiments conducted by Unger *et al.* (1988), the effect of changes in salinity, as observed in estuaries subject to tidal action, lead to a decrease in adsorption with increasing salinity. In the present experiments, the concentration of triorganotin compounds in the undisturbed artificial sediments at the end of the incubation period were slightly higher in sea water than fresh water (Figs. 3.2 & 3.3). Randall & Weber (1986), also using artificial sediments, observed higher adsorption at higher salinities and attributed this to a salting out effect, caused by the competition of Na^+ with TBT^+ for the carboxylate ions of fulvic acid. It is therefore possible that the observed behaviour of triorganotin compounds in the present study could be an artefact caused by the use of artificial, rather than natural sediments. However, Harris & Cleary (1987) found higher adsorption coefficients at higher salinities working with natural sediments from the Crouch and Tamar estuaries (U.K.). This suggests that, although the adsorption of triorganotin compounds is mainly determined by the hydrophobicity of the organic moiety, the observed behaviour is indeed a reflection of a combination of sediment parameters and chemical characteristics of triorganotins rather than a single governing factor.

3.4. Conclusions

The sediment preparation in this study made it possible to produce a chronic exposure of flounders to relatively stable environmental organotin concentrations. During the 6 week experiments in fresh water and sea water only 22 % and 19 % of TBTCI and 29 % and 24 % of TPhTCI added was degraded.

Chapter 4. Triorganotin compounds and osmo-regulation in freshwater-adapted flounders

4.1. Introduction

In fresh water the blood of the European flounder, *Platichthys flesus* (L.), is hyperosmotic with respect to the external medium, causing an increase in water influx and a loss of electrolytes. In sea-water the blood is hyposmotic with respect to the external medium inducing the loss of water and a diffusional influx of electrolytes. Osmoregulating organisms, as opposed to osmoconforming organisms, require a variety of mechanisms to regulate osmotic balance and body fluid volume, thereby maintaining constant intracellular conditions enabling efficient cellular function. Such mechanisms include, as well as strategies for ionic regulation, the control of drinking rates, urine production and membrane permeability (Motais *et al.*, 1966, 1969a; Evans, 1984).

The gills, with their large surface area and thin membranes, are not only responsible for respiration but are also the most important site of extrarenal ionic regulation. Their thin membranes not only make them more permeable for water than the rest of the body surface, but also make them obvious targets for lipophilic pollutants such as organotin compounds. The permeability of model biological membranes has been found to be sensitive to TBT (Przestalski *et al.*, 1996; Cullen *et al.*, 1997; Gabrielska *et al.*, 1997; Langner *et al.*, 1998) and TPhT (Sarapuk *et al.*, 2000) compounds. Therefore, the unidirectional flux of tritiated water (THO) was measured in order to determine any change in membrane permeability, that may have been caused by the chronic exposure of flounders to sediment-associated TBT and TPhT. The processes involved in osmo- and ionic regulation, such as drinking and urine production rates as well as Na^+/K^+ -ATPase activity were also determined and the results discussed in the light of the data acquired from blood osmolality measurements and growth rate determinations.

The aim of this chapter was to quantify the effects of TBT and TPhT on the osmoregulatory system of the euryhaline 0-group European flounder, *P. flesus*, adapted to fresh water. No experiments were performed to identify the mechanisms responsible for the observed results but several likely possibilities are discussed, supported by evidence provided by the available literature.

4.2. Results

4.2.1. Effects of triorganotin on water permeability

Chronic exposure of freshwater-adapted 0-group flounders to sediment containing 150 ng g⁻¹ tri-*n*-butyltin chloride (TBTCl) or triphenyltin chloride (TPhTCl) caused a significant reduction of the half-time of exchange ($T_{1/2}$) of tritiated water (THO), indicating a reduction in membrane permeability. The membrane permeability of the control group did not change throughout the experiment and is characterised by a stable $T_{1/2}$ (Fig. 4.1). During the first 14 days in the TBTCl group and 21 days in the TPhTCl group the $T_{1/2}$ had increased by 595 % and 561 %, from initially 41 ± 7 to 279 ± 94 minutes ($P < 0.05$) and 52 ± 10 to 288 ± 74 minutes ($P < 0.05$), respectively. However, the $T_{1/2}$ in both organotin groups increased back to normal after 35 days of exposure.

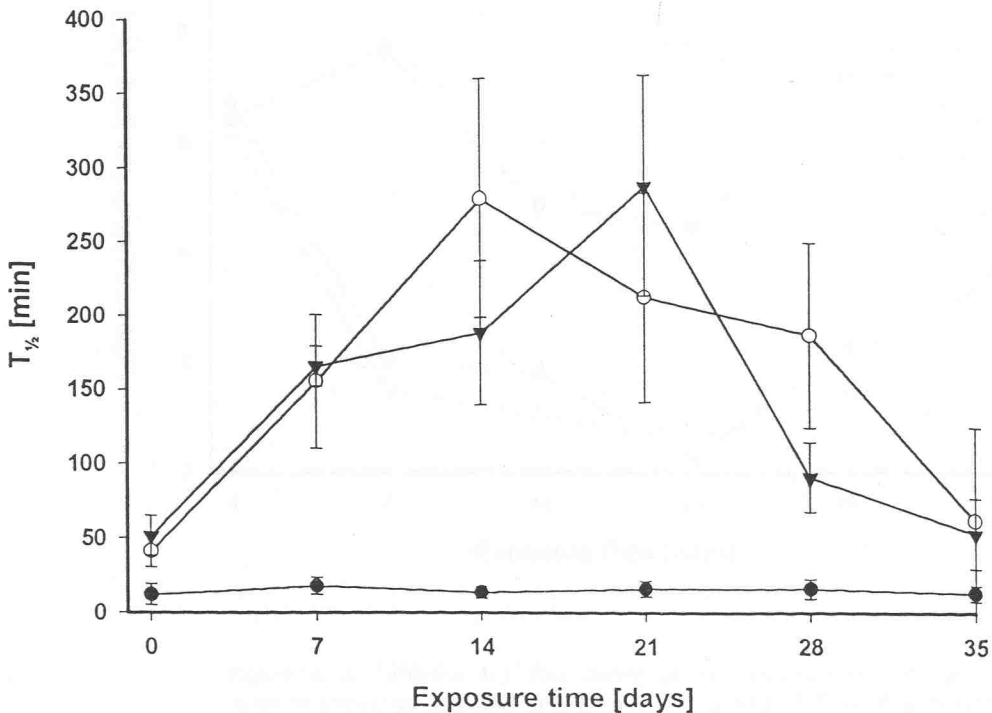


Figure 4. 1. Half-time of exchange ($T_{1/2}$) of THO during chronic exposure to 150 ng g⁻¹ sediment-associated organotin in minutes; $n = 15$; mean ± S. D. (Fish weight: 0.44 g ± 0.209; ○ TBTCl; ▼ TPhTCl; ● Control).

The reduced membrane permeability was reflected in a 90 % decrease in diffusional water flux, falling from $6.19 \pm 0.26 \mu\text{l g}^{-1} \text{h}^{-1}$ to $0.58 \mu\text{l g}^{-1} \text{h}^{-1} \pm 0.08$ ($P < 0.05$) in the TBT group and a 96 % decrease in the TPhT group, falling from $6.7 \pm 0.19 \mu\text{l g}^{-1} \text{h}^{-1}$ to $0.3 \pm 0.18 \mu\text{l g}^{-1} \text{h}^{-1}$ ($P < 0.05$; Fig. 4.2). After two weeks of exposure in the TBT group and three weeks in the TPhT group, respectively, $T_{1/2}$ began to decrease steadily, eventually reaching the level that the control group had constantly maintained throughout the experiment. It must be stressed that during most of the experiment, the THO flux across the membranes of both organotin groups was significantly lower than that of the control group ($P < 0.001$). There was no significant difference in the THO flux between the two organotin groups ($P > 0.05$). However, the diffusional water flux ($\mu\text{l g}^{-1} \text{h}^{-1}$) was generally significantly lower in the TBT group than in the TPhT group ($P < 0.05$).

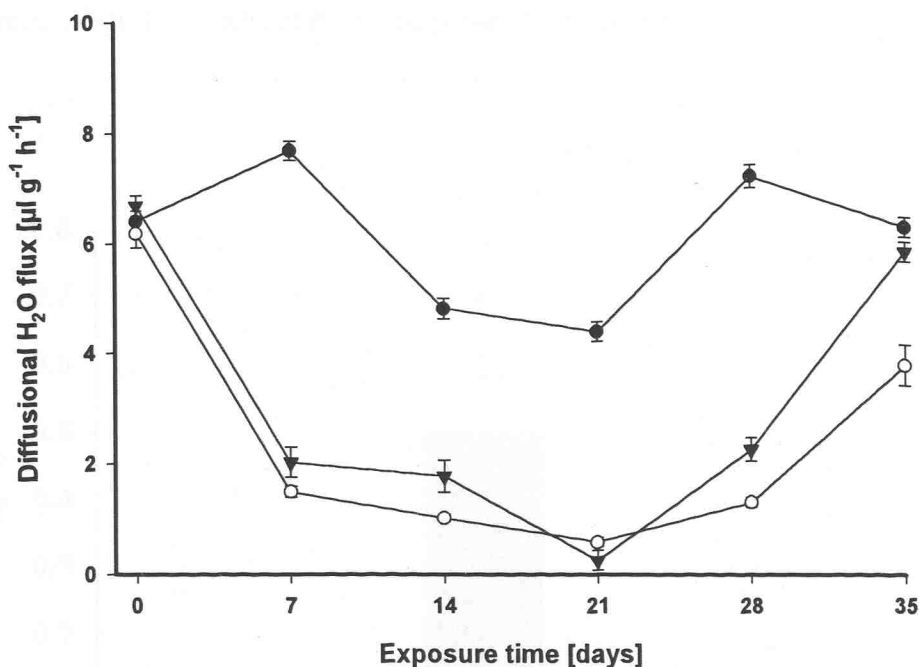


Figure 4. 2. Diffusive H_2O -flux during chronic exposure to 150 ng g^{-1} sediment-associated organotin in $\mu\text{l g}^{-1} \text{h}^{-1}$, $n = 15$; mean \pm S. D. (Fish weight: $0.44 \text{ g} \pm 0.209$; \circ TBTCl; \blacktriangledown TPhTCl; \bullet Control).

From the calculated net osmotic water flux, an estimate of the branchial surface area and the solute concentration gradient, the osmotic permeability coefficient (P_{os}) was calculated (Motais *et al.*, 1969a; Loretz, 1979) as:

$$P_{os} = \frac{flux_{net}}{A\sigma\Delta C_s}, \quad (\text{Eq 20})$$

where P_{os} is the osmotic permeability coefficient [cm s^{-1}], $flux_{net}$ is the net osmotic flux, calculated from urine minus drinking rates [mole s^{-1}], A is the branchial area [cm^2], σ is the Staverman or reflexion coefficient (≤ 1), that measures the effective semi-permeability of the membrane to the osmolyte and ΔC_s is the solute concentration gradient [mole cm^{-3}]. The diffusional permeability coefficient (P_d) (Motais *et al.*, 1969a; Loretz, 1979) was calculated as:

$$P_d = \frac{flux_{uni}}{A \cdot C_w}, \quad (\text{Eq 21})$$

where P_d is the diffusional permeability coefficient [cm s^{-1}], $flux_{uni}$ is the unidirectional flux [mmoles s^{-1}], A is the branchial surface area [cm^2] and C_w is the solute concentration gradient [mmole cm^{-3}]. The ratio P_{os}/P_d is typically close to one for fish in sea water, that tend to be less permeable than fish in fresh water, with ratios much greater than one (Loretz, 1979). The results of P_{os}/P_d are presented in Fig. 4.3.

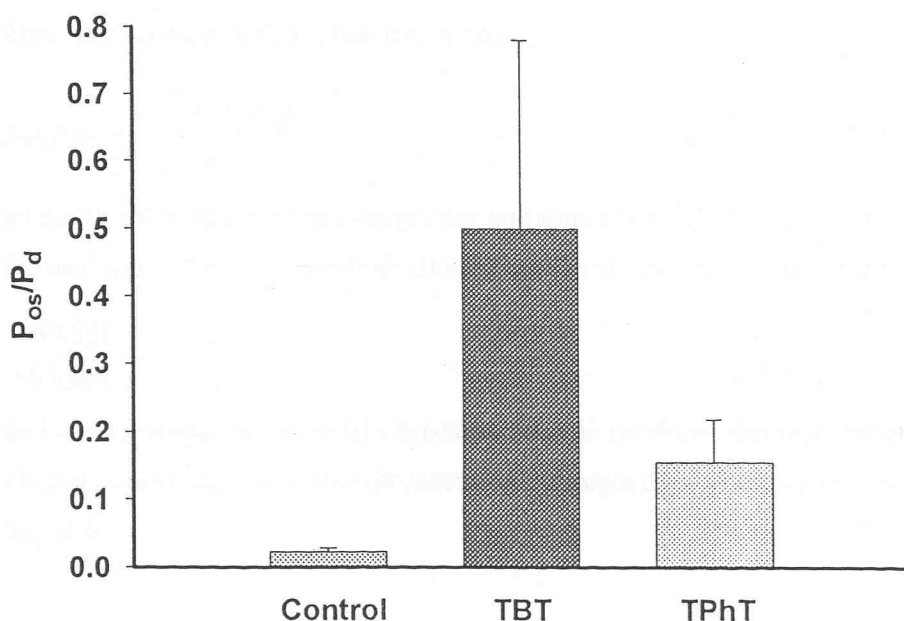


Figure 4. 3. P_{os}/P_d ratio after chronic exposure to 150 ng g^{-1} sediment-associated triorganotin compounds; fish weight: $0.44 \text{ g} \pm 0.209$ ($n = 15$; mean \pm S. D.).

The values from the TBTCI group were significantly higher ($P < 0.001$) than the control values, suggesting that the diffusional permeability in the TBTCI group was lower than in the control group. However, the ratio in the TPhTCI group did not differ significantly from the control group ($P > 0.05$).

4.2.2. Effects of triorganotins on the net water balance

The net water balance of a fish consists of several components: the diffusional water flux across its permeable membranes (gills) and the drinking and urine production rates. The application of radiotracers to osmoregulatory physiology has enabled the direct measurement of water fluxes. The net water balance can be calculated from the measurements of the half-time of exchange ($T_{1/2}$) of THO, the mole fractions of the surrounding medium and the blood and the total amount of water exchanged per unit time (Hutchinson & Hawkins, 1990).

The total water exchanged per unit time is calculated as:

$$R = \frac{100 \cdot \ln 2}{T_{1/2}} \% \text{ weight h}^{-1}.$$

Thus, the net water balance was calculated as:

$$\text{Net Flux} = \frac{R \cdot (m_1 - m_2)}{m_1} \quad (\text{Eq 22})$$

where R is the total water exchanged per unit time (% weight h^{-1}), m_1 is the mole fraction of the medium and m_2 is the mole fraction of the blood, where m_1 and m_2 are calculated from:

$$\frac{55.556}{55.556 + x} \quad (\text{Eq 23})$$

and x is the respective osmolal concentration. The results of this method for fish following chronic exposure to sediment-associated triorganotin compounds are presented in Fig. 4.4.

The direct measurement of the net water balance using the exchange of THO is based on the diffusional water fluxes across permeable membranes. The net water balance can also be estimated by calculating net osmotic permeability. The net osmotic water balance can only be determined indirectly (Figs. 4.4 & 4.7) by using the measurements of the drinking and the urine production rates (Figs. 4.5 & 4.6). Fresh water-adapted fish are hyper-

osmotic with respect to their environment and therefore a net influx would be expected. Thus the net osmotic water balance can be calculated by subtracting the values for drinking from the values for urine production rates (Smith, 1932).

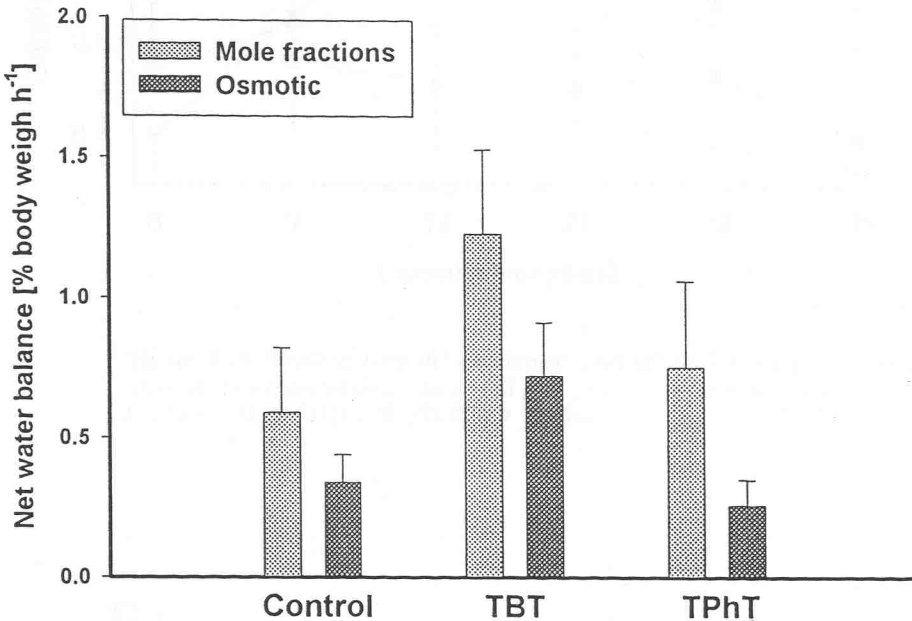


Figure 4. 4. Net water balance calculated from T_w and the mole fractions of the medium and fish blood and the net osmotic water balance calculated from urine production minus drinking rates, after five weeks of chronic exposure to 150 ng g^{-1} sediment-associated organotin; fish weight: $0.44 \text{ g} \pm 0.209$ ($n = 15$; mean \pm S. D.).

In the TBT and TPhT groups, drinking rates were significantly increased in the first three weeks and the first two weeks of chronic exposure, by 100 %, from $0.45 \pm 0.12 \text{ } \mu\text{l g}^{-1} \text{ h}^{-1}$ to $0.90 \pm 0.23 \text{ } \mu\text{l g}^{-1} \text{ h}^{-1}$ ($P < 0.001$) and by 122 %, from $0.4 \pm 0.10 \text{ } \mu\text{l g}^{-1} \text{ h}^{-1}$ to $0.89 \pm 0.23 \text{ } \mu\text{l g}^{-1} \text{ h}^{-1}$ ($P < 0.001$), respectively. They slowly decreased towards the end of the experiment, to $0.64 \pm 0.17 \text{ } \mu\text{l g}^{-1} \text{ h}^{-1}$ and $0.73 \pm 0.19 \text{ } \mu\text{l g}^{-1} \text{ h}^{-1}$, 42 % and 81 % above the initial values, respectively (Fig 4.5); the drinking rates of the control group did not change significantly ($P > 0.05$).

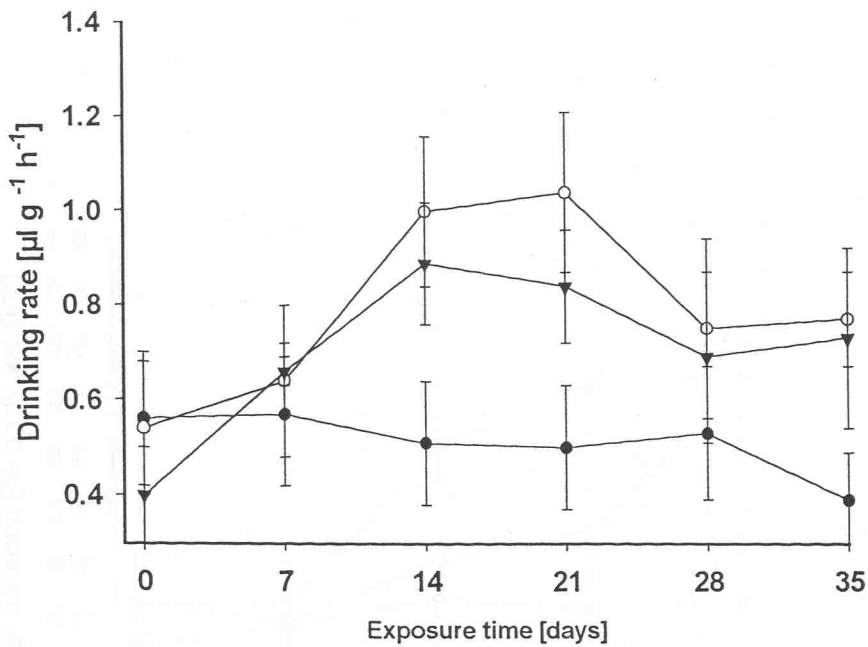


Figure 4. 5. Drinking rates during chronic exposure to 150 ng g⁻¹ sediment-associated triorganotin compounds in µl g⁻¹ h⁻¹; n = 15; mean ± S. D. (Fish weight 0.09 g ± 0.02; ○ TBTCI; ▼ TPhTCI; ● Control).

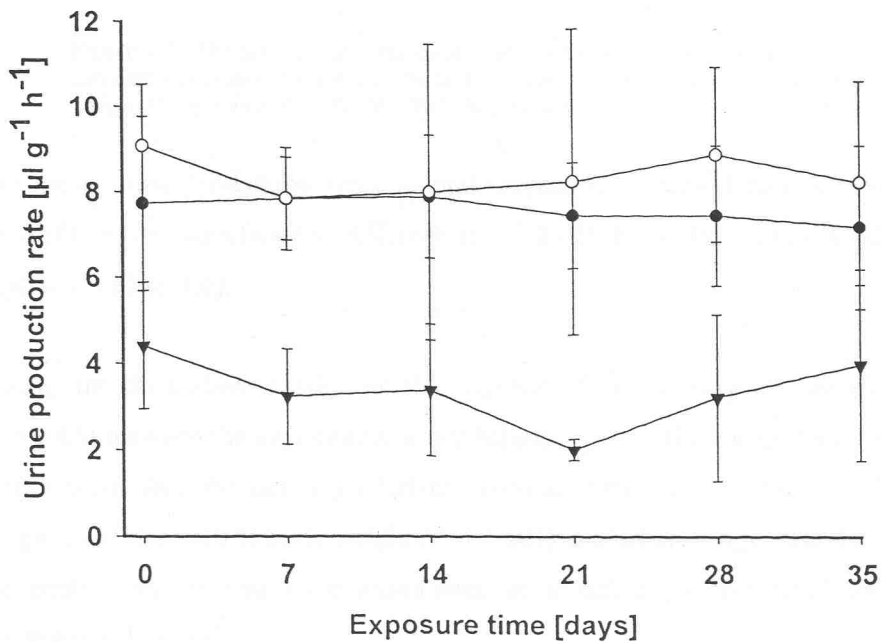


Figure 4. 6. Urine production rates during chronic exposure to 150 ng g⁻¹ sediment-associated triorganotin in µl g⁻¹ h⁻¹; n = 15; mean ± S. D. (Fish weight 0.09 g ± 0.02; ○ TBT; ▼ TPhT; ● Control).

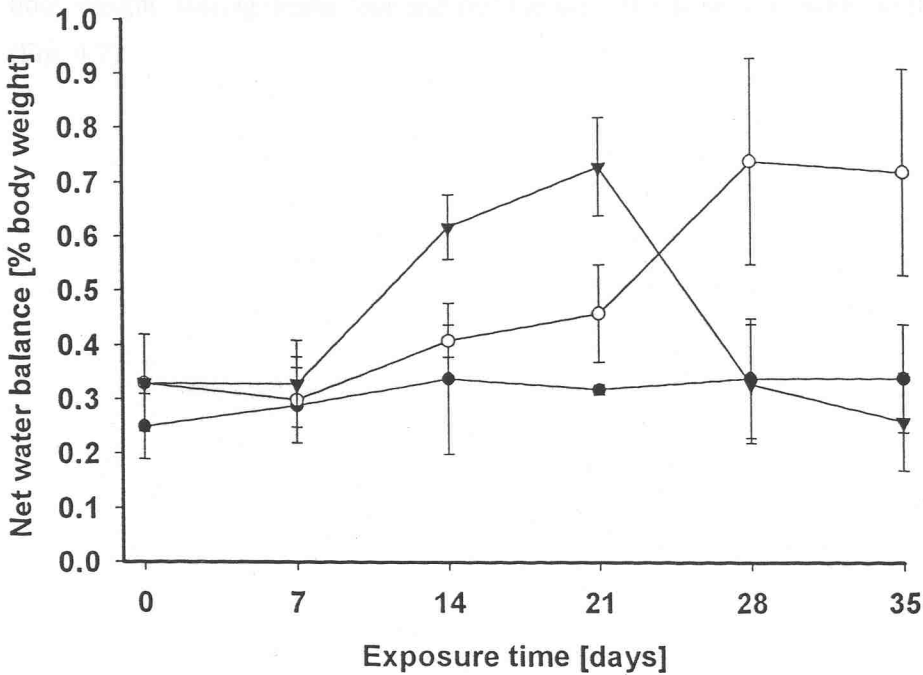


Figure 4. 7. The net osmotic water balance during chronic exposure to 150 ng g⁻¹ sediment-associated organotin in % body weight; n = 15; mean ± S. D. (Fish weight 0.09 g ± 0.02; ○ TBT; ▼ TPhT; ● Control).

The mean urine production rates in both organotin groups decreased slightly but were generally never significantly different ($P > 0.05$) from the values at the start of the experiment (Fig. 4.6).

During the first three weeks of the experiment, there was no significant difference ($P > 0.05$) between the net osmotic water balance of the TBT and control groups. However, during week four the net water balance increased by 123%, from 0.33 ± 0.09 % body weight to 0.74 ± 0.19 % body weight ($P < 0.001$) and differed significantly ($P < 0.001$) from the control values that were maintained at a stable positive level during the entire experiment (Fig. 4.7).

During the first three weeks the net osmotic water balance of the TPhT group peaked at 0.73 ± 0.19 % body weight, that is 120 % higher than the initial value of 0.33 ± 0.09 % body weight. During weeks four and five the net water balance returned to the initial value (Fig. 4.7).



4.2.3. Effects of triorganotin on sodium fluxes

Passive sodium (Na^+)-efflux rates in the control group remained unchanged during the experiment ($P > 0.05$). In the TBT and TPhT groups, however, passive Na^+ -efflux showed a 175 % and 84 % increase, from $32.21 \pm 8.32 \text{ nmol mm}^{-2} \text{ h}^{-1}$ to $88.33 \pm 22.81 \text{ nmol mm}^{-2} \text{ h}^{-1}$ ($P < 0.05$) and $38.10 \pm 11.0 \text{ nmol mm}^{-2} \text{ h}^{-1}$ to $70.32 \pm 20.30 \text{ nmol mm}^{-2} \text{ h}^{-1}$ ($P < 0.05$), respectively, over the first three weeks of exposure.

Towards the end of the experiment, the passive Na^+ -efflux decreased to $60.3 \pm 15.56 \text{ nmol mm}^{-2} \text{ h}^{-1}$ and $66.6 \pm 19.24 \text{ nmol mm}^{-2} \text{ h}^{-1}$, 88 % and 74 %, respectively, above the initial values at t_0 and were generally significantly higher ($P < 0.05$) than the values of the control group (Fig. 4.8).

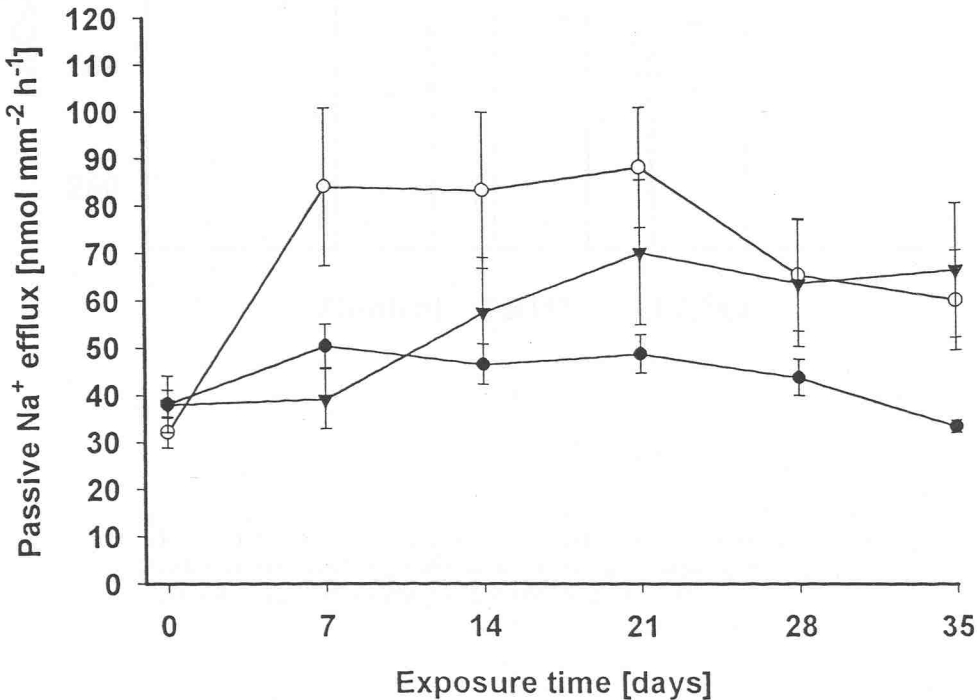


Figure 4. 8. Passive Na^+ -efflux during chronic exposure to 150 ng g^{-1} sediment-associated organotin in nmol per gill area and time; $n = 15$; mean \pm S. D. (Fish weight $0.19 \text{ g} \pm 0.05$; \circ TBT; \blacktriangledown TPhT; \bullet Control).

4.2.4. Effects of triorganotins on blood osmolality

After five weeks of exposure, the blood osmolality in both the organotin groups was significantly lower than the values determined in the control group (Fig. 4.9; $P < 0.05$). However, there was no significant difference in blood osmolality between the TBT and TPhT groups ($P > 0.05$).

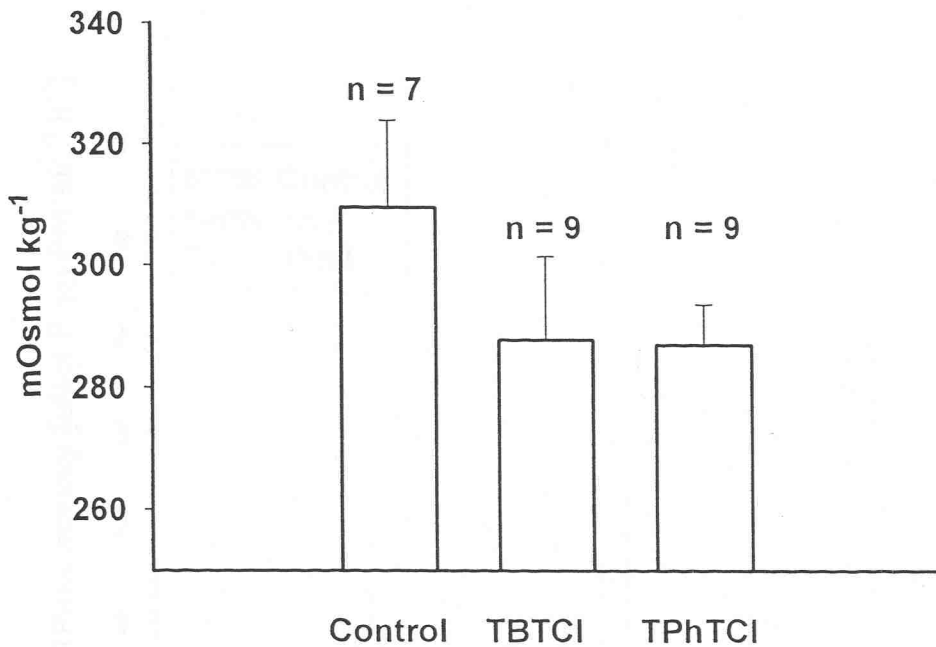


Figure 4. 9. Blood osmolality after five weeks of chronic exposure to 150 ng g⁻¹ sediment-associated organotin in mOsmol kg⁻¹ compared to the values in a control group (Fish weight 0.19 g \pm 0.05; mean \pm S. D).

4.2.5. Effects of triorganotin on the $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity of freshwater-adapted flounders

The activity of gill $\text{Na}^+\text{-K}^+$ -dependent adenosine triphosphatase ($\text{Na}^+\text{-K}^+\text{-ATPase}$) was measured in freshwater-adapted flounders during exposure to sediment-associated TBTCI and TPhTCI for 6 days (Fig. 4.10). The TBT group showed no significant change from the initial values at t_0 ($P > 0.05$) and there was also no significant difference between the TBT and control groups ($P > 0.05$). However the ATPase activity measured in the TPhT group was significantly increased ($P < 0.05$).

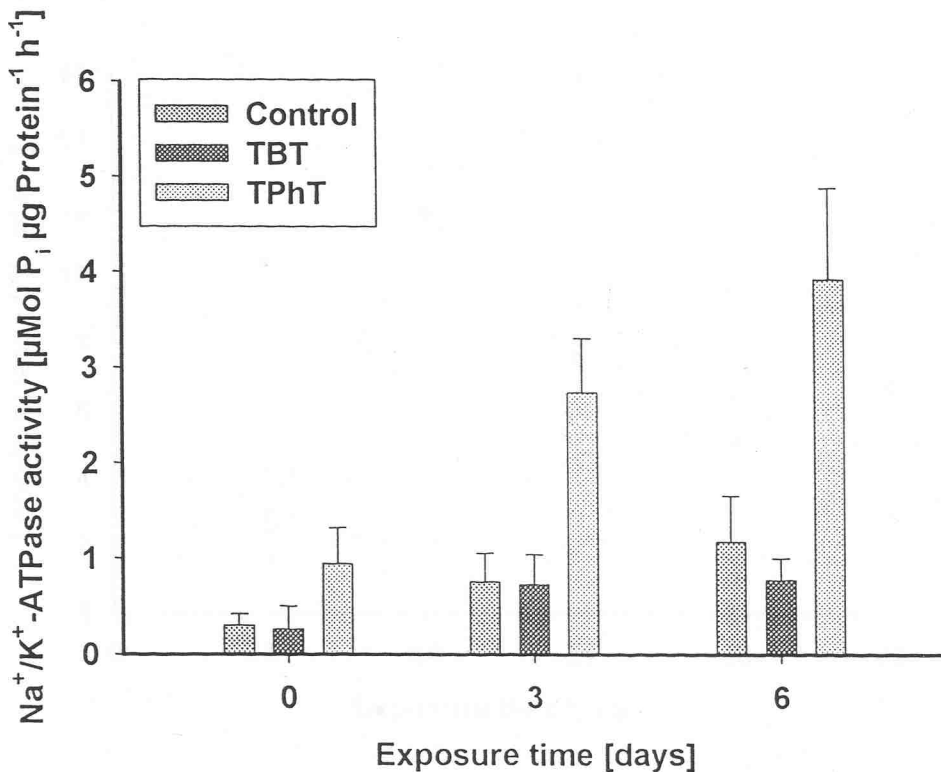


Figure 4. 10. $\text{Na}^+\text{/K}^+\text{-ATPase}$ activity in the gills of freshwater-adapted flounders during 6 days of exposure to sediment-associated organotin compounds, compared to a control group. (Fish weight $0.54 \text{ g} \pm 0.04$; mean \pm S.D.; $n = 6$)

4.2.6. Effects of triorganotin on growth rates during exposure

In order to evaluate any possible metabolic costs of counteracting the adverse effects of organotin exposure on the osmoregulatory system, the length of individual fish was measured in weekly intervals. The percentage length increase per week is plotted in

Fig. 4.11. During the first week of the experiment, the fish of the organotin and control groups all grew (length increase per week) at approximately the same rate of 3 %. The growth rate of the control fish then increased to 11.8 % after the second week, decreased to 8 % after the third week and then stabilised at 7 % for the rest of the 5 week experiment. The growth rate of the TPhT group increased only to 4.5 % after the second week but slowly increased during the remainder of the experiment and finished off just below the value of the control group at 6.5 % at the end of the exposure period. The growth rate of the TBT group, however, slowly decreased throughout the experiment and eventually reached 0.5 % growth per week at the end of the experiment.

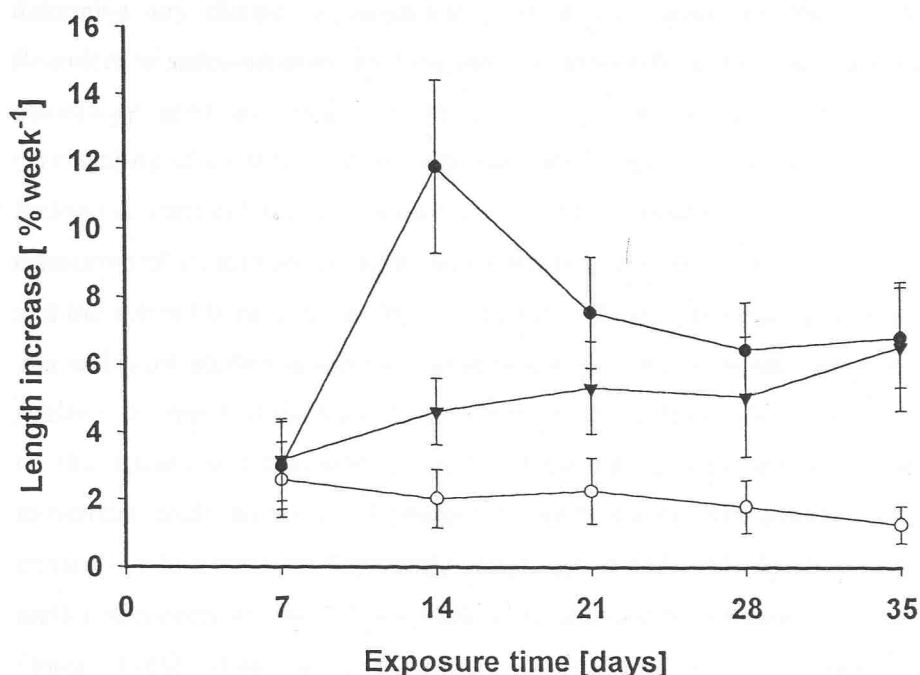


Fig. 4.11. Length increase during chronic exposure to 150 ng g⁻¹ sediment-associated TBTCI and TPhTCI expressed as % increase week⁻¹; n = 10; mean ± S. D. (○ TBT; ▼ TPhT; ● Control).

4.3. Discussion

Freshwater-adapted euryhaline fish are hyperosmotic in respect to the external medium. They therefore have not only to compensate for ion loss but also for an osmotic water influx across the gill membrane by adjusting the membrane permeability, drinking and urine production rates (Evans, 1969b). The effects of exposing freshwater-adapted 0-group flounders to environmental concentrations of sediment-associated TBTCI and TPhTCI on these physiological parameters are discussed below.

4.3.1. Effects of triorganotins on water permeability

The half-time of exchange ($T_{1/2}$) of tritiated water (THO) was measured in order to determine any change in membrane permeability caused by the chronic exposure of flounders to sediment-associated organotin compounds. Tritium and deuterium have been commonly used as tracers enabling direct measurements of the diffusional water permeability of the body surface of aquatic organisms (Motais *et al.*, 1969b; Loretz, 1979; Jackson & Fromm 1981; Lockwood *et al.*, 1982). Several problems are associated with the measurement of diffusional water permeabilities *in vivo*: (1) it is assumed that the tracer and the solvent behave in an identical fashion; (2) the presence of an osmotic gradient in this and most studies involving measurements of unidirectional fluxes in intact animals, neglects the fact that the tracer is involved in solvent flow, which may mask the diffusion of the tracer to some extent; (3) the temperature dependency of diffusional water movement could have caused changes to water fluxes. The experiments were therefore carried out in a constant temperature room set at 15°C; (4) unstirred layers have been a matter of concern in tracer flux studies using preparations of animal membranes (Dainty & House, 1966). However, the efficiency of fish gills in gas exchange depends on the lamellar surface area, the water to blood diffusion distance and the magnitude of matching gill ventilation and blood flow. Consequently, a healthy fish needs to constantly pass water across the gills to satisfy its respiratory needs and therefore gill membranes are regarded as having some of the best stirred layers (Johansen, 1982). In theory, internal unstirred layers, brought about by a mis-match between blood flow and gill ventilation, caused by blood being shunted away from the respiratory lamellae (Steen & Kryusse, 1964), could lead to an underestimation of diffusional water flux. However, the approximation of P_{os} and P_d in various fish species, led Evans (1969b) to the conclusion that unstirred layers in the membrane have little effect on the movement of water between fish and medium; (5)

fluctuating body weight during the five-week exposure experiments could have influenced $T_{1/2}$ (Evans, 1969b). Hutchinson (1984) reported no correlation between body weight and $T_{1/2}$ within the weight range of 0-group flounders; a finding confirmed by this study ($r = 0.05$). Nevertheless, care was taken to use fish of a similar size in one and the same experiment. The fish grew during the five week experiments, albeit at different rates, which may have contributed to differences between the fluxes of fish at a given time during the same experiment and stress-induced food refusal may also have played a part in weight and growth fluctuations.

The increased values in the TBT and TPhT groups (Fig. 4.1) suggest that the interaction of these compounds with the gills decreased the diffusional flux of THO across the membrane (Fig. 4.2), indicating a decrease in the apparent membrane permeability. The fact that $T_{1/2}$ returned to the initial value at the end of the 5 week experiment, suggests that the fish may be actively compensating for the effects of organotin exposure. This would occur at a metabolic cost and may be reflected in reduced growth rates (see below).

The effect of organotin compounds on membrane permeability has been widely studied using model membranes. For example, Cullen *et al.* (1997) reported a decrease in membrane 'fluidity', following the addition of TBTCI to the extraliposomal compartment of an egg phosphatidylcholine liposome preparation, that lead to a decreased efflux of encapsulated dimethylarsenic acid by passive diffusion. Heywood *et al.* (1989) recorded changes of membrane structure, such as lysis, caused by tributyltin compounds and suggested that this could lead to an increased permeability. Experiments with fluorescent probes have indicated that TBTCI locates itself in the hydrophobic core of erythrocyte membranes causing hemolysis (Falcioni *et al.*, 1996). These authors suggested that the oxygen radicals produced during this process could cause structural defects to the membrane by increasing the number of double bonds in the hydrocarbon chains, leading to modifications of membrane permeability.

The interaction of triphenyltin compounds with membrane lipid bilayers has been reported to be different from that of tri-*n*-butyltin (Langner *et al.*, 1998; Sarapuk *et al.*, 2000). Whereas TBTCI locates itself in the hydrophobic membrane core, TPhTCI was found to attach to the headgroup region of the model lipid bilayer and cause changes in the charge of model membranes, leading to a depolarisation of the membrane (Langner *et al.*, 1998; Radecka *et al.*, 1999). Visoottiviseth *et al.* (1999) found a time- and dose-dependent morphological effect of TPhT on the gill membranes of 0-group *Oreochromis nilotica*.

The nominal organotin concentrations used in this study were based on sediment concentrations found in the River Itchen. In all of the above studies, model membranes were used in conjunction with organotin concentrations that were several orders of magnitude higher than those applied in this study. This may explain the observed membrane disruption in those experiments. A reduction in membrane 'fluidity' has also been caused by other lipophilic compounds, such as cholesterol (Houslay & Stanley, 1982) and alpha-tocopherol (Fukuzawa *et al.*, 1979). Morris *et al.* (1982, 1987) found plasticisers and petroleum hydrocarbons, lipophilic pollutants, in the gill membranes of the amphipod *Gammarus duebeni*, that caused alterations to the fatty acid composition of the gill phospholipids which may also have led to changes in membrane permeability.

Although the octanol/water coefficient of both compounds is similar, this is only a measure of hydrophobicity and not necessarily a measure of bioavailability or toxicity, because octanol is not a good model for the complex structured lipid bilayer of a biological membrane. This becomes evident in the water permeability data from this study. Despite the fact that the $T_{1/2}$ for THO (Fig. 4.1) are similar in both organotin groups, when converted to actual diffusional flux rates (Fig. 4.2), the values for the TBT group were significantly lower than those for the TPhT group. The consequences of these different interactions are also reflected in the osmotic (P_{os}) and diffusive (P_d) permeability coefficients (Fig. 4.3).

The significantly increased P_{os}/P_d ratios observed in this study for flounders following the five-week exposure to TBTCI supports the interpretation of the THO flux data and suggests that TBTCI caused a reduction of diffusive membrane permeability and a shift towards osmotic permeability. Although the P_{os}/P_d ratios for the TPhT group were significantly higher than those for the control group, the shift towards osmotic permeability was far less pronounced than that in the TBT group. This could be another manifestation of the different modes of interaction of the two organotin compounds with biological membranes. The difference may also have been caused by the fact that flounders in the TPhT group lost weight during the experiment and were generally in a poorer condition than the TBT and control groups towards the end of the five-week exposure.

The P_{os}/P_d ratios for flounders of the control group in this study were much lower than the values reported previously for freshwater-adapted fish (Evans, 1969b; Motais *et al.*, 1969a; Potts *et al.*, 1967). Juvenile flounders do not develop scales until they reach 75 mm in

length (Hutchinson, pers. comm.) and therefore may be more permeable than the adult fish, that were used by the previous authors. The P_{os}/P_d ratios in this study were also much lower than the results reported by Hutchinson (1984) for 0-group individuals of the same species. This can almost certainly be attributed to gill area that constitutes an integral part of the equation for calculating P_{os} and P_d (Eqs. 18 & 19). The gill area estimates used in this study were derived from measurements performed on 0-group flounders using digital image analysis (see 2.17 & Chapter 6). The previous calculations of P_{os}/P_d ratios for 0-group flounders were, of necessity, based on gill area estimates from a fish weighing 200g (Hutchinson, 1984).

The gills account for 90 % of the diffusional water flux (Evans, 1969b, Motais *et al.*, 1969b), so a reduction in gill permeability and subsequent reduction of diffusive water influx would be likely to alter the water balance, so as to cause an increase in blood osmolality. In an osmoregulator, such as *P. flesus*, drinking rates and urine production are adjusted in order to offset any elevation of osmolality.

4.3.2. Effects of triorganotins on the net water balance

The drinking rates were estimated from the amount of imbibed ^{51}Cr -EDTA. Smith (1930) concluded that freshwater-adapted fish do not drink at all. However, since the introduction of gamma-emitting radiotracers it has become apparent that drinking does occur in freshwater-adapted fish, albeit much less than in fish adapted to sea water (Maetz, 1974). A variety of radiotracers have been used as markers to estimate drinking rates, such as colloidal ^{110}Ag (Dall, 1967), ^{125}I polyvinylpyrrolidone (Evans, 1968) ^{113}I or ^{125}I phenol red (Maetz & Skadhauge, 1968) and ^{51}Cr -EDTA (Babiker & Rankin, 1975). A problem that arises when estimating drinking rates using radiotracers is the assumption that all the tracer uptake is due to drinking. Although Hutchinson (1984) found no significant difference between the activity of the intact fish and the excised gut, any amount of tracer binding to the general body surface could lead to small errors and could cause an overestimation of the drinking rate. The fish were therefore thoroughly rinsed after loading in the medium.

The urine flow was estimated from the clearance of ^{51}Cr -EDTA. There are also a number of problems that have to be considered when interpreting the data derived from this method. According to Babiker & Rankin (1975) ^{51}Cr -EDTA underestimated the glomerular filtration rate in studies on *Tilapia nilotica* and *Anguilla anguilla* by 12.5 % and 9.5 %, respectively.

respectively, compared to ^3H inulin. ^{51}Cr -EDTA can also be actively retained by plasma proteins, that can lead to a retention of up to 5 % after dialysis (Babiker *et al.*, 1979). They also found some uptake and sequestration of ^{51}Cr -EDTA by lymphoid tissues in the kidneys, gonads and spleen. Estimation of urine flow requires measurements of a number of factors, such as U/B ratios, percentage water content, the ^{51}Cr -EDTA space as well as the $T_{1/2}$ of exponential loss of ^{51}Cr -EDTA. Taking all parameters into account, this method probably leads to an underestimation of the true rate of urine flow. Nevertheless, Babiker & Rankin (1975) concluded that this underestimation was acceptable and that ^{51}Cr -EDTA was a suitable tracer for the estimation of glomerular filtration rates in fish.

The drinking rates at t_0 (before the addition of organotin compounds) for the control, TBT and TPhT groups measured in this study, averaged 0.42, 0.45 and 0.40 $\text{ul g}^{-1} \text{h}^{-1}$ or 0.56, 0.54 and 0.67 % body weight h^{-1} , respectively. These values fall within the range reported by Hutchinson (1984) for 0-group flounders.

A healthy freshwater-adapted flounder would be expected to drink occasionally and to produce large volumes of dilute urine, in order to keep the net water influx and the ion loss at an absolute minimum and therefore the blood osmolality within a narrow range (Evans, 1979). This behaviour was observed in the control group (Figs. 4.5 & 4.6), with the exception of a slight increase of urine production towards the end of the experiment, which may be an artefact caused by handling stress (Eddy, 1981; McDonald & Milligan, 1997). However, Fletcher (1992) found stress-induced diuresis not to be a significant factor in stress experiments conducted with plaice (*Pleuronectes platessa*) and Lahlou (1967) also did not observe laboratory diuresis in *P. flesus*. The significant increase in drinking rates and failure to adjust urine production in both organotin groups in this study (Figs. 4.5 & 4.6) resembled the behaviour expected from a freshwater-adapted flounder after transfer to sea water and suggests an attempt by the fish to compensate for increasing blood osmolality, caused by the apparent reduction of membrane permeability for water. This process can also be observed in freshwater-adapted fish when subjected to osmotic stress as reported by Lahlou *et al.* (1969) for the goldfish *Carassius auratus*.

The data available in the literature suggest that between 62 % and 80 % of the water swallowed by various fresh-water and sea water-adapted euryhaline fish species is actually absorbed by the intestine (Smith, 1930; Hickman, 1968; Oide & Utida, 1968; Shehadeh & Gordan, 1969). If this is also the case for freshwater-adapted flounders, then the shift in the

osmotic water influx, caused by the enhanced drinking rates, should be reflected by a shift in the net osmotic water balance of organotin-exposed fish. This is in fact the case and is illustrated in Fig. 4.7 for TBTCI and TPhTCI exposed fish.

Theoretically, the net water balance determined by direct measurement of THO exchange and calculations using the mole fractions of the blood and external medium should equal the net water balance determined indirectly by measuring the drinking and urine production rates and subtracting the former from the latter. Although there was indeed no significant difference between the two results in the control group ($P > 0.05$; Fig. 4.4), the mean values of the control group derived from both methods did not balance each other and this was probably due to the fact that the parameters used for calculating the net water balance were obtained from separate experiments. It is therefore likely that slight differences in size and condition of the individual fish used have had an effect. Hutchinson (1984) reported slightly lower values for freshwater-adapted 0-group flounders than the results presented here, but there was also no significant difference between the results of the two calculation methods in his study. The mean net water balance for the TBT group was higher than in the control group (Fig. 4.7) and peaked towards the end of the experiment. The results for the TPhT group for most of the experiment were also higher than those of the control group, however, the peak was situated around the third week of exposure. The effect of both organotin compounds on the water balance was manifested in the decreased osmolality of the blood (Fig. 4.9). The fact that the net water balance for both organotin groups showed different patterns may have been caused by the different modes of interaction of these compounds with the gill membrane, although the poor condition of the fish after exposure to TPhTCI may also have had an effect. Furthermore, the results of the two methods of calculating the net water balance were significantly different from each other in both organotin groups ($P < 0.05$). This suggests a stress effect brought about by the exposure to TBTCI and TPhTCI.

4.3.3. Effects of triorganotins on passive sodium fluxes

Studies on the fluxes of Na^+ and Cl^- have shown that during acclimation to fresh water, euryhaline fish display a sharp reduction in the efflux of both ions in an effort to conserve ions in a hyposmotic environment (Evans, 1967; Motais, 1967; Potts *et al.*, 1967; Evans, 1969a). A further effect of the exposure of 0-group flounders to sediment-associated TBTCI and TPhTCI on the gill membranes observed in this study involves the passive efflux of Na^+ , that was significantly increased ($P < 0.05$; Fig. 4.8).

The gills, and in particular, the mitochondria-rich chloride cells are believed to be a significant site of ionic uptake in fresh water fish (Avella *et al.*, 1987; Laurent & Perry, 1990; Perry *et al.*, 1992; Uchida *et al.*, 1996). Evidence from Perry *et al.* (1992) showed that the rate of Na^+ uptake and the chloride cell area fraction of the gill membrane were highly correlated. If TBTCI and TPhTCl are interacting with gill membranes and changing them, as suggested by the results of the THO flux experiments in this study, then it is possible that these changes also affect the chloride cells. Grinwis *et al.* (1998) reported substantial gill membrane damage, such as lesions and lamellar fusion following the 14-day exposure of flounders to tri-*n*-butyltin oxide (TBTO; $7 \mu\text{g l}^{-1}$). However, Pinkney *et al.* (1989a) found no treatment-related morphological changes in the gill membranes of *Fundulus heteroclitus* at far lower concentrations of TBTO ($2 \mu\text{g l}^{-1}$). In both of these studies, TBT was administered as an aqueous suspension rather than sediment-bound as in the present study. Although the concentrations used in the present experiments were far lower than those used by Grinwis *et al.* (1998) and Pinkney *et al.* (1989a), the mode of exposure may be a more important factor than the actual level of contamination. Alternatively, the interaction of TBT and TPhT with the branchial membranes may have had an effect on intercellular junctions. These are believed to be dynamically regulated and rapidly changeable structures necessary for fish that exhibit rapid euryhalinity (Madara *et al.*, 1987; Karnaky, 1992).

4.3.4. Effects of triorganotins on the $\text{Na}^+ - \text{K}^+$ -ATPase activity of freshwater-adapted flounders

Experiments with the $\text{Na}^+ - \text{K}^+$ -ATPase activity of freshwater-adapted flounders exposed to sediment-associated organotin compounds may offer additional clues to the understanding of the changes in ionic fluxes observed in this study. Active sodium absorption from fresh water is mediated by coupled Na^+ / H^+ (Maetz, 1971) and/or $\text{Na}^+ / \text{NH}_4^+$ exchanges (Kerstetter *et al.*, 1970; Wood & Randall, 1973; Kerstetter & Keeler, 1976; Payan, 1978; Heisler, 1984). It is widely accepted that membrane-bound $\text{Na}^+ - \text{K}^+$ -ATPase of the chloride cell, is involved in this coupled Na^+ -transport in freshwater-adapted fish (Avella *et al.*, 1987; Laurent & Perry, 1990; Perry *et al.*, 1992; Uchida *et al.*, 1996), although there is some evidence that suggests, that pavement cells may also be involved in this process (Perry, pers. comm.). *In vitro* experiments have shown that TBTCI significantly inhibits the activity of Na^+ / K^+ -dependent adenosine triphosphatase (Na^+ / K^+ -ATPase) (Aldridge, 1976; Pinkney *et al.*, 1989b). However, Pinkney *et al.* (1989b) failed to find any significant

changes in Na^+/K^+ -ATPase activity, *in vivo*, during exposure of striped bass (*Morone saxatilis*) to an aqueous suspension of TBT. The Na^+/K^+ -ATPase activity measured in this study for freshwater-adapted flounders exposed to TBTCI for 6 days showed a similar result with values not significantly different from those of a control group.

The gill Na^+/K^+ -ATPase activity of freshwater-adapted flounders exposed to TPhTCI was significantly increased compared to the corresponding values of a control group (Fig. 4.10). The results of gill Na^+/K^+ -ATPase activity correspond with the data derived from the passive Na^+ efflux experiments (Fig. 4.8), that suggests that the lower passive Na^+ efflux observed in the TPhT group may be due to the increased Na^+/K^+ -ATPase activity. There are no observations reported in the literature concerning any effects TPhTCI may have on Na^+/K^+ -ATPase.

However, the nature of the different interactions of TBTCI and TPhTCI with biological membranes may again help to explain the results of the Na^+ efflux and Na^+/K^+ -ATPase activity experiments. If TBTCI is capable of penetrating deep into the gill membrane, but TPhTCI only interacts with the membrane surface, then the observed failure of the Na^+/K^+ -ATPase in the TBT group to respond to the changes in the membrane may be because TBTCI is actually reaching and inhibiting the basal border membrane-bound Na^+/K^+ -ATPase, whereas TPhTCI is not. Whatever the cause, it is likely that the increased loss of Na^+ may be contributing further to the decreased blood osmolality observed in both organotin groups (Fig. 4.9).

4.3.5. Effects of triorganotins on blood osmolality

The reduced blood osmolality in the organotin groups could be a reflection of the increased osmotic water influx rates caused by stress-induced increase in drinking as a consequence of permeability changes to the gill membranes following the interaction with re-mobilised sediment-associated triorganotin compounds. The results presented here also suggest that benthic fish, that are in contact with contaminated sediments, are more likely to suffer adverse effects to their osmoregulatory system than pelagic species. This also suggests that this source of exposure may be a more important factor than organotin compounds in the water column, especially as far higher concentrations in water seemed to have little effect on blood osmolality as shown by previous studies (Chliamovitch & Kuhn, 1977; Pinkney *et al.*, 1989b).

4.3.6. Effects of triorganotin on growth rates during exposure

The osmotic and ionic concentrations of fish body fluids usually differ from those of the surrounding medium, so the fish is required to expend a certain amount of energy in order to meet the metabolic costs of ionic and osmotic regulation. Apart from external signs such as infection, injury or behaviour, growth and weight gain are commonly used as indicators of a fishes condition. From this point of view, there would appear to be a metabolic cost attached to the changes produced by exposure to TBTCI and TPhTCI, that was manifested as a minimal increase in body length compared to the controls, as shown in Fig. 4.11. This observation is consistent with the findings of a study by Seinen *et al.* (1981), who observed significant growth retardation and weight loss in rainbow trout yolk sac fry during chronic exposure to 1 ppb TBTCI.

4.4. Conclusions

1) The results presented here lead to the conclusion that tri-*n*-butyltin chloride and triphenyl chloride in sediments are capable of significantly disrupting the osmoregulatory functions of a freshwater-adapted estuarine fish, at concentrations currently found in local sediments.

2) The half-time of exchange of THO in flounders exposed to organotin compounds was significantly increased, suggesting a reduction in membrane permeability for water.

3) The significantly lowered positive net diffusional water flux following five weeks of exposure to sediment-associated organotin compounds appears to be a consequence of the reduced membrane permeability.

4) The drinking rates increased significantly indicating a reaction to osmotic stress.

5) The urine production remained unchanged during the whole experiment, possibly an attempt by the fish to conserve ions.

6) The net water balance increased in both organotin groups, whereby the peaks for both groups occurred at different times during the experiment. This was possibly caused by structural differences between the two molecules and their different interaction with biological membranes.

7) The blood osmolality of both organotin groups was significantly lower than that of a control group at the end of the five week experiment.

8) The passive Na⁺ efflux increased during the experiment. The increased loss of Na⁺ was most likely linked to structural changes of the gill membrane, possibly affecting the surface area of chloride cells and/or the epithelial intercellular junctions.

9) There was no change of gill Na⁺-K⁺-ATPase activity in freshwater-adapted fish exposed to TBTCI. However, gill Na⁺-K⁺-ATPase activity was significantly enhanced during exposure to TPhTCI. This suggests, that TBTCI is inhibiting Na⁺-K⁺-ATPase whereas the

enzyme is not inhibited in the TPhT group and is reacting to the osmotic stress brought about by the exposure to TPhTCl.

10) From the data presented here, and the available evidence in the literature, it can be concluded, that although the exposure of 0-group flounders to TBTCI and TPhTCl exhibit similar effects on membrane permeability and blood osmolality, the effect on ionic regulation appears to be of a different nature. The reason for this may be found in the different chemical structures of the two compounds and their reported behaviour during interaction with biological membranes.

Chapter 5. Triorganotin compounds and ionic regulation in flounders during sea water adaptation

5.1. Introduction

Sea water adaptation of euryhaline fish involves radical changes in the physiology and morphology of gill membranes in order to cope with the transition from an hyposmotic to an hyperosmotic environment (Foskett *et al.*, 1981; Madsen *et al.*, 1994; Uchida *et al.*, 1996; Van der Heijden *et al.*, 1997; Marshall *et al.*, 1999). In particular, the rapid change in active Na^+ -flux from an ion-conserving influx to an ion efflux, facilitated by the membrane-bound enzyme, Na^+/K^+ -dependent ATPase, has been observed by many authors including Bornancin & De Renzis (1972), Forrest *et al.* (1973), Potts *et al.* (1973), Thomson & Sargent (1977) and Carroll *et al.* (1995). In addition, chloride cells, the sites of active ion exchange, display considerable changes in density and distribution within the gill epithelial membranes during adaptation to sea water as has been shown by Thomson & Sargent (1977), Girard & Payan (1980), King & Hossler (1991) Uchida *et al.* (1996) Van der Heijden *et al.* (1997) Hiroi *et al.* (1998) Hirai *et al.* (1999) Wong & Chan (1999) and Sakamoto *et al.* (2000). Several authors have also reported a significant correlation between the ionic transport rate and the number of chloride cells and provided clear evidence that chloride cells are the site of active NaCl -excretion in sea water (Karnaky *et al.*, 1979, 1984; Foskett & Scheffey., 1982).

In the epithelial membranes of seawater-adapted gills of euryhaline fish, chloride cells are connected to other fully developed or immature chloride cells, the so-called accessory cells, by shallow tight junctions and to pavement cells by deep tight junctions (Hootman & Philpott, 1980; Laurent & Dunel, 1980; Karnaky, 1992; see Fig. 5.1). The Na^+/K^+ -ATPase located in the tubular system of the chloride cells, creates a sodium gradient across the tubular system plasma membrane. The gradient in turn drives a sodium chloride carrier (secondary active transport) that is also located on the tubular system membrane. Cl^- enters the cell via this pathway, diffuses to the apical crypt and exits to the external medium (sea water) through a chloride channel. Na^+ exits passively to the sea water side of the cell via the “leaky” shallow tight junctions between the chloride and/or accessory cells. The driving force for the Na^+ movement is the transepithelial electrical potential gradient established by the ATPase mediated movement of Cl^- (Potts, 1968; Evans, 1980, 1993; Karnaky, 1992, 1998).

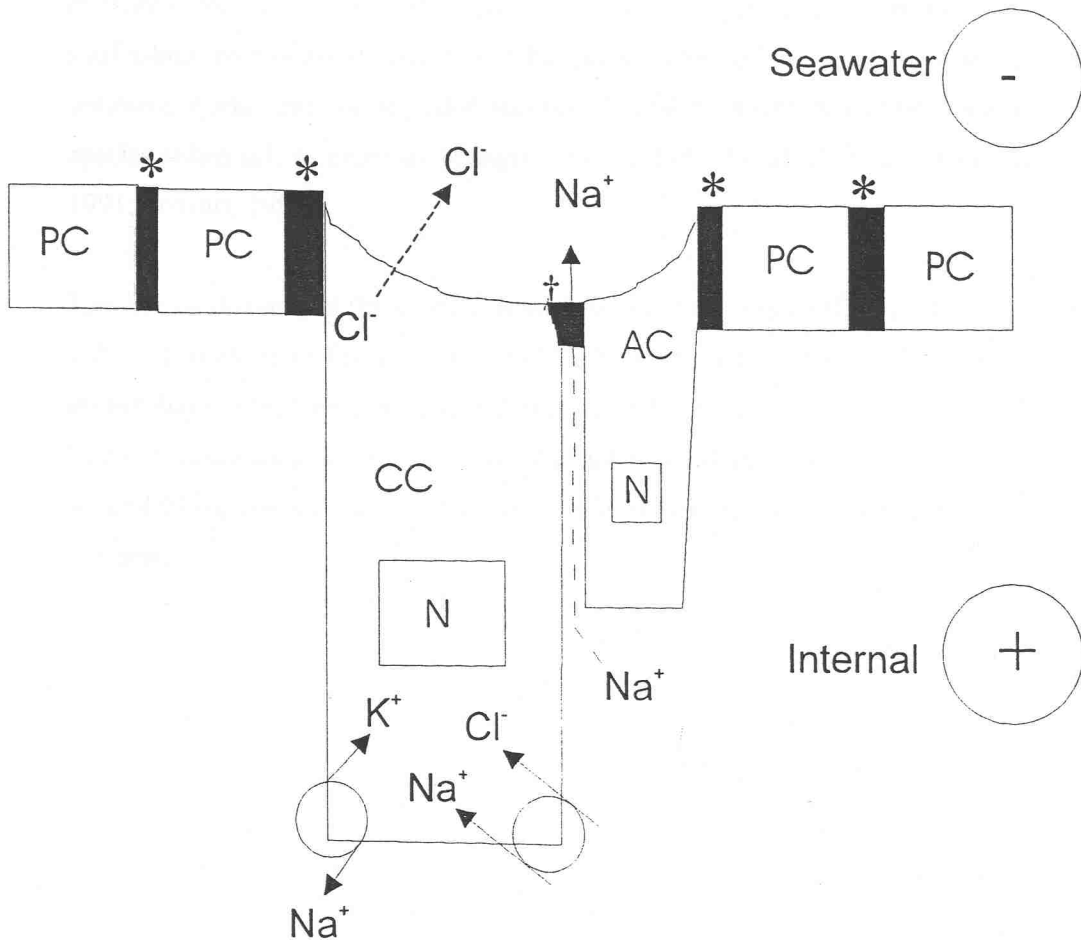


Figure 5. 1. Schematic model of a chloride-accessory cell complex of a seawater-adapted euryhaline fish. Pavement cell (PC); chloride cell (CC); accessory cell (AC); nucleus (N); deep tight junctions (*), shallow tight junction (†); after Karnaky (1992).

Depending on the species and their degree of euryhalinity, sea water adaptation of freshwater-adapted fish can take anything from a few hours (Marshall *et al.*, 1999) to several days (Forrest *et al.*, 1973). This process can be severely disrupted by a wide variety of organic and inorganic pollutants, including organotin compounds (Coleman *et al.*, 1977; Pinkney *et al.*, 1989b; Wendelaar Bonga *et al.*, 1989, 1990, 1992; Sola *et al.*, 1995; Devos *et al.*, 1998; Galvez *et al.*, 1998; Hogstrand *et al.*, 1999). Although the above studies were carried out under different conditions and using different types of pollutants, they were all performed with sublethal water concentrations, but no effects of polluted sediments on osmotic and ionic regulation have been reported. Therefore, the approach in the present study was to use environmental levels of sediment-associated organotin compounds in order to observe the histopathological and physiological effects on the gill membranes of a

benthic euryhaline fish, the European flounder, *Platichthys flesus* (L.). This is particularly relevant since, despite the partial ban of organotin compounds as the biocidal ingredient in antifouling formulations and the subsequent reduction in water concentrations, the sediment levels have not degraded accordingly and are easily remobilised making benthic species vulnerable to exposure (Langston *et al.*, 1987; Waldock *et al.*, 1990; Waite *et al.*, 1991; Stewart, 1996).

The aim of this part of the study was to demonstrate and quantify any effects remobilised sediment-associated TBTCI and TPhTCI have on the physiology and associated gill morphology of 0-group flounders at levels currently found in local Southampton sediments. The exact mechanisms responsible for the observed effects were not investigated, although several likely possibilities are discussed, supported by evidence provided by the available literature.

5.2. Results

5.2.1. Effect on active Na⁺-efflux

The average rates of active Na⁺-efflux in freshwater-adapted 0-group flounders at t_0 (before the exposure to organotin compounds) measured $50 \pm 10 \text{ nmol mm}^{-2} \text{ h}^{-1}$ and showed no significant difference between the three groups ($P > 0.05$; Fig. 5.2).

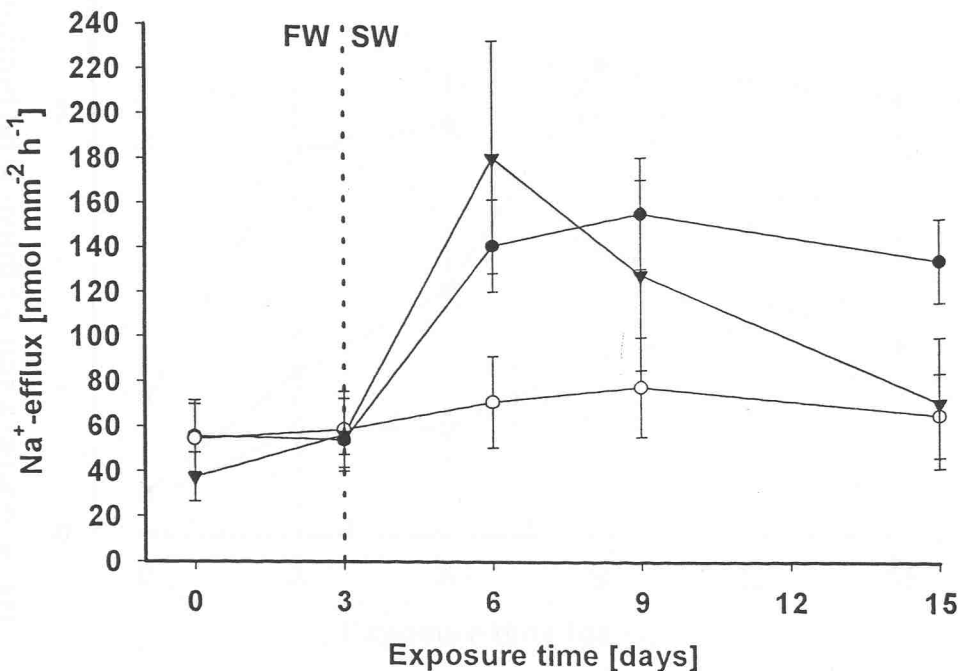


Figure 5. 2. Active Na⁺-efflux during chronic exposure of freshwater-adapted (FW) 0-group flounders to 150 ng g^{-1} sediment-associated organotin before and after transfer to sea water (SW) in nmol per gill area and time; $n = 15$, mean \pm S.D. (Fish weight $0.19 \text{ g} \pm 0.05$; ○ TBT; ▼ TPhT; ● Control).

After three days of exposure to TBTCl and TPhTCl in fresh water, there was still no difference between the organotin groups and the control group ($P = 0.891$). The fish were then transferred to full strength sea water. Three days after transfer the active Na⁺-efflux rates in the control and TPhT groups increased dramatically to 140 ± 40 and $180 \pm 52 \text{ nmol mm}^{-2} \text{ h}^{-1}$ ($P < 0.001$), respectively, but were not significantly different from each other ($P = 0.356$). The TBT group displayed only a minimal increase to $65 \pm 20 \text{ nmol mm}^{-2} \text{ h}^{-1}$ ($P = 0.329$) and was significantly lower than the rates measured in the other two groups ($P < 0.001$). While the active Na⁺-efflux rates in the control group remained stable

thereafter, the efflux rates in the TPhT group decreased to the same level as the TBT group at the end of the 15 day experiment.

5.2.2. Effect on gill Na^+/K^+ -ATPase activity

The Na^+/K^+ -ATPase activity of freshwater-adapted 0-group flounders was measured before and after transfer to sea water (Fig. 5.3).

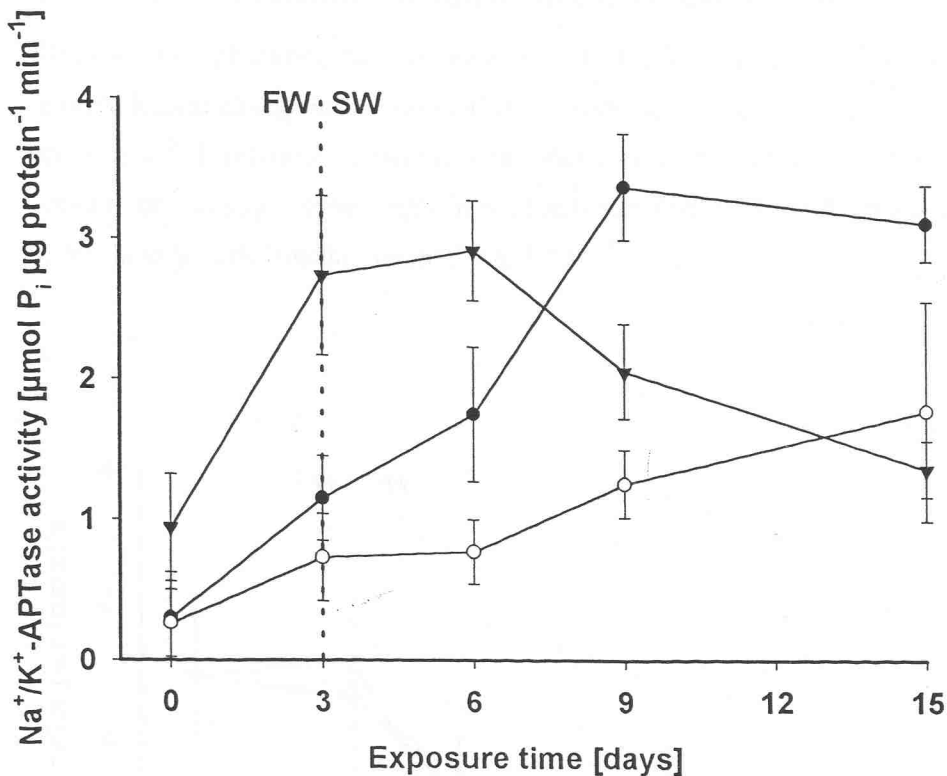


Figure 5. 3. Na^+/K^+ -ATPase activity in the gills of freshwater-adapted (FW) 0-group flounders during chronic exposure to sediment-associated organotin compounds, before and after transfer to sea water in $\mu\text{mol P}_i \mu\text{g protein}^{-1} \text{min}^{-1}$ (SW); mean \pm S.D.; $n = 6$ (Fish weight $0.54 \text{ g} \pm 0.04$; ○ TBT; ▼ TPhT; ● Control).

At t_0 (before organotin exposure) the Na^+/K^+ -ATPase activity was similar to that measured in a previous experiment using freshwater-adapted flounders (see Fig. 4.10), averaging $0.50 \pm 0.31 \mu\text{mol P}_i \mu\text{g protein}^{-1} \text{min}^{-1}$. Three days following the addition of TPhTCl the TPhT group displayed a significant increase ($P < 0.002$) in Na^+/K^+ -ATPase activity, which is consistent with the previous results that were discussed in Chapter 4 (see 4.3.4 & Fig. 4.10). The Na^+/K^+ -ATPase activity of the TBT and control groups showed no significant increase ($P > 0.05$). Three days after transfer to sea water, the Na^+/K^+ -ATPase activity in the control group increased to $1.75 \pm 0.48 \mu\text{mol P}_i \mu\text{g protein}^{-1} \text{min}^{-1}$, increased further after

6 days ($P < 0.001$) to $3.37 \pm 0.38 \mu\text{mol P}_i \mu\text{g protein}^{-1} \text{min}^{-1}$ and thereafter remained stable for the rest of the experiment. The organotin groups showed a completely different behaviour. The Na^+/K^+ -ATPase activity of the TPhT group levelled off after the transfer to sea water and then gradually fell to $1.36 \pm 0.20 \mu\text{mol P}_i \mu\text{g protein}^{-1} \text{min}^{-1}$. The Na^+/K^+ -ATPase activity of the TBT group showed no significant ($P > 0.05$) increase throughout the whole experiment, even after transfer to sea water.

5.2.3. Effect on chloride cell distribution in the gill epithelium

During the physiological acclimation of freshwater-adapted euryhaline fish, a morphological change in the gill epithelia became apparent. This change is most visible in the size and distribution of chloride cells. Before the addition of organotin compounds the number of lamellar chloride cells in freshwater-adapted 0-group flounders averaged 2.5 ± 0.59 chloride cells lamella⁻¹ (Figs. 5.4 & 5.10).

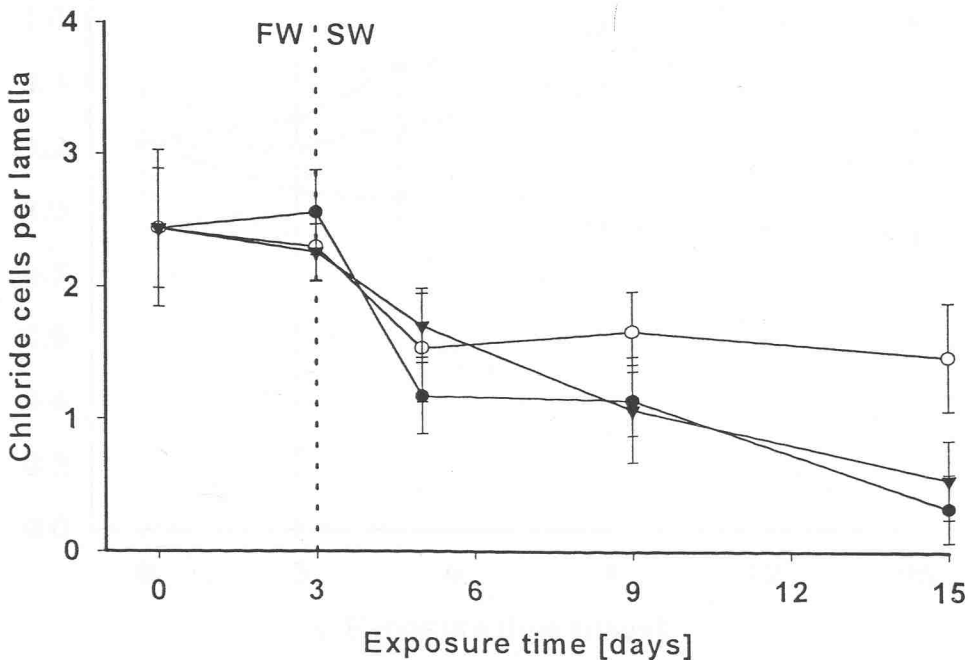


Figure 5. 4. Number of lamellar chloride cells of freshwater-adapted (FW) 0-group flounders during chronic exposure to sediment-associated organotin compounds, before and after transfer to sea water (SW); $n = 6$; mean \pm S.D. (Fish weight $0.15 \text{ g} \pm 0.04$; ○ TBT; ▼ TPhT; ● Control).

The exposure to TBTCI and TPhTCI in fresh water had no significant effect on the number of lamellar chloride cells, $P = 0.2761$ and $P = 0.1023$, respectively. However, three days after the transfer to sea water a significant reduction in lamellar chloride cells was observed in the control group (1.18 ± 0.29 chloride cells lamella⁻¹; $P < 0.001$) and decreased further still to 0.33 ± 0.26 chloride cells lamella⁻¹ towards the end of the experiment (Figs. 5.4 & 5.11). The number of lamellar chloride cells in the TBT group levelled off at 1.5 ± 0.41 chloride cells per lamella, remained unchanged for the rest of the experiment and was significantly higher than the number of lamellar chloride cells in the control group ($P < 0.001$; Figs. 5.4 & 5.12). The TPhT group displayed a significant reduction of lamellar chloride cells and gradually reached 0.55 ± 0.30 chloride cells lamella⁻¹, that was not significantly different from the number of lamellar chloride cells found on gill sections of the control group at the end of the experiment ($P = 0.9691$; Figs. 5.4 & 5.13).

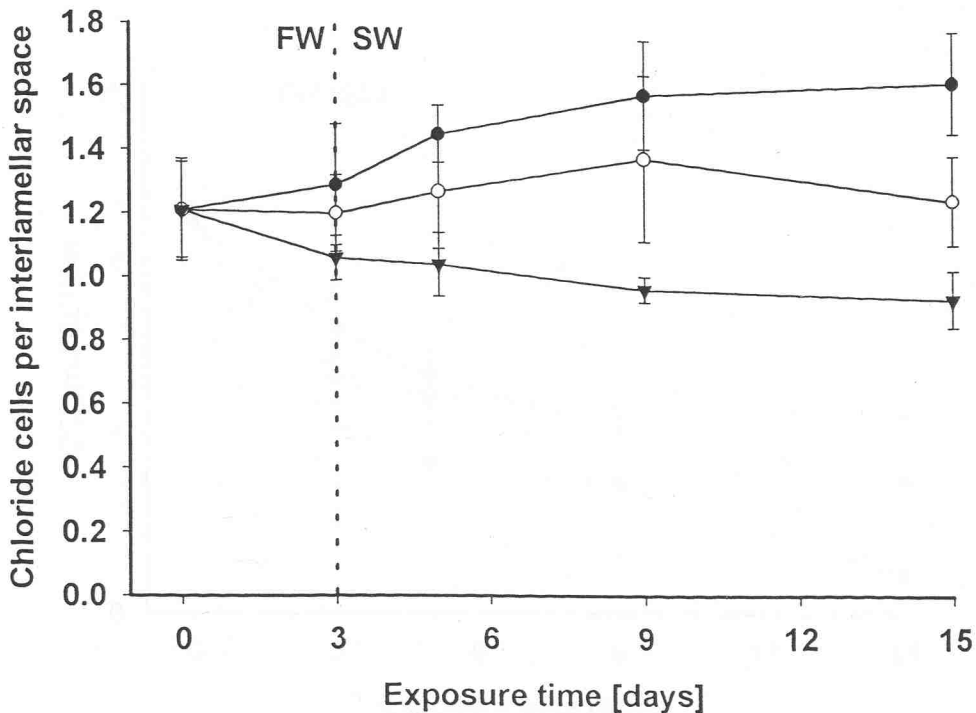


Figure 5. 5. Number of interlamellar chloride cells of freshwater-adapted (FW) 0-group flounders during chronic exposure to sediment-associated organotin compounds, before and after transfer to sea water (SW); $n = 6$, mean \pm S.D. (Fish weight $0.15 \text{ g} \pm 0.04$; ○ TBT; ▼ TPhT; ● Control).

The interlamellar epithelia were also examined and the number of chloride cells determined. The interlamellar chloride cell count averaged 1.2 ± 0.15 chloride cells per interlamellar space in freshwater-adapted 0-group flounders at t_0 (before organotin exposure; Figs. 5.5 & 5.10).

Three days after exposure to TBTCI and TPhTCI there was no significant difference in the numbers of interlamellar chloride cells between the three groups (control: 1.29 ± 0.12 ; TBT: 1.20 ± 0.19 ; TPhT: 1.06 ± 0.07 chloride cells per interlamellar space $P > 0.05$; Fig. 5.5). On day two, following the transfer to full strength sea water, there was still no significant change in the number of interlamellar chloride cells in both the organotin and control groups ($P > 0.05$; Fig. 5.5). However, at the end of the experiment, after 12 days in sea water the number of interlamellar chloride cells was significantly higher in the control group (1.61 ± 0.16 chloride cells per interlamellar space) than in the TBT group ($1.24 \pm$

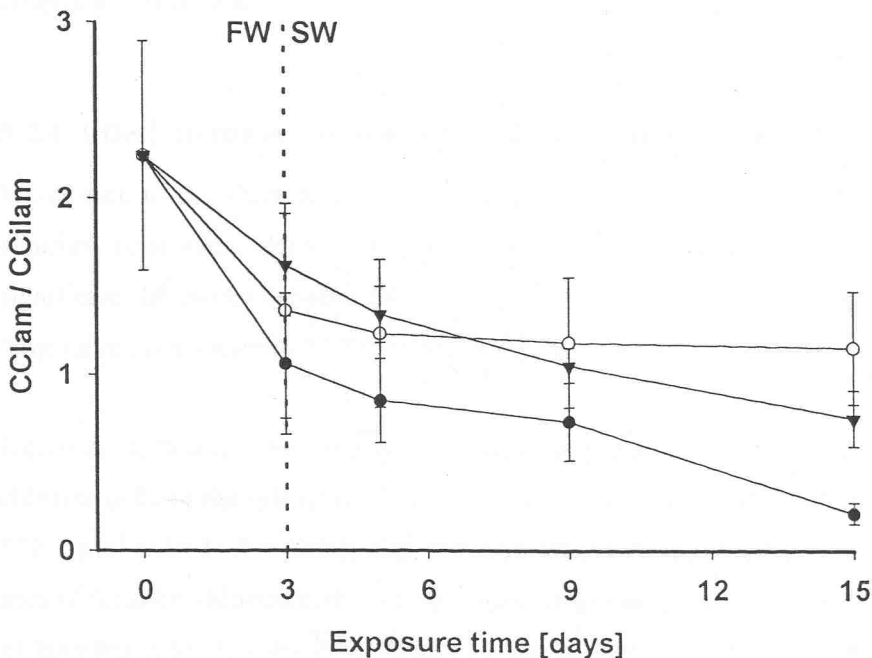


Figure 5. 6. The ratio of lamellar (CCLam) vs. interlamellar (CCilam) chloride cells of freshwater-adapted (FW) 0-group flounders during chronic exposure to sediment-associated organotin compounds, before and after transfer to sea water (SW); $n = 6$; mean \pm S.D. (Fish weight $0.15 \text{ g} \pm 0.04$; ○ TBT; ▼ TPhT; ● Control).

0.14 chloride cells per interlamellar space ; $P < 0.001$; Figs. 5.5 & 5.11 - 12). At the end of the experiment, the interlamellar chloride cell count for the TPhT group dropped sharply to 0.93 ± 0.09 chloride cells per interlamellar space and was significantly lower than either the TBT or the control groups ($P < 0.05$; Figs. 5.5 & 5.13).

The ratio of lamellar to interlamellar chloride cells is an indicator of the extent of morphological changes taking place in the gill epithelia during adaptation to sea water and the effect of exposure to sediment-associated organotin compounds on the distribution of chloride cells (Fig. 5.6).

The number of chloride cells, both lamellar and interlamellar, had declined in the TPhT group, but seemingly no significant change had occurred in the TBT group. Plotting the ratio of the number of lamellar to interlamellar chloride cells against time, revealed that the two organotin groups showed a similar pattern of chloride cell distribution during adaptation to sea water (Fig. 5.6). The ratio for the control group was significantly lower than that of both organotin groups ($P < 0.001$), as the lamellar chloride cells all but disappeared (Fig. 5.6).

5.2.4. Effect on the size of lamellar and interlamellar chloride cells

In addition to the chloride cell distribution, the sizes of both lamellar and interlamellar chloride cells were determined by measuring their respective areas. There was no significant difference between the areas of the control and the two organotin groups after three days of exposure to TBTCI and TPhTCI ($P > 0.05$; Fig. 5.7).

However, following the transfer to full-strength sea water, the mean area of lamellar chloride cells in the gill epithelia of the control group dropped from $257.0 \mu\text{m}^2 \pm 11.0$ to $172.5 \mu\text{m}^2 \pm 15.1$; $P = 0.012$; Fig. 5.7) and remained significantly smaller than the mean area of lamellar chloride cells in both organotin groups ($P < 0.05$; Fig 5.7). The mean area of lamellar chloride cells in the organotin groups did not change significantly throughout the experiment ($P = 0.767$; Fig. 5.7).

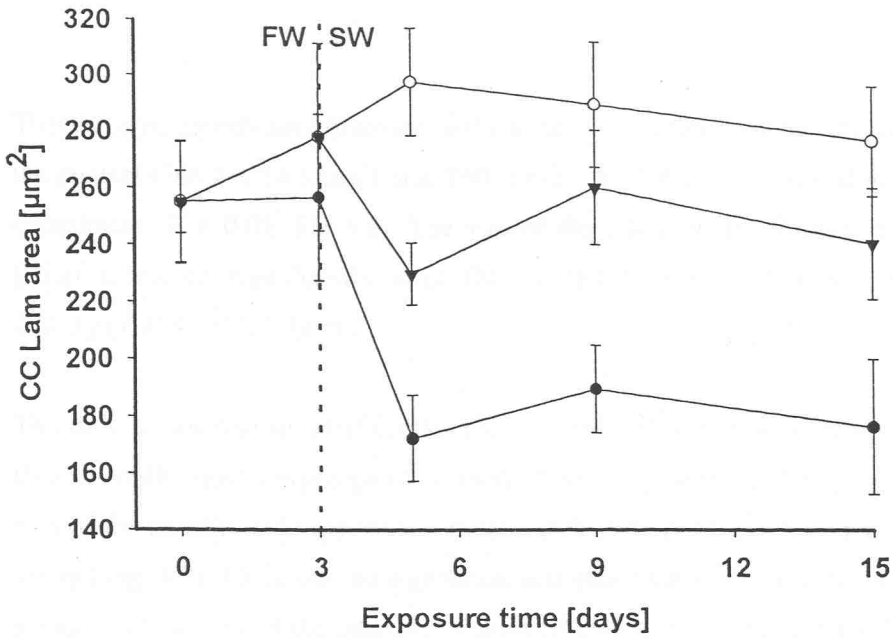


Figure 5. 7. The area of lamellar chloride cells (CC Lam) of freshwater-adapted (FW) 0-group flounders during chronic exposure to sediment-associated organotin compounds, before and after transfer to sea water in μm^2 (SW); $n = 6$; mean \pm S.D. (Fish weight $0.15 \text{ g} \pm 0.04$; \circ TBT; \blacktriangledown TPhT; \bullet Control).

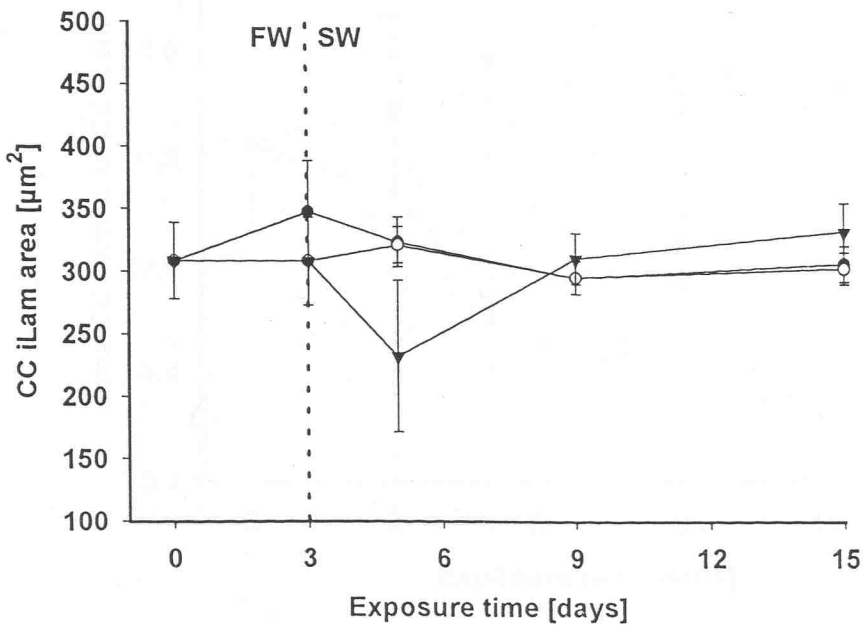


Figure 5. 8. The area of interlamellar chloride cells (CC iLam) of freshwater-adapted (FW) 0-group flounders during chronic exposure to sediment-associated organotin compounds, before and after transfer to sea water in μm^2 (SW); $n = 6$; mean \pm S.D. (Fish weight $0.15 \text{ g} \pm 0.04$; \circ TBT; \blacktriangledown TPhT; \bullet Control).

There was no significant difference in the mean area of interlamellar chloride cells between the control ($306.2 \pm 14.5 \mu\text{m}^2$) and TBT ($302.5 \pm 12.9 \mu\text{m}^2$) groups throughout the entire experiment ($P > 0.05$; Fig 5.8). The size of the interlamellar chloride cells of the TPhT group increased significantly after the transfer to full-strength sea water ($332.15 \pm 22.90 \mu\text{m}^2$ $P < 0.001$; Fig. 5.8).

The ratio of lamellar to interlamellar chloride cell areas, that was significantly lower than those of both organotin groups ($P < 0.05$), clearly demonstrated the reduction of the mean area of the lamellar chloride cells in favour of the interlamellar chloride cells in the control group (Fig. 5.9). There was no significant difference between the ratios for both organotin groups, indicating that the changes in chloride cell areas expected after the transfer to sea water were significantly disrupted by the chronic exposure of 0-group flounders to environmental concentrations of sediment-associated TBTCI and TPhTCI.

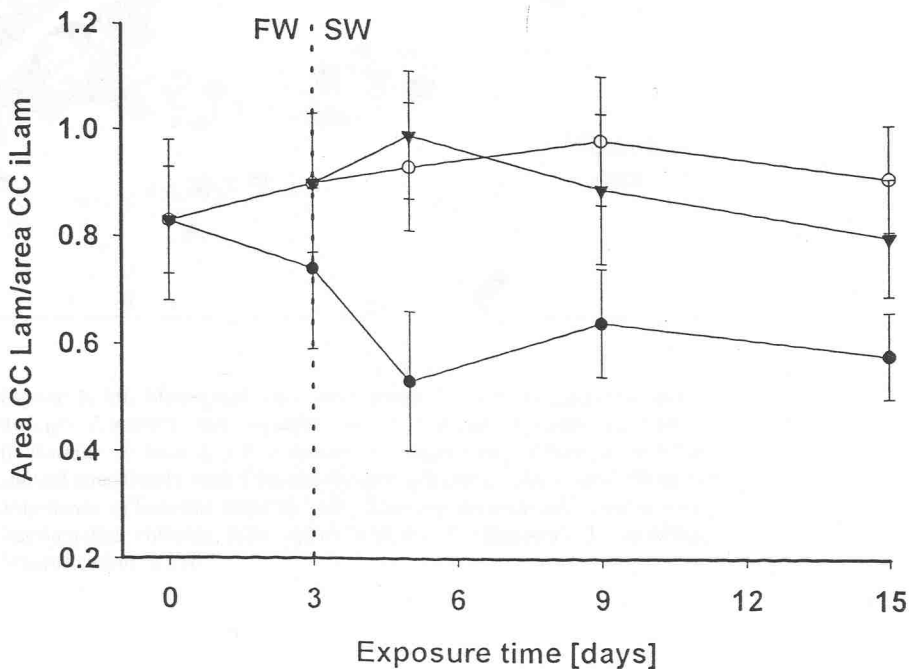


Figure 5. 9. The ratio of lamellar (area CC Lam) and interlamellar (area CC iLam) area of freshwater-adapted (FW) 0-group flounders during chronic exposure to sediment-associated organotin compounds, before and after transfer to sea water (SW); $n = 6$; mean \pm S.D. (Fish weight $0.15 \text{ g} \pm 0.04$; ○ TBT; ▼ TPhT; ● Control).

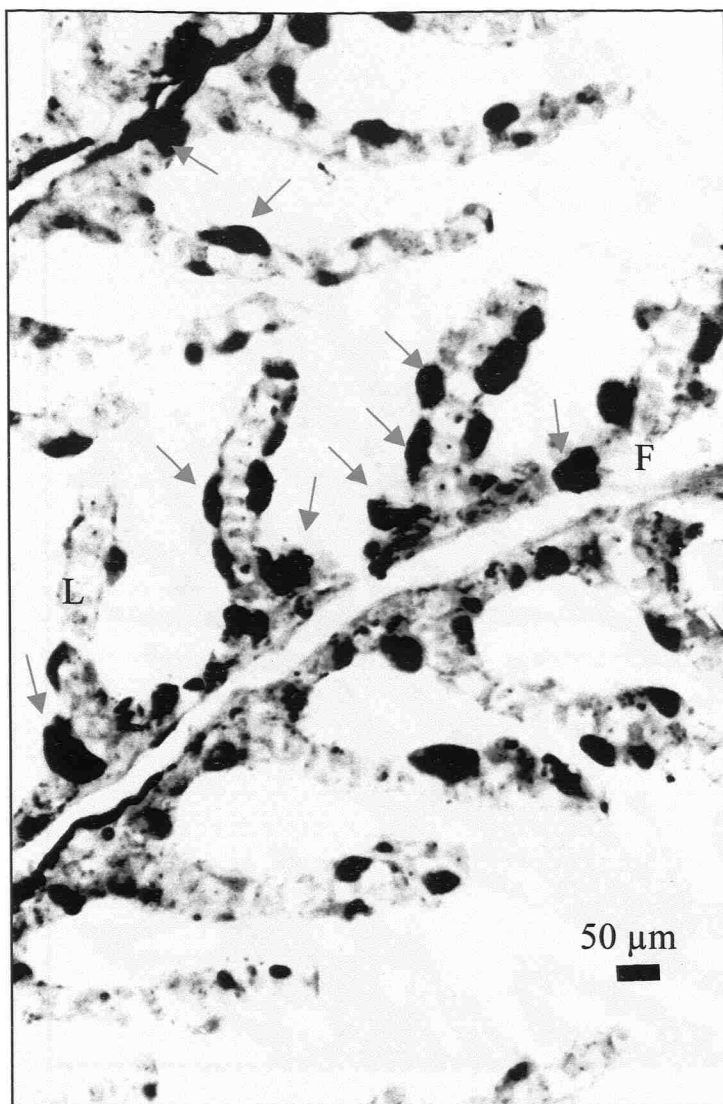


Figure 5. 10. Micrograph of a representative 5 μm longitudinal section through filaments and lamellae of an 0-group flounder adapted to freshwater at time t_0 (no exposure to organotins). Chloride cells are stained specifically with Champy-Maillet's fixative (black dots). Note the abundance of lamellar chloride cells. [lamellar chloride cells (red arrows); interlamellar chloride cells (green arrows); F (filament); L (lamella)]. Magnification: x 250

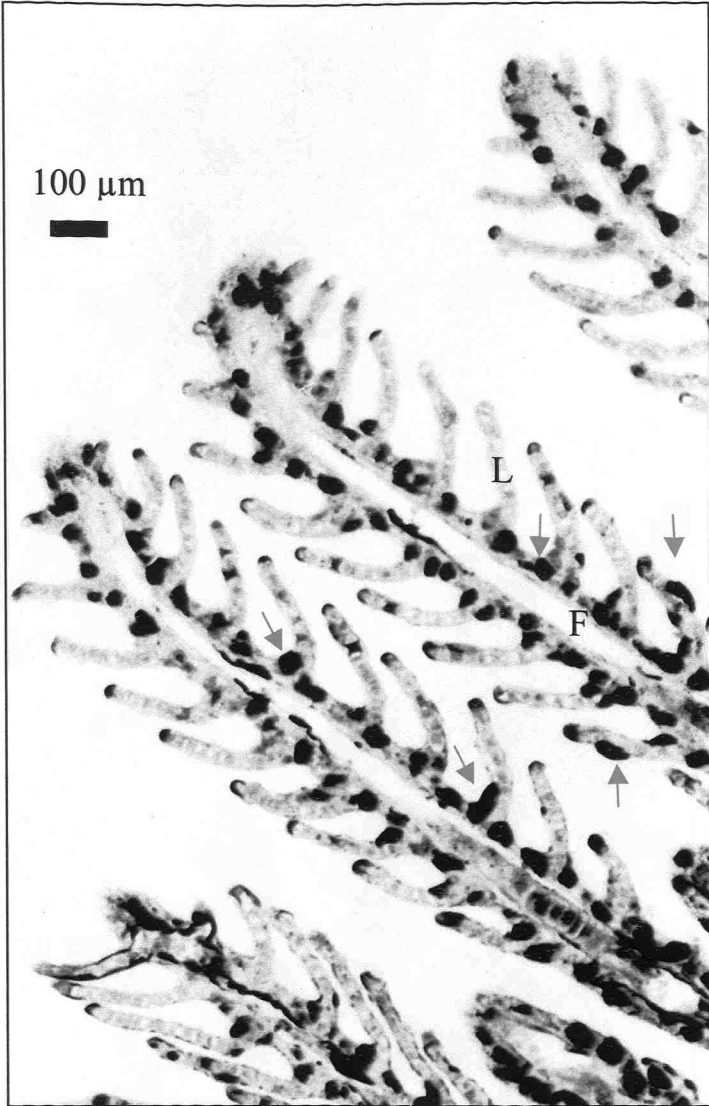


Figure 5. 11. Micrograph of a representative 5 μm longitudinal section through filaments and lamellae of a freshwater-adapted 0-group flounder 13 days after transfer to full strength seawater (no exposure to organotins). Chloride cells are stained specifically with Champy-Maillet's fixative (black dots). [lamellar chloride cells (red arrows); interlamellar chloride cells (green arrows); F (filament); L (lamella)]. Magnification x 125.

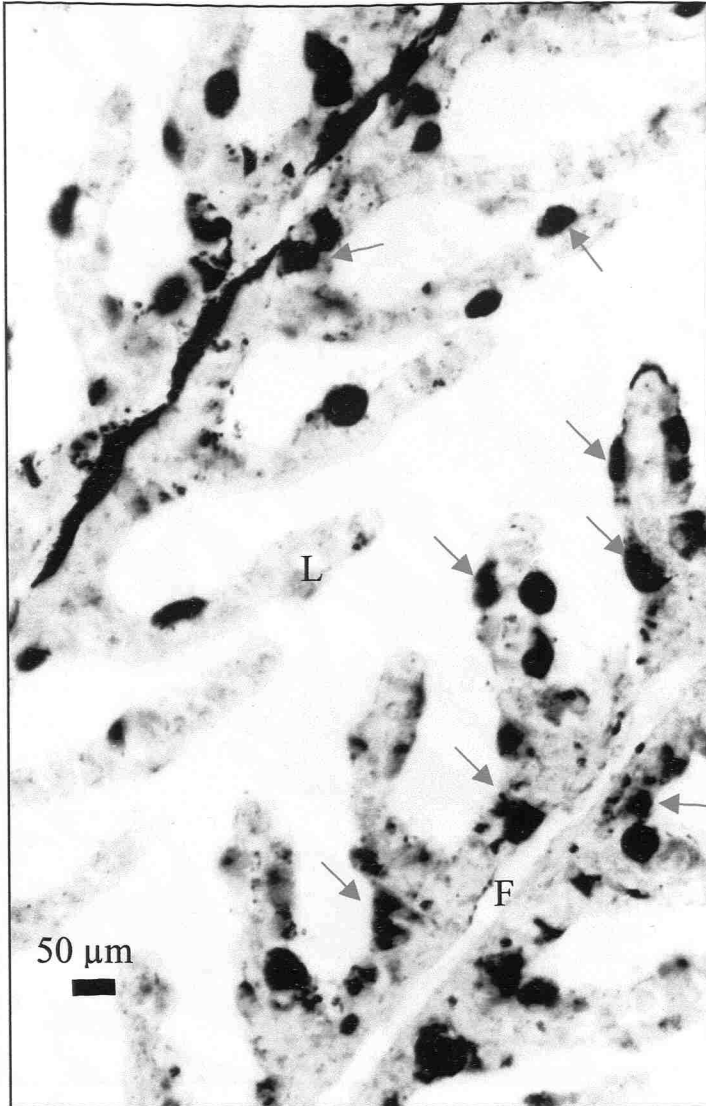


Figure 5. 12. Micrograph of a representative 5 μm longitudinal section through filaments and lamellae of a freshwater-adapted 0-group flounder 13 days after transfer to full strength seawater and exposed to 150 ng g^{-1} TBTCI for a total of 15 days. Chloride cells are stained specifically with Champy-Maillet's fixative (black dots). [lamellar chloride cells (red arrows); interlamellar chloride cells (green arrows); F (filament); L (lamella)]. Magnification x 250.

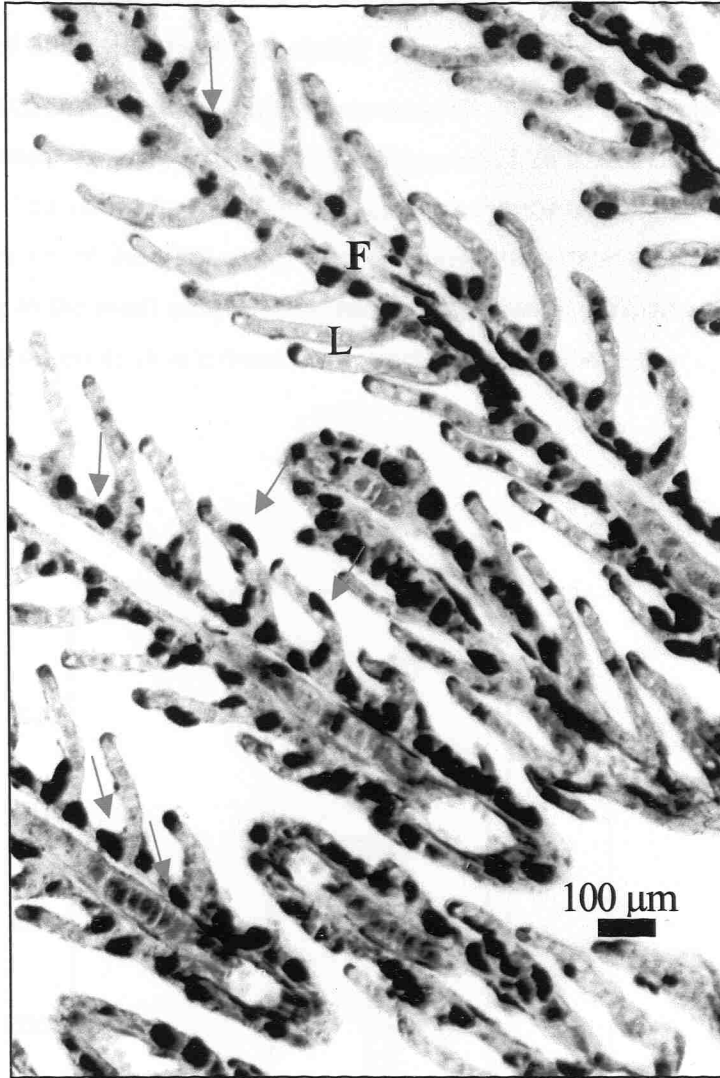


Figure 5. 13. Micrograph of a representative 5 μm longitudinal section through filaments and lamellae of a freshwater-adapted 0-group flounder 13 days after transfer to full strength seawater and exposed to 150 ng g^{-1} TPhTCl for a total of 15 days. Chloride cells are stained specifically with Champy-Maillet's fixative (black dots). [lamellar chloride cells (red arrows); interlamellar chloride cells (green arrows); F (filament); L (lamella)]. Magnification x 125.

5.2.5 Effects on blood osmolality

The blood osmolality of freshwater-adapted 0-group flounders exposed to sediment-associated organotin compounds was measured 12 days after the transfer to sea water (Fig. 5.14). The values for the control group were lower than those for both organotin groups. The values of the TBT group were also lower than those of the TPhT group. However, owing to the small sample sizes, reliable statistical significance could not be determined and these results should therefore be treated with caution and used for information only.

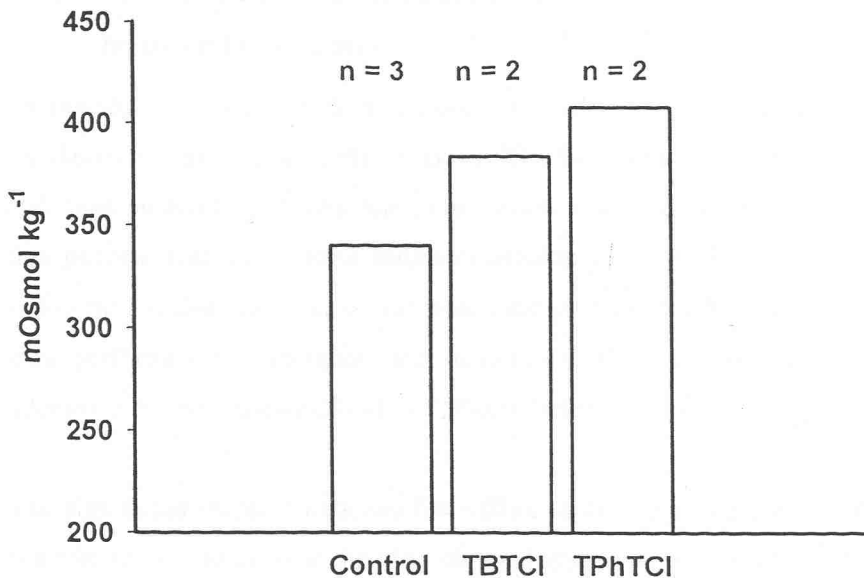


Figure 5. 14. Blood osmolality of freshwater-adapted 0-group flounders, 12 days after transfer to full-strength sea water, during chronic exposure to 150 ng g⁻¹ sediment-associated organotin compounds (mOsmol kg⁻¹), compared to the values in a control group (mean values).

5.3. Discussion

The euryhalinity of European flounders (*P. flesus*), characterised by osmo- and ionic regulatory mechanisms, enables them to cope with fluctuating salinities typically confronted in estuarine environments (Motais *et al.*, 1966; Evans, 1984). The data presented in this chapter deal with the effects of chronic exposure of freshwater-adapted 0-group flounders to environmental concentrations of sediment-associated organotin compounds on selected physiological and morphological parameters of ionic regulation during acclimation to full-strength sea water.

5.3.1. Effect on active Na^+ -efflux and Na^+/K^+ -ATPase activity following the transfer to sea water

On transfer of freshwater-adapted 0-group flounders to sea water, the demands on the ionic regulatory system are abruptly reversed. The fish is now in a hyperosmotic environment and must switch from active ion conservation to active ion excretion. In order to observe this process and any effects sediment-associated organotin compounds may have had, radioactive sodium isotopes (^{22}Na) were used to follow the Na^+ -efflux and an enzyme assay was performed to determine the activity of the ouabain-sensitive, Na^+/K^+ -activated adenosine triphosphatase (Na^+/K^+ -ATPase), before and after the transition to sea water.

The significant increase in active Na^+ -efflux measured in the control group, following the transfer to sea water, was the physiological response expected from a healthy euryhaline fish (Fig. 5.2.; De Renzis & Bornancin, 1984; Evans *et al.*, 1999; Marshall *et al.*, 1999). The active Na^+ -efflux in all three groups (control, TBT and TPhT) in this study was strongly correlated with the Na^+/K^+ -ATPase activity, before and after transfer to sea water, $r = 0.91$, $r = 0.81$ and $r = 0.99$, respectively. This correlation, during the adaptation to hyperosmotic environments, is a well established observation in euryhaline fish, such as the European eel, *Anguilla anguilla* (Bornancin & De Renzis, 1972), the yellow eel, *Anguilla rostrata* (Jampol & Epstein, 1970; Butler & Carmicheal, 1972; Forrest *et al.*, 1973), the killifish, *Fundulus heteroclitus* (Epstein *et al.*, 1967; Towle *et al.*, 1977), the Mozambique tilapia, *Oreochromis mossambicus* (Uchida *et al.*, 2000) and the rainbow trout *Oncorhynchus mykiss* (Kamiya & Utida, 1969). However, Kirschner (1969), Stagg & Shuttleworth (1982) and Hutchinson (1984), using various techniques, all failed to detect any difference in Na^+/K^+ -ATPase activity between sea water- and freshwater-adapted

flounders. This is in contrast to the results presented here (Fig. 5.3) and may be explained by the different experimental approaches. The aforementioned studies measured Na^+/K^+ -ATPase activity in flounders acclimated to a range of salinities but the enzyme activities in this study were obtained from flounders undergoing the early stages of adaptation to sea water, after direct transfer from fresh water.

It would appear that, Na^+/K^+ -dependent ATPase activities were reduced when 0-group flounders were exposed to TBTCI in both fresh water and sea water. Based on the observations for freshwater-adapted flounders (see 4.2.5 & Fig. 4.10), it was expected that TPhTCI-exposed fish would not show any sign of enzyme inhibition. However, the fact that the Na^+/K^+ -ATPase activity in the TPhT group slowly declined following the transfer to sea water, suggests otherwise. A possible explanation is that the duration of exposure, which was nearly two weeks longer than that of the fish in the fresh water experiments, may play an important part in the toxicology of TPhTCI (Visoottiviseth, *et al.*, 1999). This, together with the data concerning the different patterns of interaction between TBTCI and TPhTCI with biological membranes (Langner *et al.*, 1998; Radecka *et al.*, 1999; Sarapuk *et al.*, 2000), may help to understand observations made by several other authors concerning the slightly different toxicity of TPhTCI compared to TBTCI (Snoeij *et al.*, 1985; Fent & Meier, 1994; Morcillo & Porte, 1997).

Hutchinson (1984) noted a strong correlation between the blood sodium ion concentration and Na^+/K^+ -ATPase activity in 0-group flounders subjected to fluctuating salinities. Despite the distinct correlation between the blood sodium concentration, different active sodium efflux and the Na^+/K^+ -ATPase activity, and the fact that Na^+ -fluxes can be influenced by the K^+ -concentration in the external medium (De Renzis & Bornancin, 1984), the data presented here provided additional correlative evidence but no further direct evidence of any association between the two.

One disadvantage of the experimental approach applied here is that the fish were too small for gill biopsies to be taken, that would otherwise enable the observation of the Na^+/K^+ -ATPase activity of a single individual throughout the entire adaptational process, as was done for the measurement of active Na^+ -efflux in this study. Therefore, different individuals were used for every stage of the experiment. However, no pooling of tissue was necessary, because all the filaments of each individual together yielded enough material to perform the enzyme assay. The Na^+/K^+ -ATPase of the control group measured in this study

at t_0 (in fresh water before the transfer to sea water) were lower than the corresponding values for flounders given by Kirschner (1969) and Hutchinson (1984); the values following transfer to sea water are however within the range given by these authors. Hutchinson (1984) used 0-group flounders of a similar size to the ones examined here and Kirschner (1969) used adult fish, therefore, any differences in the weight of 0-group flounders used can be disregarded as an explanation for the different results. McCormic & Naiman (1984), working on the brook trout, *Salvelinus fontinalis*, also found no correlation between Na^+/K^+ -ATPase activity and fish size.

Motais *et al.* (1966) reported a 400 % difference between the active Na^+ -efflux rates of adult sea water- and freshwater-adapted flounders and Hutchinson (1984) observed a 500 % difference in 0-group flounders. These values are comparable with the 400 % difference in flux rates of freshwater-adapted 0-group flounders (control group) found in the present study after transfer to sea water.

The Na^+ -efflux rates in this study were found to be highly dependent on gill area, so that the size of the fish was a major concern when comparing the flux rates of the control and organotin groups. Accordingly, fish of a similar size were used for the different groups in the Na^+ -efflux experiments (Fig. 5.2). Hutchinson (1984) also found a correlation between weight and Na^+ -efflux in 0-group flounders. However, Carroll *et al.* (1995) found no weight-specific Na^+ -efflux in adults of other flatfish (*Limnada limnada* and *Pleuronectes platessa*), that may be linked to the stenohalinity of these two species (Evans, 1969b).

5.3.2. Effect on the size and distribution of lamellar and interlamellar chloride cells during adaptation to sea water

In addition to the physiological changes that occur in freshwater-adapted euryhaline fish during adaptation to sea water (see above), major morphological changes to branchial epithelia became apparent, particularly regarding the size and distribution of mitochondria-rich or chloride cells (Shirai & Utida, 1970; Thomson & Sargent, 1977; Avella *et al.*, 1987; King & Hossler, 1991; Uchida *et al.*, 1996; Van der Heijden *et al.*, 1997; Hiroi *et al.*, 1999; Wong & Chan, 1999; Sakamoto *et al.*, 2000; Uchida *et al.*, 2000).

On transfer to sea water, freshwater-adapted 0-group flounders, in the untreated control group, displayed a significant reduction in the number and size of lamellar chloride cells

and an increase in number and size of interlamellar chloride cells. The almost complete disappearance of lamellar chloride cells, following the transfer to sea water, has also been reported by other authors such as Uchida *et al.* (1996) for chum salmon fry, *Oncorhynchus keta*, Hirai *et al.* (1999) for the Japanese sea bass, *Lateolabrax japonicus* and Avella *et al.* (1987) for the rainbow trout, *O. mykiss*. This supports the views of Foskett & Scheffey (1982), Zadunaisky (1984), Uchida *et al.* (1996) and Perry (1997) that lamellar chloride cells are involved in ionic uptake in hyposmotic environments, become obsolete during sea water adaptation and are therefore degenerated. However, this process may not be a generic feature of euryhalinity, but one of several survival strategies of fish exposed to changing salinities. For instance, Shirai & Utida (1970), working on the Japanese eel, *A. japonica*, and Pisam *et al.* (1987) working with the guppy, *Lebistes reticulatus*, identified two types of interlamellar chloride cells, distinct in ultrastructure and activity, depending on the salinity of the surrounding medium, but no lamellar chloride cells. Madsen *et al.* (1994) found no change at all in the distribution of chloride cells between lamellar and interlamellar epithelia in the gills of the striped bass, *Morone saxatilis*, during sea water adaptation.

During adaptation to sea water the number of interlamellar chloride cells increased significantly in the gill tissue samples from the control group ($P < 0.05$; Fig. 5.5 & Figs. 5.10 & 5.11). This has been the typical morphological change observed during sea water adaptation in various other euryhaline species (Petrik, 1968; Shirai & Utida, 1970; Utida *et al.*, 1971; Maetz, 1974; Thomson & Sargent, 1977; Pisam *et al.*, 1987; McCormick, 1990; Madsen *et al.*, 1994; Hirai *et al.*, 1999). In particular, the development of multicellular interlamellar chloride cell complexes is frequently observed during adaptation to sea water, consisting of fully developed chloride cells in association with smaller accessory cells (Girard & Payan, 1980; Hootman & Philpott, 1980; Laurent, 1982).

The chronic exposure of freshwater-adapted 0-group flounders to environmental levels of sediment-associated tri-*n*-butyltin chloride had a marked effect on the redistribution of lamellar chloride cells during sea water adaptation, whereas exposure to triphenyltin chloride did not result in any anomalies of lamellar chloride cell distribution (Figs. 5.4; 5.12 & 5.13). However, exposure to both organotin compounds had an inhibiting effect on the proliferation of interlamellar chloride cells (Fig. 5.5 & Figs. 5.10 & 5.12 - 5.13). The number of interlamellar chloride cells did not increase significantly ($P > 0.05$) and was significantly lower than the numbers observed in the control group, 12 days following the

transfer to sea water ($P < 0.05$). As shown in Figs. 5.7 & 5.8, the size of interlamellar chloride cells in the control group did not change significantly ($P > 0.05$), suggesting that the development of multicellular chloride cell complexes (large chloride cell surrounded by smaller accessory cells) lead to an increase of the number of enlarged interlamellar chloride cells, but the average size was reduced because of the increased number of smaller accessory cells. The unchanged size of the average interlamellar chloride cell size in the organotin groups indicates that the development of accessory cells was inhibited or delayed by the exposure to TBTCI and TPhTCI.

Chloride cells are thought to develop from accessory cells situated in close proximity to mature chloride cells in the primary gill epithelium (Olivereau, 1970; Sardet *et al.*, 1979; Hootman & Philpott, 1980; Rojo *et al.*, 1997). Chloride cells, evolving from accessory cells in the primary epithelium (filaments) migrate to the secondary epithelium (lamellae) during fresh water adaptation (Bindon *et al.*, 1994a, b; Hirai *et al.*, 1999). They replace necrotic cells damaged by changing environmental factors and apoptotic cells approaching the end of their life cycles (Wendelaar Bonga & Van der Meij, 1989; Rojo *et al.*, 1997). The above account of chloride cell development seems to corroborate the observations made in this study, although Laurent & Dunel (1980) have challenged this and believe that accessory cells are a cell type specific to seawater-adapted fish, but were not able to identify an alternative developmental precursor of chloride cells. Unfortunately, the exact elucidation of this process was beyond the scope of this project and will therefore not be discussed any further. Nevertheless, the process of gill epithelial restructuring during sea water adaptation is important to understand, because of the way TBTCI and TPhTCI interact with epithelial membranes. As discussed in Chapter 4, the interaction of TBTCI and TPhTCI with biological membranes is quite different, so that the undisturbed chloride cell re-distribution in TPhT exposed fish, may be a consequence of TPhT not penetrating deep into the epithelium but rather absorbing in the headgroup region of the lipid bilayer (Langner *et al.*, 1998; Radecka *et al.*, 1999) and thus not interfering with membrane restructuring during sea water adaptation.

Chloride cell development has been demonstrated to be under hormonal control, with cortisol stimulating chloride cell differentiation and prolactin the dedifferentiation and reduction of chloride cells (Foskett *et al.*, 1983; Laurent & Perry, 1990; Madsen, 1990; Fuentes *et al.*, 1996; Mancera & McCormick, 1999; Seidelin & Madsen, 1999). Therefore, an alternative or additional explanation for the morphological anomalies observed during

sea water adaptation in this study may be that TBT and TPhT are disrupting these endocrine processes. This seems all the more possible, since TBT and, more recently TPhT, have given rise to great concern, regarding the link between these compounds and endocrine disruption in other aquatic organisms. The observed abnormalities vary from subtle changes to permanent alterations, including disturbed sex differentiation with feminised or masculinised sex organs, changed sexual behaviour, altered immune function and changes in diffusional water permeability (Hawkins & Hutchinson, 1990; Cadee *et al.*, 1995; Gibbs & Bryan, 1996; Mensink *et al.*, 1996, 1997; Swennen *et al.*, 1997; Matthiessen & Gibbs, 1998; Sumpter, 1998; Morcillo & Porte, 2000).

The average blood osmolality of 0-group flounders, 12 days following the transfer to sea water, measured 340 ± 6 mOsmol kg^{-1} . These values lie within the range of osmolalities previously given for seawater-adapted 0-group and adult flounders: Hutchinson (1984) reported values of 360 ± 10 mOsmol kg^{-1} , Alkindi *et al.* (1996) 312 ± 1.5 mOsmol kg^{-1} , Harding *et al.* (1997) 320 ± 10 mOsmol kg^{-1} and Stagg (1992) 322 ± 6 mOsmol kg^{-1} . An increase in blood osmolality was observed in 0-group flounders exposed to environmental levels of sediment-associated TBTCI and TPhTCI (Fig. 5.14). It would appear that the increased blood osmolality was caused by the inhibition of Na^+/K^+ -ATPase activity (Fig. 5.3) and the resulting reduction of active Na^+ -efflux (Fig. 5.2) during sea water adaptation. However, the small sample sizes available for the analysis of blood osmolality in this study did not allow the determination of reliable statistical significance of the different results. Therefore, although the results of the blood osmolality analysis, in connection with organotin exposure during sea water adaptation, were interesting, the data should be treated cautiously.

5.4. Conclusions

1) The results presented here lead to the conclusion that tri-*n*-butyltin chloride and triphenyl chloride in sediments are capable of significantly disrupting the osmoregulatory functions of an estuarine fish during sea water adaptation, at concentrations currently found in local sediments.

2) Na^+/K^+ -ATPase activity in the gill tissue of 0-group flounders was significantly inhibited by TBTCI and TPhTCI during sea water adaptation. The results suggest that the effects of environmental concentrations of TPhT on the Na^+/K^+ -ATPase activity are not just dose-related but are also time-dependent, in contrast to TBT, where the effects were almost immediately observed upon the transfer of freshwater-adapted flounders to sea water.

3) As a consequence of the inhibited Na^+/K^+ -activity, the active Na^+ -efflux, following the transfer to sea water, was significantly reduced in the TBT group. Again, the effects on Na^+ -efflux in the TPhT group were delayed.

4) Na^+/K^+ -ATPase activity was significantly correlated with the active Na^+ -efflux rates of 0-group flounders and presents further indirect evidence for the involvement of Na^+/K^+ -ATPase in ionic regulation.

5) The major re-arrangement of chloride cells in the secondary or lamellar epithelium expected in a freshwater-adapted 0-group flounder on transfer to sea water, was significantly inhibited by the chronic exposure to environmental concentrations of sediment-associated TBTCI. Exposure to TPhTCI had no effect on lamellar chloride cell distribution.

6) The proliferation of chloride cells in the primary or interlamellar epithelium was inhibited by both TBTCI and TPhTCI.

7) The lamellar chloride cells in flounders exposed to both organotin compounds were larger than those of the control group. Furthermore, although the number of lamellar chloride cells in the TPhT group had reduced significantly, the few remaining lamellar chloride cells did not change in size as was the case in the control group, where chloride cell size was significantly reduced.

8) The size of interlamellar chloride cells in both organotin groups did not change during sea water adaptation as was the case in the control group, indicating the developmental inhibition of accessory cells for the formation of multicellular complexes typical for seawater-adapted euryhaline fish.

9) In summary, the different interaction characteristics of TBTCI and TPhTCI with biological membranes reported by previous authors, seem to be reflected in the results presented in this study and may explain the observation of different effects of these compounds on physiological and morphological aspects of sea water adaptation in 0-group flounders.

Chapter 6 - Gill biometry of *Platichthys flesus*

6.1. Introduction

In fish, the gills are not only responsible for gas exchange but are also the site of extrarenal ion excretion. These are surface area-dependent processes, and therefore accurate gill surface area estimates are essential for studying the physiology of gas exchange and ionic fluxes across the gills (Motais *et al.*, 1966; Hughes & Morgan, 1973). Such measurements are of particular significance in studies of the physiology of estuarine fish and require calculations of total gill surface area that are dependent on the following parameters: total filament length, the frequency of lamellae and the unilateral lamellar surface area; such measurements can now be conveniently made using digital image analysis techniques. Prior to the availability of such technology, studies by Gray (1954) and Hughes (1966) examined a number of species, and estimated lamellar area by treating individual lamellae as simple triangles. Also, total filament length was estimated by measuring every n^{th} filament and multiplying the results by the total number of filaments rather than examining every filament. Muir & Hughes (1966) devised a weighted method for the determination of gill dimensions in fish, that has since been widely adopted and which was adapted by Al-Kadhomy (1985) for use with larval and post-metamorphic European flounder *Platichthys flesus* (L.)

During preliminary experiments for Na^+ influx, it became apparent that the weight of the fish fluctuated a great deal during the 6 week experiments. In order to rule out any flux artefacts caused by weight gain or loss, it was decided to express Na^+ fluxes as μmoles sodium per unit gill area per hour ($\mu\text{moles}\cdot\text{mm}^{-2}\cdot\text{h}^{-1}$) rather than $\mu\text{moles g}^{-1}\text{ h}^{-1}$. The aim of this chapter was therefore to see if the results of PC-based digital image analysis of gills of 0-group *P. flesus* were comparable with existing gill area data, because accurate measurements were required for a wider study of hydromineral fluxes in this species.

6.2. Results

The gill area of 0-group flounders was found to be a highly correlated allometric function of body weight (Fig. 6.1 a; $r = 0.96$). The same was found for total filament length (Fig. 6.1 b; $r = 0.96$). The average bilateral surface area and the frequency of lamellae (mm^{-1}) showed no correlation with body weight, $r = 0.11$ and $r = 0.08$, respectively (Fig. 6.1 c & d).

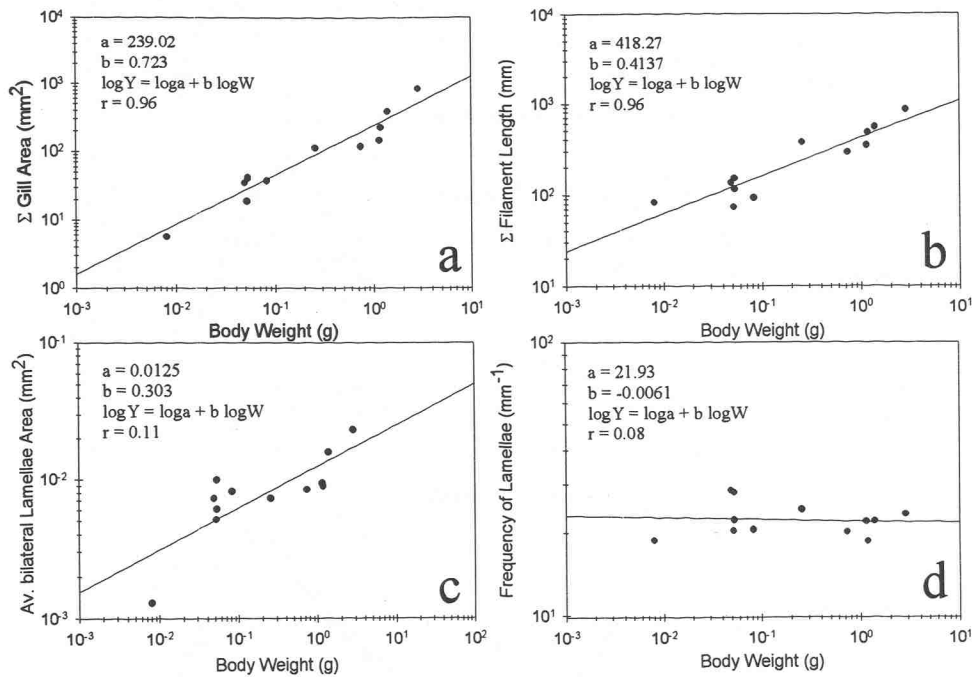


Figure 6. 1. Linear logarithmic plots of A) total gill area, B) total filament length, C) average bilateral lamellar area and D) frequency of lamellae vs body weight.

The measured parameters were also compared on the two hemibranchs of each gill arch as the ratio anterior hemibranch / posterior hemibranch ($h1 : h2$). The total length of the filaments of the anterior hemibranchs on the gill arches of the upper gill pouch was approximately 70% greater than on the posterior hemibranch, whereas on the gill arches of the lower gill pouch, total filament length of the anterior hemibranchs was about 37% larger than that of the posterior hemibranchs (Fig. 6.2).

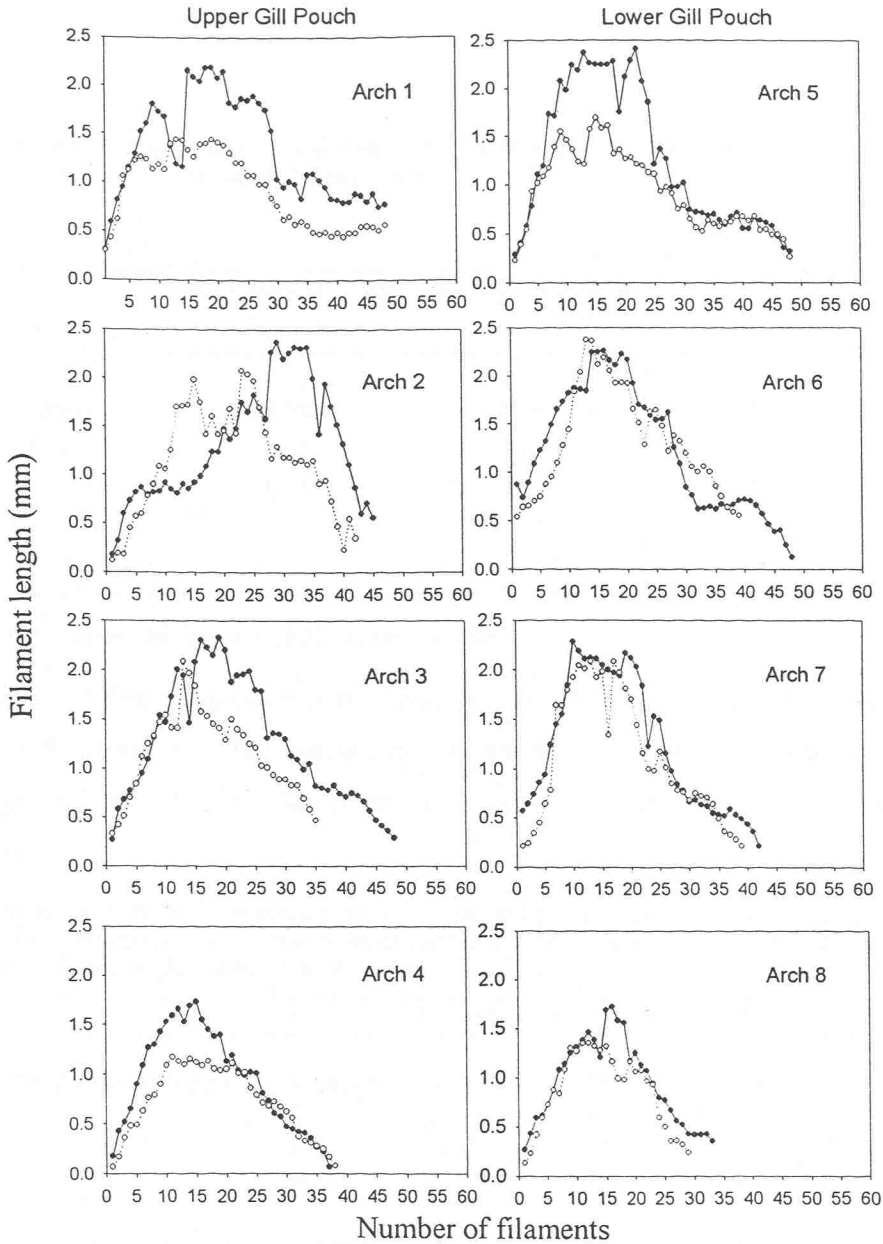


Figure 6. 2. 0-group *Platichthys flesus*. Lengths of the gill filaments on the anterior (h1; ●) and posterior (h2; ○) hemibranchs of all eight gill arches plotted against the number of filaments in a 2.86 g (wet weight) specimen.

The poor correlation between body weight and the h1 : h2 ratios of bilateral lamellae area and the frequency of lamellae, suggests that while the gill area increases during growth, the proportions remain more or less the same and are independent of weight (Table 6.1).

Table 6. 1. The hemibranch ratios (h1 : h2)* of each gill arch for the parameters measured. n = 17; wet weight 0.008g - 2.860g.

| | Total Filament Length | Interlamellar Space | Av. Lamellar Area |
|---------|-----------------------|---------------------|-------------------|
| | h1 : h2 | h1 : h2 | h1 : h2 |
| Average | 1.58 | 0.96 | 1.59 |
| S.E.M. | 0.25 | 0.18 | 0.36 |
| r | 0.15 | 0.0016 | 0.12 |

*h1: anterior hemibranch; h2: posterior hemibranch

The data in Table 6.2 show that the upper gill pouch contained larger gill arches than those of the lower one. The average ratio of surface areas of gills from the upper and lower pouches was 1.53 ± 0.88 ; linear regression showed no correlation between weight and ratios.

Table 6. 2. The ratio of measurements of upper gill pouch arches (A1 - A4) to lower gill pouch arches (A5 - A8); r = correlation coefficient; P = probability; S. E. = standard error; n = 17; wet weight 0.008g - 2.860g.

| | | A1 : A5 | A2 : A6 | A3 : A7 | A4 : A8 |
|---------------------------------|---------|---------|---------|---------|---------|
| Total filament length | Average | 1.26 | 1.00 | 1.14 | 1.12 |
| | S.E. | 0.30 | 0.24 | 0.27 | 0.26 |
| | r | 0.02 | 0.03 | 0.20 | 0.10 |
| | P | 0.93 | 0.92 | 0.38 | 0.58 |
| Frequency of lamellae | Average | 1.05 | 0.98 | 0.99 | 0.93 |
| | S.E. | 0.25 | 0.23 | 0.23 | 0.22 |
| | r | 0.21 | 0.30 | 0.12 | 0.05 |
| | P | 0.42 | 0.22 | 0.63 | 0.84 |
| Average bilateral lamellar area | Average | 6.42 | 8.16 | 5.04 | 3.72 |
| | S.E. | 1.51 | 1.92 | 1.19 | 0.88 |
| | r | 0.27 | 0.19 | 0.11 | 0.08 |
| | P | 0.30 | 0.48 | 0.67 | 0.76 |
| Total gill area | Average | 1.39 | 1.22 | 1.25 | 1.33 |
| | S.E. | 0.33 | 0.29 | 0.29 | 0.31 |
| | r | 0.03 | 0.30 | 0.27 | 0.21 |
| | P | 0.92 | 0.12 | 0.29 | 0.44 |

Table 6.3 compares gill areas obtained in the present study, taking into account the dimorphism between upper and lower gill arches, with the gill area estimates that would be obtained from the same basic suite of measurements using calculations previously applied to flounders.

Table 6. 3. Comparison of total gill areas for *P. flesus* estimated by (1) doubling upper and lower gill pouch (UGP x 2) areas and (2) addition of separate upper and lower gill pouch estimates (UGP + LGP); S. E. (standard error).

| Weight (g) | UGP x2 (mm ²) | UGP + LGP (mm ²) | Difference (%) |
|---------------|------------------------------|---------------------------------|-------------------|
| 0.008 | 7.50 | 5.88 | 22 |
| 0.029 | 2.49 | 1.29 | 48 |
| 0.030 | 0.22 | 0.20 | 6 |
| 0.049 | 38.09 | 34.56 | 9 |
| 0.052 | 19.37 | 18.63 | 4 |
| 0.052 | 41.81 | 40.72 | 3 |
| 0.053 | 54.78 | 46.21 | 16 |
| 0.083 | 38.40 | 37.37 | 3 |
| 0.257 | 138.83 | 113.00 | 19 |
| 0.740 | 131.14 | 119.98 | 9 |
| 0.980 | 75.98 | 50.98 | 33 |
| 1.100 | 231.95 | 147.83 | 36 |
| 1.150 | 161.60 | 146.60 | 9 |
| 1.190 | 228.18 | 227.02 | 1 |
| 1.400 | 424.63 | 388.69 | 8 |
| 2.580 | 321.54 | 265.57 | 17 |
| 2.860 | 896.88 | 845.17 | 6 |
| | | Average: | 15 |
| | | S.E. | 4 |

The latter method of estimation simply multiplied the total surface area of the arches of the upper gill pouch by two, resulting in significantly ($P < 0.001$) higher values for total gill area than the value obtained by direct measurement of all gill arches of both upper and lower gill pouches.

Comparisons of total filament length are shown in Table 6.4; these demonstrate that the estimates derived from Gray's (1954) method were significantly smaller ($P = 0.038$) than direct measurements by an average factor of 23.82%. Calculations using Al-Kadhomi's

(1985) method produced estimates that were also significantly smaller ($P < 0.001$) by an average factor of 18.07%.

Table 6. 4. Estimates of total filament length calculated according to Gray (1954) and Al-Kadhomiyy (1985) compared with direct measurements using digital image analysis; S. E. (standard error).

| Body Weight (g) | Body Length (mm) | Total Filament Length | | | | |
|--------------------|---------------------|--|----|--|----|--------------------|
| | | Gray's Calculation (mm) Deviation* (%) | | Al-Kadhumiyy's Calculation (mm) Deviation* (%) | | This Study (mm) |
| 0.008 | 10.00 | 53.15 | 19 | 57.25 | 12 | 65.32 |
| 0.029 | 10.40 | 15.00 | 34 | 17.28 | 24 | 22.89 |
| 0.030 | 11.20 | 29.98 | 32 | 35.45 | 20 | 44.25 |
| 0.049 | 18.80 | 78.33 | 32 | 95.47 | 17 | 115.26 |
| 0.052 | 13.30 | 41.10 | 35 | 55.38 | 12 | 63.21 |
| 0.052 | 18.80 | 70.38 | 29 | 72.91 | 26 | 98.93 |
| 0.053 | 17.70 | 89.04 | 32 | 105.67 | 19 | 130.16 |
| 0.083 | 17.50 | 56.25 | 29 | 67.37 | 15 | 79.63 |
| 0.257 | 31.10 | 236.48 | 27 | 243.26 | 25 | 322.35 |
| 0.740 | 38.00 | 194.25 | 23 | 202.71 | 19 | 250.86 |
| 0.980 | 40.00 | 84.35 | 12 | 84.58 | 12 | 95.64 |
| 1.150 | 42.00 | 246.02 | 18 | 262.40 | 12 | 298.31 |
| 1.190 | 48.50 | 344.01 | 15 | 338.68 | 17 | 406.41 |
| 1.400 | 54.00 | 389.82 | 18 | 383.94 | 19 | 474.56 |
| 1.700 | 60.00 | 330.75 | 15 | 334.86 | 14 | 391.09 |
| 2.580 | 40.00 | 393.07 | 18 | 385.37 | 19 | 478.43 |
| 2.860 | 71.00 | 611.64 | 18 | 569.82 | 23 | 744.03 |
| | | Average: | 24 | | 18 | |
| | | S.E. | 6 | | 4 | |

* Deviation from measurements obtained in this study

In this study, the average bilateral lamellar area was found to be significantly smaller (60.45 %; $P < 0.001$; Fig. 6.3) than previously reported values. There was no significant difference ($P > 0.05$) in the frequency of lamellae per millimetre between this study and previous observations.

The results of the calculations of total gill area of *P. flesus* were on average significantly smaller (58%; $P = 0.03$) in the present study than the values reported by Al-Kadhomiyy (1985) (Fig. 6.4).

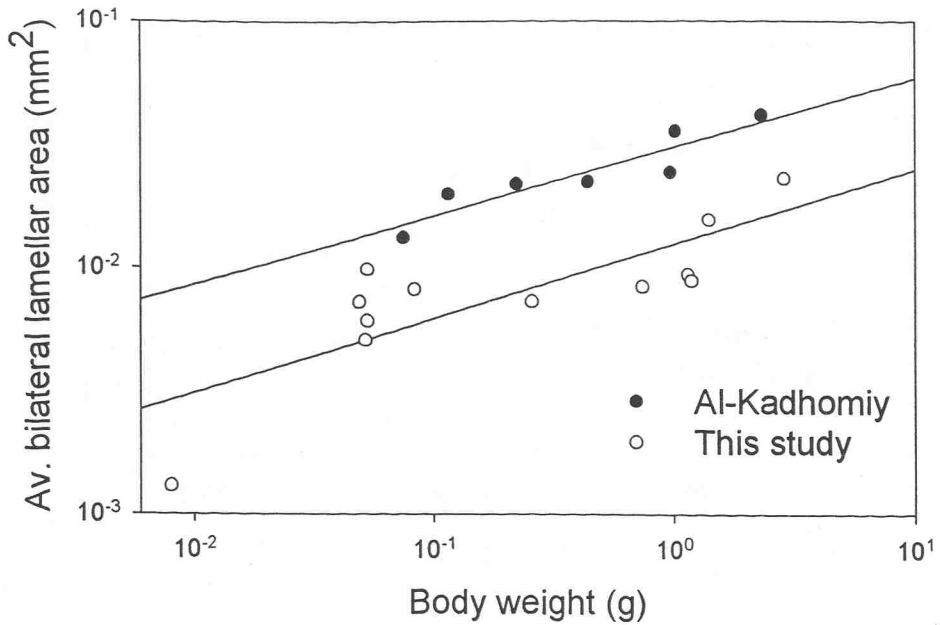


Figure 6. 3. Average bilateral lamellar area plotted against body weight. Data from Al-Kadhomiyy (1985) are compared with measurements from this study.

Separate experiments were performed in order to estimate the degree of shrinkage during fixation and storage. The maximum shrinkage during the 6 week storage period was found to be 10.98 %. This was not significantly different from the shrinkage observed after a 24 hour fixation period (Table 6.5).

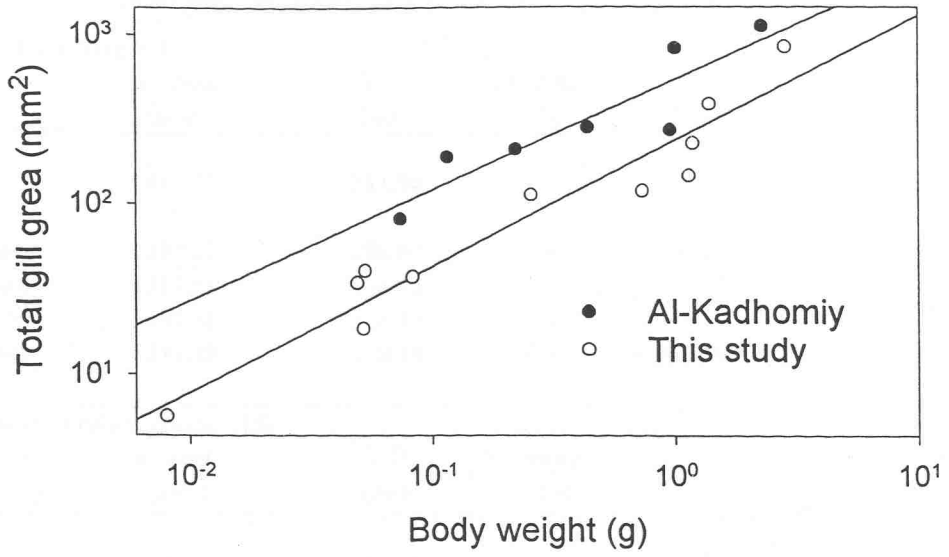


Figure 6. 4. Total gill area plotted against body weight. Data from Al-Kadhomiyy (1985) are compared with measurements from this study.

Table 6. 5. Differential changes over time in gill measurements derived from *P. flesus* gill arches preserved in cold Bouin's solution; probabilities (*P*) obtained from one-way ANOVA; mean values \pm SD.

| filament length (n = 5) | | | | |
|-----------------------------------|--------------------------------|----------------------------|------------------|-------------------|
| time | average (μm) | S. D. (μm) | shrinkage (%) | |
| 0 | 1418.75 | 234.94 | | |
| 24 hours | 1322.23 | 185.69 | 6.80 | <i>P</i> =0.009* |
| 72 hours | 1217.54 | 134.52 | 7.29 | |
| 2 weeks | 1197.38 | 128.42 | 7.99 | |
| 6 weeks | 1211.49 | 138.19 | 9.31 | <i>P</i> =0.563** |
| unilateral lamellar area (n = 19) | | | | |
| time | average (μm^2) | S. D. (μm) | shrinkage (%) | |
| 0 | 3526.02 | 858.92 | | |
| 24 hours | 3000.90 | 508.32 | 14.89 | <i>P</i> =0.026* |
| 72 hours | 2998.07 | 687.83 | 14.97 | |
| 2 weeks | 2965.80 | 673.92 | 15.89 | |
| 6 weeks | 2942.98 | 688.34 | 16.54 | <i>P</i> =0.399** |
| interlamellar space (n = 16) | | | | |
| time | average (μm) | S. D. (μm) | shrinkage (%) | |
| 0 | 38.89 | 2.59 | | |
| 24 hours | 37.14 | 2.64 | 4.75 | <i>P</i> =0.049* |
| 72 hours | 37.07 | 2.66 | 5.79 | |
| 2 weeks | 37.05 | 2.22 | 6.92 | |
| 6 weeks | 36.99 | 1.87 | 7.77 | <i>P</i> =0.450** |

*) ANOVA for shrinkage during 24 hours of storage

**) ANOVA for shrinkage between 24 hours and 6 week storage period

6.3. Discussion

A radical change in methods of gill area measurement can be attributed to McDonald *et al.* (1991) who introduced digital image analysis of drawings into a study of gill areas of fresh water fish. Subsequent studies by Stevens (1992) on red drum, *Sciaenops ocellatus*, gill areas and by Jakubowski (1997) working on Baikalian deep-water sculpins, *Abyssocottus korotneffi* and *Cottinella bouleengeri*, have used the new technology in combination with the basic calculations of Hughes (1966). The present study has followed the same path in combining new technology with well established methods but with a view to testing, with some rigour, the validity and supposed greater accuracy of this approach. This is particularly relevant given Jakubowski's (1993) study on various fish species, which indicated that there was a serious over-estimation of gill area of up to three and a half times in previous studies where every n^{th} filament was measured and an average used to calculate total filament length, rather than measuring the length of every filament.

The most common form in which gill area is expressed for comparative purposes is in the form of an allometric relationship to body weight; the relationships derived from the methodology in the present study are shown in Figure 6.1 and a comparison with that from a previous study of the same species is presented in Figure 6.4. It is quite clear from these data that by measuring every filament rather than every n^{th} filament there is a significant decrease in the gill area estimate, confirming Jakubowski's (1993) conclusions. Analysing the data further it would seem that part of this difference is caused by the over-estimation of bilateral lamellar area, as shown in Figure 6.3. In the present study, the measurement of every lamella on a filament takes into account the gradual change in size of the lamellae along the length of a filament whereas previous studies have simply taken an average of three lamellae from the base, tip and middle of the filament. It should also be borne in mind that the hemibranchs of most fish species are not uniform; in the case of *P. flesus* the anterior hemibranchs are generally larger than the posterior ones (Fig. 6.2; Table 6.1).

It, however, should be noted that the factors tending to over-estimate gill area using established techniques may to some extent be offset by an under-estimate of total filament length, as revealed in Table 6.4. It would seem that there is a further advantage in using digital image analysis of video pictures of the gills as a large number of filament lengths can be measured relatively quickly, compared to the time-consuming process using an eye

piece graticule which of necessity forced workers to limit their sub-sampling to a practical minimum.

The biometry of *P. flesus* gills is further complicated by the asymmetry of the gill pouches in the post-metamorphic body form (Yazdani, 1976; Al-Kadhomi, 1985). Table 6.2 shows that the surface area of the upper gill arches can be as much as 40 % larger than the lower ones. The recognition of this difference is essential in improving the accuracy of gill area measurements since previous studies on flatfish have not taken them into account and simply multiplied the information from the upper chamber by a factor of two thereby further contributing to an over-estimate. The degree of over-estimation is quantified by comparing the values derived from measurements that take into account the asymmetry with those calculated by multiplying by two.

The final factor which the present study has attempted to quantify is the effect of tissue shrinkage during preservation. Hughes (1984) reported shrinkage of the length of filaments fixed in Bouin's solution of up to 3 % over 24 hours; he concluded that this would lead to a proportionate shrinkage of the interlamellar spaces. It was assumed that shrinkage induced changes in these two parameters would effectively cancel each other out. He also noted that it was not possible to obtain reliable shrinkage estimates for individual lamellae with the equipment available to him. My measurements demonstrate that this assumption is invalid as interlamellar spacing does not shrink in proportion to the filament length in *P. flesus*, as shown by Table 6.5. The assumption that shrinkage is proportionate produces an over-estimate of the number of lamellae per filament therefore compounding the over-estimate of gill area attributed to the other factors described above.

6.4. Conclusions

To conclude, a truly rigorous treatment of gill area estimation must take account of gill pouch asymmetry (if appropriate), differential shrinkage of gill components on preservation and the need for maximal sampling and accuracy of measurement. The data presented here address these requirements and so are likely to produce a more accurate estimation of the gill area of 0-group *P. flesus*; this is a significantly smaller value than the value calculated in the only other comparable study by Al-Kadhomi (1985). In a wider context this approach would seem preferable when estimating the gill areas of small fish.

Chapter 7 - General discussion

In the preceding chapters, the effects of environmental concentrations of sediment-associated tri-*n*-butyltin chloride (TBTCl) and triphenyltin chloride (TPhTCl) on various aspects of ionic- and osmoregulation in 0-group European flounder, *Platichthys flesus* (L.), in steady-state freshwater-adapted fish and in freshwater-adapted fish during adaptation to sea water, have been investigated. The aim of this study was to quantify these effects and to put them into context with adaptive strategies for survival in estuarine environments that exhibit frequent changes in salinity. In particular, the lack of information on the ecotoxicology of sediment-bound organotin compounds, at concentrations currently found in local Southampton estuaries, on osmoregulation was addressed. Furthermore, the effect of TPhT on Na⁺/K⁺-ATPase activity, a key enzyme in ionic regulation, was investigated. This has particular significance, because, although several studies on the physiological and histopathological impact of TPhT on fish have been carried out (Stockdale *et al.*, 1970; Fent & Meier, 1994; Fent, 1996; Fent *et al.*, 1996; Schwaiger *et al.*, 1996; Kuroshima *et al.*, 1997; Morcillo & Porte, 1997), the available data in the literature concerning the direct interaction with this enzyme in fish only cover the effects of TBT exposure (Aldridge, 1976; Pinkney *et al.*, 1989b), making this the first study to investigate the *in vivo* effects of TPhT on Na⁺/K⁺-ATPase activity of estuarine fish.

The environmental significance of the present study lies in the determination of the *in vivo* effects of chronic exposure of 0-group flounders to environmental levels of sediment-associated TBT and TPhT on osmoregulation, something that has previously only been attempted in exposure experiments with aqueous organotin preparations (Chliamovitch & Kuhn, 1977; Pinkney *et al.*, 1989b). The purpose of this discussion, therefore, is to bring together the findings of the present project and to consider these results in the light of the current knowledge of the ecotoxicology of organotin compounds and to identify the limitations of this project in order to probe the scope for further research in this field.

Regulation of extracellular and intracellular fluids that are hyposmotic in the marine environment and hyperosmotic in fresh water is a characteristic of teleost fish. Therefore, fish must possess an array of regulatory mechanisms in order to maintain such gradients and, in the case of euryhaline species, these mechanisms must allow a switch from hyposmotic to hyperosmotic regulation and *vice versa*. These mechanisms include (1) variations of the permeability of the body surface to water and ions (Evans, 1969a, b; 1984;

Motais *et al.*, 1969a, b; Isaia & Hirano, 1975; Isaia *et al.*, 1979; Bolt *et al.*, 1980; Dawson *et al.*, 1984) and (2) the active transport of ions against electrochemical gradients (Motais *et al.*, 1966; Bornancin & De Renzis, 1972; Smith, 1973; Maetz, 1974; Payan & Girard, 1984; Avella & Bornancin, 1990; Kirschner, 1991; Carroll *et al.*, 1995; Marshall *et al.*, 1997). Although not investigated here, it is known that these ionic- and osmoregulatory processes are under endocrine control (Bentley, 1971; Foskett *et al.*, 1983; Acher *et al.*, 1997; Mancera & McCormick, 1998, 1999; Yada & Ito, 1999), a fact that could be of significance to this study when interpreting the effects of endocrine-disrupting pollutants, such as organotin compounds, on osmoregulation (see Hawkins & Hutchinson, 1990; Cadee *et al.*, 1995; Gibbs & Bryan, 1996; Mensink *et al.*, 1996, 1997; Swennen *et al.*, 1997; Matthiessen & Gibbs, 1998; Sumpster, 1998; Morcillo & Porte, 2000).

The most important aspect of laboratory toxicology experiments is to create the right environment and choose the appropriate parameters in order for the responses of the test animals to correspond to those of naturally occurring organisms (Chapman, 1983). In the present study, a considerable amount of time was allocated to the preparation of the experimental sediments, so that the sediment chemistry produced realistic concentrations and behaviour of the organotin compounds and that the conditions were generally acceptable to flounders. This method of sediment preparation enabled the chronic exposure of flounders to environmental levels of organotin compounds, that were sufficiently stable throughout the duration of the experiments. However, sediment toxicity tests with artificial sediments, as used in this study, are merely models for studying sediment-organism interaction of pollutants under controlled conditions and do not necessarily take into account the 'real world' of contaminated sediments, i.e. the effect of characteristic *in situ* organisms (Adams *et al.*, 1992). Nevertheless, the degradation rates of TBT and TPhT in the present artificial sediment preparations are in reasonable agreement with those of organotin-spiked natural sediments (Meador *et al.*, 1997; Arnold *et al.*, 1998; Stronkhorst *et al.*, 1999).

The effects of environmental levels of sediment-associated organotin compounds on selected osmoregulatory mechanisms of 0-group flounders adapted to fresh water and during acclimation to sea water were examined and quantified.

A marked reduction in the apparent membrane permeability was observed in both TBT and TPhT-exposed fish, that was characterised by a significant increase in the half-time of exchange of THO. Although the unidirectional water fluxes in both organotin groups were

significantly reduced, they only partially reflected the observations of the THO flux experiments. The reports of previous laboratory investigations carried out on the interaction of TBT and TPhT compounds with model biological membranes (Heywood, *et al.*, 1989; Langner *et al.*, 1998; Sarapuk *et al.*, 2000), suggest that the apparent membrane permeability and unidirectional fluxes in exposed fish observed in this study, could have been caused by the different interaction of these compounds with the gill membranes.

At the whole animal level, alterations in water fluxes will be manifested as changes in drinking and urine production rates. Drinking occurs sporadically in freshwater-adapted 0-group flounders and a large amount of dilute urine is produced in order to offset osmotic water influx and to maintain osmotic homeostasis (Evans, 1979). In this study, the drinking and urine production rates of freshwater-adapted 0-group flounders before the exposure to organotin compounds, and during the entire 5-week experiment in the control group, fell within the range reported by Hutchinson (1984). The drinking rates in both organotin groups increased significantly but the urine production showed no significant change. This led to an increase in the net osmotic water balance and as a consequence a reduced blood osmolality was observed (Hartl *et al.*, 2000). No significant changes of these parameters occurred in the respective control groups.

Survival in hyposmotic environments not only requires the maintenance of a stable water balance but also the regulation of intra- and extracellular ion concentrations (Uchida *et al.*, 1996, 2000; Perry, 1997). The regulation of the Na^+ balance was investigated in this study. Active Na^+ -influx in freshwater-adapted fish is facilitated by the membrane-bound Na^+/K^+ -ATPase located in the lamellar chloride cells (Hootman & Philpott, 1979; McCormick, 1990; Dang *et al.*, 2000). In order to quantify the effects of TBT and TPhT on the Na^+ balance, the passive Na^+ -efflux along its concentration gradient and the Na^+/K^+ -ATPase activity were measured. The rapid increase in passive Na^+ -efflux and the corresponding insignificant change in Na^+/K^+ -ATPase activity in the TBT group suggest that TBT is inhibiting the Na^+/K^+ -ATPase and thereby permitting Na^+ to be lost to the environment, that would otherwise have been retained, as was observed in the control group. This result is significant for three reasons: (1) the observations were made *in vivo* on fish exposed to environmental levels of sediment-associated TBT, (2) the concentrations in the water covering the sediments remained below the detection limit of 20 ng TBT l^{-1} , that was an order of magnitude lower than previous studies, such as that by Pinkney *et al.* (1989b), who have examined the effects of chronic exposure of fish to aqueous TBT suspensions and (3)

the *in vivo* exposure to sediment-associated TBT caused a significant reduction of Na^+/K^+ -ATPase activity, whereas the above mentioned aqueous preparations had no significant effect. The Na^+/K^+ -ATPase activity and passive Na^+ -efflux were also measured in TPhT-exposed flounders. The TPhT group displayed a significant increase in Na^+/K^+ -ATPase activity and the passive Na^+ -efflux, that was initially unchanged, began to rise towards the end of the experiment and eventually reached the same rate as in the TBT group, which was significantly higher than the control group. Analysis of the data presented in this study, taking into account the current knowledge of the interactions of different organotin compounds with biological membranes, suggests that TBT had immediately penetrated deep into the gill membrane and was therefore able to inhibit the membrane bound Na^+/K^+ -ATPase, whereas TPhT was not. These results, again, reflect the different interaction characteristics of TBT and TPhT with model biological membranes.

The estuarine habitat and the catadromous nature of flounders affords the ability to adapt to fluctuating salinities. On transfer to sea water, a freshwater-adapted euryhaline fish would be expected to exhibit a rapid increase in Na^+/K^+ -ATPase activity accompanied by an increase in active Na^+ -efflux (Bornancin & De Renzis, 1972; Forrest *et al.*, 1973; Potts *et al.*, 1973; Thomson & Sargent, 1977; Carroll *et al.*, 1995) and extensive morphological re-organisation of the gill membranes, involving the redistribution of chloride cells (Thomson & Sargent, 1977; Girard & Payan, 1980; King & Hossler, 1991; Uchida *et al.*, 1996; Van der Heijden *et al.*, 1997; Hiroi *et al.*, 1998; Hirai *et al.*, 1999; Wong & Chan, 1999; Sakamoto *et al.*, 2000). This response to the transfer to sea water was observed in fish from the control and TPhT groups. However, the TBT group showed no significant increase in Na^+/K^+ -ATPase activity and accordingly, the increase in active Na^+ -efflux, normally expected for euryhaline fish adapting to sea water, was not observed in the TBT exposure experiments. In addition, the expected re-distribution of lamellar chloride cells was far less extensive in the TBT group than in the control and TPhT groups. The average area of the few remaining lamellar chloride cells of the control group was significantly reduced, whereas the respective chloride cells in both organotin groups did not show a significant areal reduction. The number of interlamellar chloride cells increased in the control group but showed no significant changes in the two organotin groups. The average area of the interlamellar chloride cells showed no change in all three groups in this study, although an increase would have been expected in the control group (Hiroi *et al.*, 1998, 1999; Kelly *et al.*, 1999; Wong & Chan, 1999). It would appear that the failure to detect any increase in average interlamellar chloride cell area in the control group was caused by the proliferation

of accessory cells during the development of multicellular chloride cell complexes, characteristic of seawater-adapted fish. These accessory cells, that are generally smaller than mature chloride cells and would have reduced the average area measurements, were not distinguishable from mature chloride cells with the Champy-Maillet method. Nevertheless, the fact that the number of interlamellar chloride cells increased significantly during sea water adaptation in the control group, suggests that the expected development of multicellular complexes took place and highlights the suitability of the experimental setup in producing a relatively stress free environment for flounders of the control group, that allowed the normal morphological changes associated with sea water adaptation.

The gills are the main site of extrarenal hydromineral flux regulation (Meyer, 1951; Evans, 1982; Perry, 1997; Towle, 1999) and therefore accurate estimates were necessary in order to normalise the observed fluxes to realistic gill area. This became particularly evident when comparing the net water balance of 0-group flounders (weight range 0.008 - 2.860 g) calculated in this study with previously reported values for 0-group flounders that were based on the gill area of a 200 g fish (Hutchinson, 1984). Therefore, modern technology, in the form of computer assisted digital image analysis, was applied to the traditional methods for estimating gill surface area, pioneered by Hughes (see Hughes, 1966, 1972, 1984). This approach (Hartl *et al.*, 2000) generated a much improved gill area estimate for 0-group flounders and found previously published values for this species (Gray, 1954; Al-Kadhomy, 1985) to be seriously over estimated.

7.1. Final conclusion

The results presented in this study lead to the conclusion that tri-*n*-butyltin chloride and triphenyl-*n*-chloride in sediments are capable of significantly disrupting both the physiological as well as morphological components of osmoregulatory functions of an estuarine fish, at concentrations currently found in local sediments. The results also suggest that benthic fish which are in contact with contaminated sediments are more likely to suffer adverse effects to their osmoregulatory system than pelagic species. This indicates that this source of exposure may be a more important factor than organotin in the water column, especially as far higher concentrations in water seemed to have little effect on blood osmolality as shown by previous studies (Chliamovitch and Kuhn, 1977; Pinkney *et al.*, 1989b).

This may have implications for wild flounders, both at the level of the individual and the population. A flounder that has to allocate resources to counteract the toxicological effects of triorganotin exposure, will have less energy left to invest in growth and reproduction. Smaller individuals are more susceptible to predation and are likely to produce fewer offspring, thereby reducing the fitness of the entire population.

Furthermore, the physiological and morphological responses of flounders exposed to environmental levels of sediment-associated triorganotin compounds demonstrated in this project, such as reduced ATPase activity, decreased blood osmolality and the disruption of the re-organisation in the of gill membrane during adaptation to changing salinity, maybe useful biomarkers indicating the exposure of benthic fish species to these compounds and should be developed further.

7.2. Suggestions for future research

Organotin compounds display strong hydrophobic characteristics and therefore tend to accumulate in the lipid tissues of many aquatic species (Kannan *et al.*, 1996; Wade & Sericano, 1998) and have been shown to cause a decrease in the fluidity of model membranes (Cullen *et al.*, 1997). Morris *et al.* (1982; 1987) demonstrated the significance of fatty acid composition of the gill phospholipids on membrane permeability and alterations caused by lipophilic pollutants in the amphipod *Gammarus duebeni*. Exposure experiments with a variety of other pesticides have caused changes in cell junction morphology, making the “tight” junctions “leaky” and allowed an increased efflux of Na^+ along its concentration gradient (Nagano *et al.*, 1982; Hugla *et al.*, 1996). Thus, the altered fluxes across the gill membrane, found in this study, could be a direct consequence of modifications to the lipid composition and structure of gill membranes caused by the interaction of remobilised, sediment-associated, organotin compounds.

Unlike liver tissue with its microsomal mixed function oxygenase system, the gills do not possess any kind of enzyme system for degrading organic pollutants. A localised concentration of organotin compounds in the gills could be sufficient to alter the metabolism of the whole animal, although the total body burden was found to be low in preliminary experiments. Therefore, it would be useful to apply gas chromatography with flame photometric detection (GC-FPD) in order to determine TBTCI and TPhTCI concentrations in the gill membranes of flounders in relation to the total body burden. Furthermore, the determination of the ratio of saturated to unsaturated fatty acids in phospholipids of the gill membrane of flounders exposed to sediment-associated organotin

compounds, using gas chromatography with flame ionisation detection (GC-FID), would be of interest. This ratio can be used as an indirect measure of membrane permeability to water: the greater the degree of saturation the lower the permeability (Morris *et al.*, 1987). In addition the ultrastructure of tight/leaky gill epithelial cell junctions during organotin exposure, determined by transmission electron microscopy (TEM), would enable the further investigation of the enhanced passive Na^+ -efflux in fresh water-adapted flounders observed in this study.

Appendix I:

Procedure for organotin analysis (Waldock *et al.*, 1989)

Standards:

| | |
|---------------------|---|
| TPTCl | 0.1157g in 100ml methanol (internal standard) |
| TBTCl | 0.1240g in 100ml methanol |
| MBTCl ₃ | 0.1000g in 100ml methanol |
| DBTCl ₂ | 0.1005g in 100ml acetic acid/methanol *) |
| TPhTCl | 0.2324g in 100ml acetic acid/methanol *) |
| DPhTCl ₂ | 0.1338g in 100ml acetic acid/methanol *) |
| MPhTCl ₃ | 0.1109g 100ml methanol |

*) dissolve crystals in 30-40 ml of glacial acetic acid and top up to 100 ml with methanol.

Preparation of 'on column' concentrations (example for TPTCl):

$0.1157\text{g} \cdot 100\text{ml}^{-1} = 1.157 \text{ mg} \cdot \text{ml}^{-1}$
Dilute by 100 = $11.57 \mu\text{g} \cdot \text{ml}^{-1}$ (working standard)
 $50 \mu\text{l}$ of working standard = $0.578 \mu\text{g} = 578 \text{ ng} \cdot 50 \mu\text{l}^{-1}$
Replacement of -Cl (35,5g) with -H (1g)
 $578 \cdot 0.875 = 505.75/2000 = 0.253 \text{ ng} \cdot \mu\text{l}^{-1}$ on column

Prepare 0.1 % NaOH in Methanol

Determine water content in sediment (best done during sampling).

| | | |
|----------------|------------|-------------|
| Water content: | 0.5 - 1 ml | add 2ml |
| | 1.0 - 2 ml | add 1 ml |
| | ≥ 2 ml | add nothing |

Multiply by 4 in order to get $\text{H}_2\text{O} : \text{NaOH} = 1 : 4$

Prepare an RF sample for each standard ($50 \mu\text{l}$) + $50 \mu\text{l}$ internal standard
Prepare blank: Water + Methanol/NaOH + $50 \mu\text{l}$ TPTCl (internal standard)
Prepare samples the same as blanks + sediment

Shake for 1 hour, add 2 ml hexane + 1 heaped microspatula of NaBH
Shake again - open carefully and release gas (H_2)
Continue shaking for 15 min
Centrifuge at 4000 rpm for 5 to 10 min
Draw off Hexane phase and place in GC-tubes (not above wavy line)

Analysis on GC-FPD

Carrier Gas: H_2
Make up Gas: N_2
Column used: 25 m standard methylsilicone

Retention factor (RF) calculations (example for MBT):

$$RF = \frac{MBT(H^+)}{MBT(St)} \div \frac{TPT(H^+)}{TPT(St)}$$

Sample concentration (example for MBT):

$$[MBT]ng \cdot g^{-1} = \left[\left(\frac{MBT(H^+)}{TPT(H^+)} \cdot TPT(St) \right) \div RF \right] \div dryweight(g)$$

Appendix II

Na⁺/K⁺ - ATPase activity analysis modified from Mayer-Gostan & Lemaire (1991)

Membrane isolation

Excise gill arches - scrape epithelium from the underlying cartilage

Suspend scrapings in an hypotonic saline solution (pH 7.4):

25 mmole NaCl

1 MMOLE DITHIOTHREITOL

1 mmole Hepes-Tris

Disrupt scrapings in a homogeniser (20 strokes)

Centrifuge homogenate (cold) at 2212 g (3,000 rpm) for 10 minutes – discard pellet

Centrifuge supernatant (cold) at 2212 g (3,000 rpm) for 10 minutes – discard supernatant

Resuspend pellet in 0.5 mmole EDTA

Centrifuge (cold) at 2212 g (3,000 rpm) for 30 minutes – discard supernatant

Rinse pellet twice in isotonic sucrose solution (0.3 Mole) and re-suspend in reaction medium (store at -20°C).

Determination of protein concentration in suspension (Bradford, 1976)

Protein reagent

Dissolve 100 mg Coomassie Brilliant Blue (CBB) G-250 in 50 ml 95 % ethanol

Add 100 ml 85 % (w/v) phosphoric acid

Dilute the solution to a final volume of 1 litre (final concentrations: 0.01 % CBB, 4.7 % ethanol, 8.5 % phosphoric acid)

Standard preparation (bovine serum albumin)

Produce Protein solutions 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg 0.1 ml⁻¹

Pipette 0.1 ml of each solution into an Eppendorf vial

Add 5 ml of protein reagent - vortex

Measure absorbance at 595 nm after 2 minutes and before 1 hour in 3 ml cuvettes against 3 ml reagent blank

Plot the weight of protein against corresponding absorbance.

Microprotein assay

Pipette 0.1 ml of protein solution into Eppendorf vial

Add 5 ml over protein reagent - vortex

Measure absorbance at 595 nm in 1 ml cuvettes against 1 ml reagent blank

Na⁺/K⁺ - ATPase activity

Inorganic phosphate measurement

Inorganic phosphate standard preparation

Stock solutions

- (1) 5.72 % Ammonium molybdate in 6 N HCL
- (2) 2.32 % polyvinyl alcohol
- (3) 0.0812 % malachite green
- (4) distilled water

Colour reagent

Mix 5 ml of (1), 5 ml of (2), 10 ml (3) and 2.5 ml of (4).

Produce an inorganic phosphate standard series of blank (0), 20, 40, 60, 80, 100 μMol

Pipette 100 μl of each solution into an Eppendorf vial

Add 150 μl of colour reagent - vortex

Measure absorbance at 620 nm in microplate reader

Plot the inorganic phosphate concentration against corresponding absorbance.

ATPase assay

Produce a buffered reaction medium containing of:

100 mmole NaCl

0.1 mmole H_2EDTA

5 mmole MgCl_2

20 mmole Hepes-Tris (pH 7.4)

Incubate membrane extracts for 5 minutes on ice

Pipette 50 μl subsamples into the microplate wells

Add 1mMol Ouabain to half of the wells

Activate the reaction by adding 50 μl ATP.

Incubate and shake the plate for 10 minutes at 37°C.

The enzyme reaction is terminated by adding the colour reagent (malachite green)

Measure inorganic phosphate at 620 nm in both groups.

ATPase activity is expressed as the absorbance difference between the ouabain-free and the ouabain group in $\mu\text{Mol Pi } \mu\text{g}^{-1}$ protein

Appendix III Staining mixtures and fixatives

Bouin's fixative

| | |
|-------------------------------|----------------------|
| Saturated aqueous picric acid | 75 ml |
| Formalin | 25 ml |
| Acetic acid | 5 ml |
| Urea | 10 g.l ⁻¹ |

Champy-Maillet's stain

| | |
|------------------|-------|
| Osmium tetroxide | 0.4 % |
| Zinc iodide | 2.4 % |

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The biometry of gills of 0-group European flounder

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The gill surface area of 0-group, post-metamorphic *Pleuronectes flesus* L. was examined using digital image analysis software and expressed in relation to body mass according to the equation $\log Y = \log a + c \log W$ ($a = 239.02$; $c = 0.723$). The components that constitute gill area, total filament length, interlamellar space and unilateral lamellar area were measured. The measurement of the length of every filament on all eight arches showed that commonly used methods of calculation can lead to an under-estimation of up to 24% of total filament length. Direct measurements of unilateral lamellar area with digital image analysis showed that previously reported gill area data for the same species was over-estimated by as much as 58%. In addition, in this species the neglect of gill pouch asymmetry after metamorphosis, can bring about a 14% over-estimation of total gill area.

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Key words: gill area; *Pleuronectes*; digital image analysis.

INTRODUCTION

In fish, the gills are not only responsible for gas exchange but are also the site of extrarenal ion excretion. These are surface area dependent processes, and therefore accurate gill surface area estimates are essential for studying the physiology of gas exchange and ionic fluxes across the gills (Motais *et al.*, 1966; Hughes & Morgan, 1973). Such measurements are of particular significance in the physiology of estuarine fish that are frequently used as model species in studies of hydromineral regulation in fluctuating conditions (Potts & Eddy, 1973; Potts *et al.*, 1973; Hutchinson & Hawkins, 1990; Carrol *et al.*, 1995) and more generally, as sentinel organisms in water quality evaluations (Larson *et al.*, 1981). To determine the fluxes involved in these processes it is necessary to obtain accurate calculations of total gill surface area which in turn are dependent on the following parameters: total filament length, the frequency of lamellae and the unilateral lamellar surface area. Such measurements can now be conveniently made using digital image analysis techniques. Prior to the availability of such technology, studies by Gray (1954) and Hughes (1966) examined a number of species, and estimated lamellar area by treating individual lamellae as simple triangles. Also, total filament length was estimated by measuring and averaging every n th filament and then multiplying the results by the total number of filaments rather than examining every filament.

Muir & Hughes (1969) devised a weighted method for the determination of gill dimensions in fish that was subsequently improved by Hughes & Morgan (1973).

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Since then this method has been adopted widely and was adapted by Al-Kadhomi (1985) for use with larval and post-metamorphic European flounder *Pleuronectes flesus* L.

The aim of the present work was to see if PC based digital image analysis of gills of 0-group *P. flesus* could provide accurate areal measurements required for a study of hydromineral fluxes in this species.

MATERIALS AND METHODS

0-group flounders were caught at Woodmill, River Itchen, Southampton (Hutchinson & Hawkins, 1993). Flounders ($n=17$, wet weight: 0.008–2.860 g) were killed by an MS222 overdose (5 mg l^{-1}), followed by the destruction of the brain in accordance with the Animal (Scientific Procedures) Act of 1986 (HMSO, 1986) and processed immediately. All eight gill arches from each specimen were removed and preserved in cold Bouin's fixative, following the protocol described by Hughes (1984), who pointed to the fact that there is unavoidable shrinkage during fixation with Bouin's solution. In the present study this shrinkage was quantified by extracting five individual filaments of different initial sizes that were placed in water on a cavity slide and filament length, interlamellar space and unilateral lamellar area were measured using the methodology described below. The water was replaced by Bouin's solution, and the slide covered and refrigerated. Further measurements were made after 24 and 72 h; 1, 2 and 6 weeks. All measurements presented have been corrected for shrinkage. The transfer of gill tissue from Bouin's to tap water showed no measurable distortion, compared with tissue transferred to an isotonic saline solution.

The biometry of fresh and preserved material was determined by adapting the weighted method described by Hughes & Morgan (1973), making use of digital image analysis software (SigmaScan Pro 4.0) to obtain the primary measurements under manual control. A single areal standard was required to calibrate the system, this was provided by the grid of a Neubauer haemocytometer; linear measurements were checked using a stage micrometer. Images were captured with an Olympus BH-2 light microscope linked by a Panasonic F10 CCD video camera to a desktop PC using Matrox Rainbow Runner. The length of each filament was measured from base to tip, taking the curvature of some filaments caused by fixation into account. The filaments of each hemibranch were grouped into several sections according to length. A medium-sized filament of each section was selected and every interlamellar space and the areas (Fig. 1) of one side of every lamella on both sides of individual filaments were measured, averaged and used to calculate the surface area of the particular group. This was achieved by separating the filament from the gill arch and placing it with one side facing upwards into a cavity slide filled completely with water and sealed with a cover slip. After measurements were made on the one side, the filament was turned over on to the other side. Large filaments could be turned over with a dissecting needle but with very short filaments it was often enough to move the cover slip along the plane of the slide, the resulting movement of the water in the cavity then turned the filament on to the opposite side. Measurements had to be carried out on all eight gill arches because of the asymmetry of the upper and lower gill pouches. The total surface area (A) of a given hemibranch was determined by $n=(L/d')^2$ and $A=2bLn$, where $2b$ is the bilateral surface area of lamellae, L is the total filament length, d' is the interlamellar spacing and n is the frequency of lamellae mm^{-1} filament. The surface area data of all the hemibranchs were summed to give the total gill surface area for each fish.

The formulae of previous studies were applied to the present data set. Gray (1954) averaged every 10th filament to calculate total filament length. Al-Kadhomi (1985) calculated total filament length by dividing the filaments of a hemibranch into groups according to length and averaging the first and the last filament of each group. To demonstrate the effect of gill asymmetry on total gill area, the results from doubling the area of the upper gill pouch were compared with the area calculation derived from the

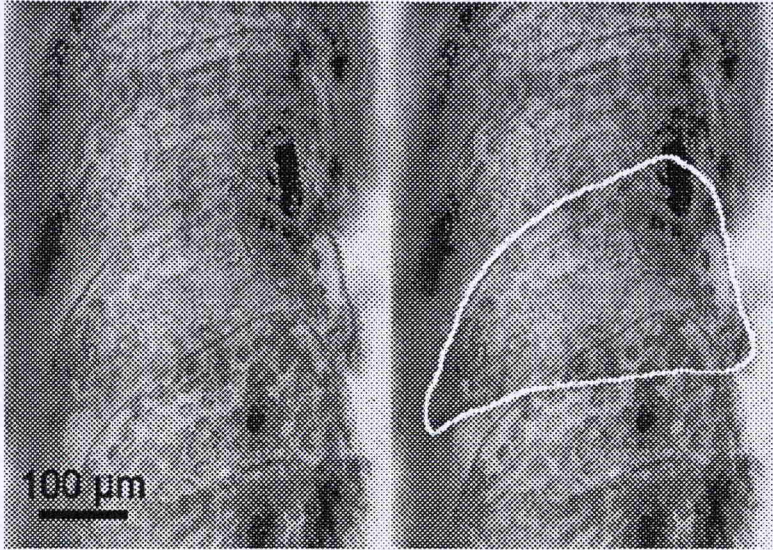


FIG. 1. Two micrographs of the same section of a filament (fish weight: 0.98 g). The unilateral lamellar area was measured on screen from grabbed digital images by hand tracing the shapes of intact lamellae (white line; right). The enclosed area was then integrated by a PC running 'Sigma Scan Pro 4.0'.

sum of all eight gill arches. The gill area data (Y) was analysed in relation to body weight (W) with the allometric equation $Y = aW^c$ and using linear logarithmic transformation $Y = \log a + c \log W$. A paired t -test was used to compare the results of the calculations of total filament length using both Gray's (1954) and Al-Kadhomy's (1985) methods with the measurements obtained in this study. The data for average bilateral lamellar area estimates in this study and in Al-Kadhomy's (1985) were analysed with a Student's t -test and total gill area results of both studies were compared with a one-way ANOVA.

RESULTS

The gill area of 0-group flounders was highly correlated ($r = 0.96$) with body weight using the standard allometric equation [Fig. 2(a)]. Total filament length [Fig. 2(b)] was similarly correlated ($r = 0.96$). The average bilateral surface area and the frequency of lamellae (mm^{-1}) showed no correlation with body weight, $r = 0.11$ and $r = 0.08$, respectively [Fig. 2(c), (d)]. The measured parameters were also compared on the two hemibranchs of each gill arch as the ratio anterior hemibranch: posterior hemibranch ($a : p$). The total length of the filaments of the anterior hemibranchs on the gill arches of the upper gill pouch was 70% greater than on the posterior hemibranch, whereas on the gill arches of the lower gill pouch, total filament length of the anterior hemibranchs was 37% larger than that of the posterior hemibranchs (Fig. 3). The poor correlation between body weight and the $a : p$ ratios of bilateral lamellar area ($r = 0.12$) and the frequency of lamellae ($r < 0.001$), suggests that while the gill area increases during growth, the proportions remain more or less the same and are independent of weight.

The upper gill pouch contained larger gill arches than those of the lower one (Table I). The average ratio of the areas of upper pouch arches : lower pouch arches was 1.53 ± 0.88 ; log-transformed linear regression showed no correlation between weight and ratios. The gill area estimates determined by multiplication

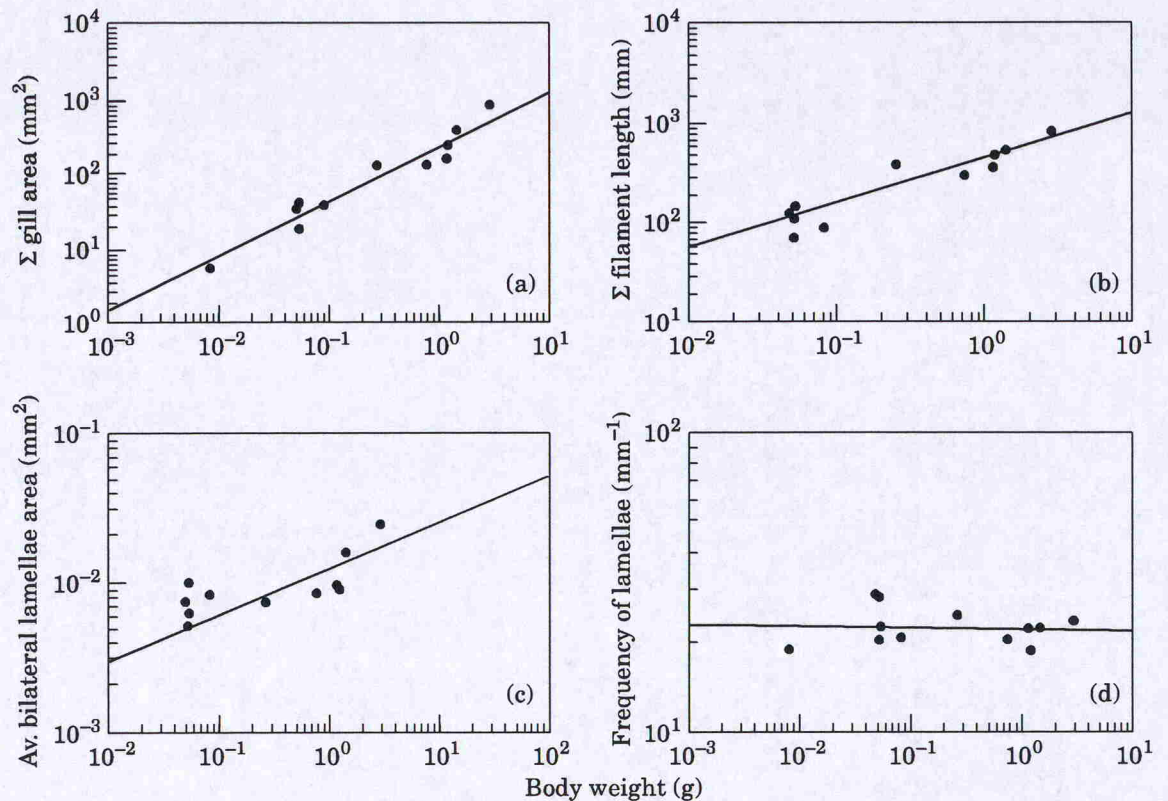


FIG. 2. Linear logarithmic plots of (a) total gill area, (b) total filament length, (c) average bilateral lamellar area and (d) frequency of lamellae from individual fish v. body weight. (a) $a=239.02$, $b=0.723$, $\log Y=\log a+b \log W$, $r=0.96$. (b) $a=418.27$, $b=0.4137$, $\log Y=\log a+b \log W$, $r=0.96$. (c) $a=0.2125$, $b=0.302$; $\log Y=\log a+b \log W$, $r=0.11$. (d) $a=21.93$, $b=-0.0061$, $\log Y=\log a+b \log W$, $r=0.08$.

and those obtained by separate measurements of upper and lower gill pouches were significantly different ($P < 0.001$) (Table II).

Separate experiments were performed to estimate the degree of shrinkage during fixation and storage. The maximum shrinkage of total gill area during the 6 week storage period was 11%. This was not significantly different from the shrinkage observed after a 24 h fixation period (Table III). A detailed analysis of total filament length and interlamellar spacing revealed a significant shrinkage of 9% ($P=0.009$) and 7% ($P=0.04$), respectively. The unilateral lamellar area shrank significantly by 16% ($P=0.02$).

DISCUSSION

A radical change in methods of gill area measurement can be attributed to McDonald *et al.* (1991), who introduced digital image analysis of drawings into a study of gill areas of freshwater fish. Subsequent studies by Stevens (1992) on red drum *Sciaenops ocellatus* (L.) gill areas and by Jakubowski (1997) working on Baikalian deep-water sculpins *Abyssocottus korotneffi* Berg and *Cottinella boulengeri* Berg, have used the new technology in combination with the basic calculations of Hughes (1966). The present study has followed the same path in combining new technology with well established methods. The validity and

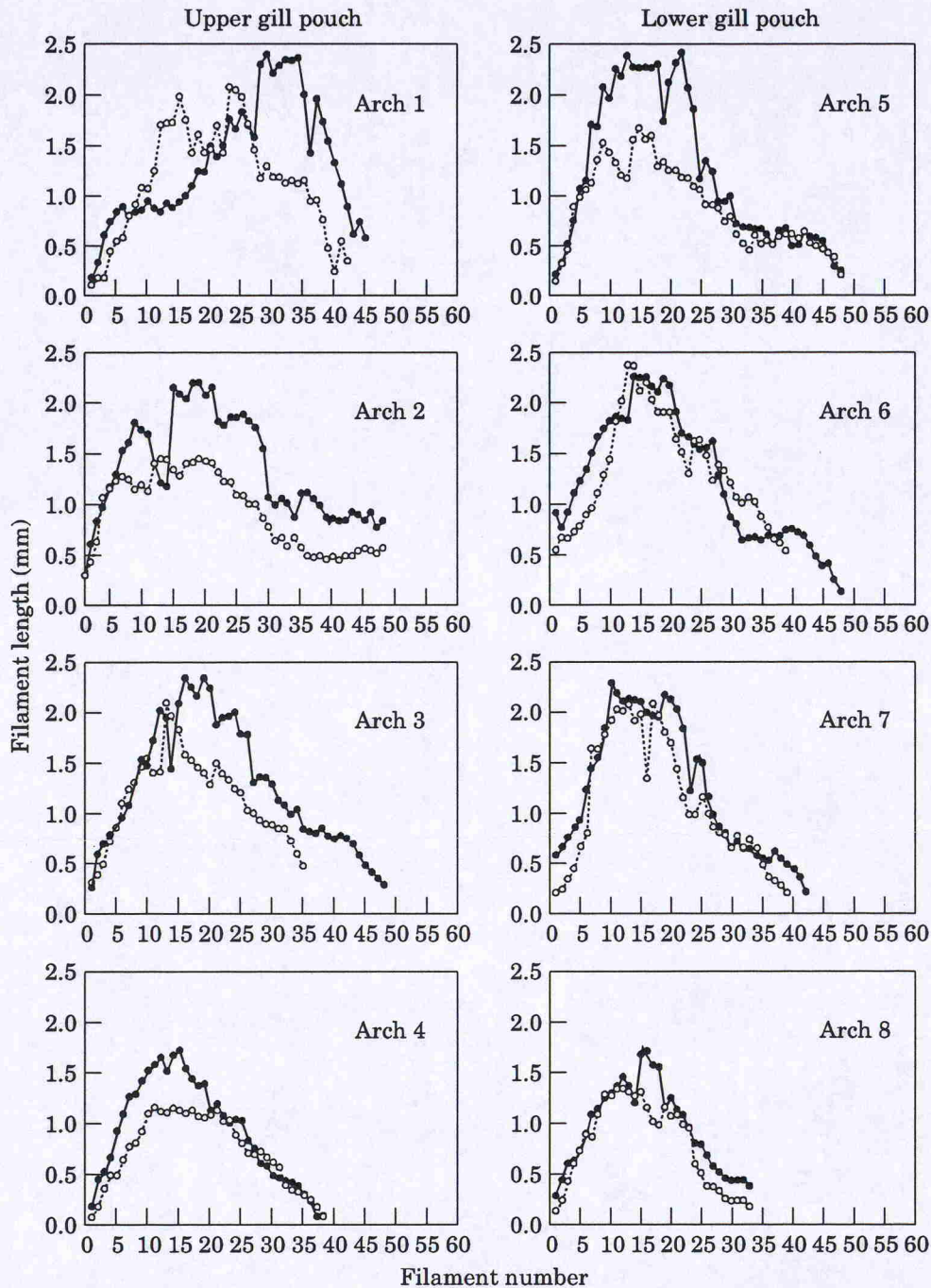


FIG. 3. Lengths of the gill filaments on the anterior (●) and posterior (○) hemibranchs of all eight gill arches plotted against the number of filaments in a 2.86 g (wet weight) specimen.

supposed greater accuracy of this approach was tested, with some rigour, given Jakubowski's (1993) study, which indicated that values given in the literature for the gill area of various fish species had been seriously over-estimated. The most common expression of total gill area for comparative purposes is in the form of an allometric relationship to body weight (Fig. 2). It should also be borne in mind that the hemibranchs of most fish species are not uniform. In the

TABLE I. The ratio of measurements on arches of the upper gill pouch (A1–A4) to those on arches of the lower gill pouch (A5–A8); $n=17$; wet weight 0.008–2.860 g

| | | A1 : A5 | A2 : A6 | A3 : A7 | A4 : A8 |
|---|---------|---------|---------|---------|---------|
| Total filament length (mm) | Average | 1.26 | 1.00 | 1.14 | 1.12 |
| | S.E. | 0.30 | 0.24 | 0.27 | 0.26 |
| | r | 0.02 | 0.03 | 0.20 | 0.10 |
| | P | 0.93 | 0.92 | 0.38 | 0.58 |
| Frequency of lamellae (mm^{-1}) | Average | 1.05 | 0.98 | 0.99 | 0.93 |
| | S.E. | 0.25 | 0.23 | 0.23 | 0.22 |
| | r | 0.21 | 0.30 | 0.12 | 0.05 |
| | P | 0.42 | 0.22 | 0.63 | 0.84 |
| Average bilateral lamellar area (μm^2) | Average | 6.42 | 8.16 | 5.04 | 3.72 |
| | S.E. | 1.51 | 1.92 | 1.19 | 0.88 |
| | r | 0.27 | 0.19 | 0.11 | 0.08 |
| | P | 0.30 | 0.48 | 0.67 | 0.76 |
| Total gill area (mm^2) | Average | 1.39 | 1.22 | 1.25 | 1.33 |
| | S.E. | 0.33 | 0.29 | 0.29 | 0.31 |
| | r | 0.03 | 0.30 | 0.27 | 0.21 |
| | P | 0.92 | 0.12 | 0.29 | 0.44 |

S.E., Standard error.

case of *P. flesus* the anterior hemibranchs are generally larger than the posterior ones (Fig. 3).

Total gill area of *P. flesus* was significantly smaller (58%; $P=0.03$) in the present study (Fig. 4) than that reported by Al-Kadhomy (1985). Comparisons of total filament length (Fig. 5) showed that both the estimates, derived from the methods of Gray (1954) and Al-Kadhomy (1985), were significantly smaller than direct measurements by 23% ($P=0.038$) and 18% ($P=0.03$), respectively. Values for average bilateral lamellar area and the frequency of lamellae obtained were also compared with the data of Al-Kadhomy (1985). In the present study the average bilateral lamellar area was significantly smaller by 60% ($P<0.001$; Fig. 6). There was no significant difference ($P>0.05$) in the frequency of lamellae per mm of filament. This underestimation of lamellar area was not an artefact caused by curling of lamellae, as only intact lamellae were measured. A detailed analysis of the data suggests that part of the differences described above were caused by an apparent over-estimation of bilateral lamellar area in previous studies. The measurements of unilateral lamellar area in this study were carried out on filaments in a cavity slide filled with water sealed with a cover slide, while previous methods applied to the same age group and species were performed on histological sections. One problem that can arise from the method described here is that optical diffraction at the boundaries of the different media (glass and water) can cause distortions to the image through moving parts of the object out of the focal plane at the edges of the cavity. However, these edge distortions were observably negligible.

TABLE II. Comparison of total gill areas for *P. flesus* estimated by (1) doubling the areas of the upper and lower gill pouches (UGP \times 2) and (2) addition of area estimates of the upper and lower gill pouches (UGP+LGP)

| Weight (g) | UGP \times 2 (mm ²) | UGP+LGP (mm ²) | Difference (%) |
|---------------|--------------------------------------|-------------------------------|-------------------|
| 0.008 | 7.50 | 5.88 | 22 |
| 0.029 | 2.49 | 1.29 | 48 |
| 0.030 | 0.22 | 0.20 | 6 |
| 0.049 | 38.09 | 34.56 | 9 |
| 0.052 | 19.37 | 18.63 | 4 |
| 0.052 | 41.81 | 40.72 | 3 |
| 0.053 | 54.78 | 46.21 | 16 |
| 0.083 | 38.40 | 37.37 | 3 |
| 0.257 | 138.83 | 113.00 | 19 |
| 0.740 | 131.14 | 119.98 | 9 |
| 0.980 | 75.98 | 50.98 | 33 |
| 1.100 | 231.95 | 147.83 | 36 |
| 1.150 | 161.60 | 146.60 | 9 |
| 1.190 | 228.18 | 227.02 | 1 |
| 1.400 | 424.63 | 388.69 | 8 |
| 2.580 | 321.54 | 265.57 | 17 |
| 2.860 | 896.88 | 845.17 | 6 |
| | | Average: | 15 |
| | | S.E. | 4 |

In the studies by Gray (1954) and Al-Kadhomy (1985) the measurement of every n th filament rather than every filament led to a significant under-estimation of total gill filament length, though this would tend to offset the accompanying over-estimates of total gill area. In the present study the measurement of every lamella on a filament took into account the gradual change in size of the lamellae along the length of a filament whereas previous studies have taken an average of lamellae from the base, tip and middle of the filament. Yazdani & Alexander (1967) and Al-Kadhomy (1985) recognized that the asymmetry of the gill pouches in the post-metamorphic body form of *P. flesus* would complicate its gill biometry. The recognition of this difference is essential in improving the accuracy of gill area measurements (Table I) since it is not apparent from previous studies on flatfish that gill pouch asymmetry was taken into account. Gray (1954) multiplied the information from the upper chamber by a factor of two, which over-estimates total gill area (Table II). Al-Kadhomy (1985) recognized the problem posed by gill pouch asymmetry, but applying the calculations that were described by this author to the present data set produces gill area estimates that are similar to those of Gray (1954) and which are significantly greater than those obtained with the methods used in the present study.

The final factor, which the present study has attempted to quantify, is the effect of tissue shrinkage during preservation. Hughes (1984) reported shrinkage of the length of filaments fixed in Bouin's solution of up to 3% over 24 h; he

TABLE III. Differential changes over time in gill measurements derived from *P. flesus* gill arches preserved in cold Bouin's solution; probabilities obtained from one-way ANOVA; mean values \pm S.D.

| Time | Average (mm) | S.D. (mm) | Shrinkage (%) | |
|-------------------------------------|--------------|-----------|---------------|-------------|
| Filament length ($n=5$) | | | | |
| 0 | 1418.75 | 234.94 | | |
| 24 h | 1322.23 | 185.69 | 6.80 | $P=0.009^a$ |
| 72 h | 1217.54 | 134.52 | 7.29 | |
| 2 weeks | 1197.38 | 128.42 | 7.99 | |
| 6 weeks | 1211.49 | 138.19 | 9.31 | $P=0.563^b$ |
| Unilateral lamellar area ($n=19$) | | | | |
| 0 | 3526.02 | 858.92 | | |
| 24 h | 3000.90 | 508.32 | 14.89 | $P=0.026^a$ |
| 72 h | 2998.07 | 687.83 | 14.97 | |
| 2 weeks | 2965.80 | 673.92 | 15.89 | |
| 6 weeks | 2942.98 | 688.34 | 16.54 | $P=0.399^b$ |
| Interlamellar space ($n=16$) | | | | |
| 0 | 38.89 | 2.59 | | |
| 24 h | 37.14 | 2.64 | 4.75 | $P=0.149^a$ |
| 72 h | 37.07 | 2.66 | 5.79 | |
| 2 weeks | 37.05 | 2.22 | 6.92 | |
| 6 weeks | 36.99 | 1.87 | 7.77 | $P=0.450^b$ |

^aANOVA for shrinkage during 24 h of storage. ^bANOVA for shrinkage between 24 h and 6 weeks of storage.

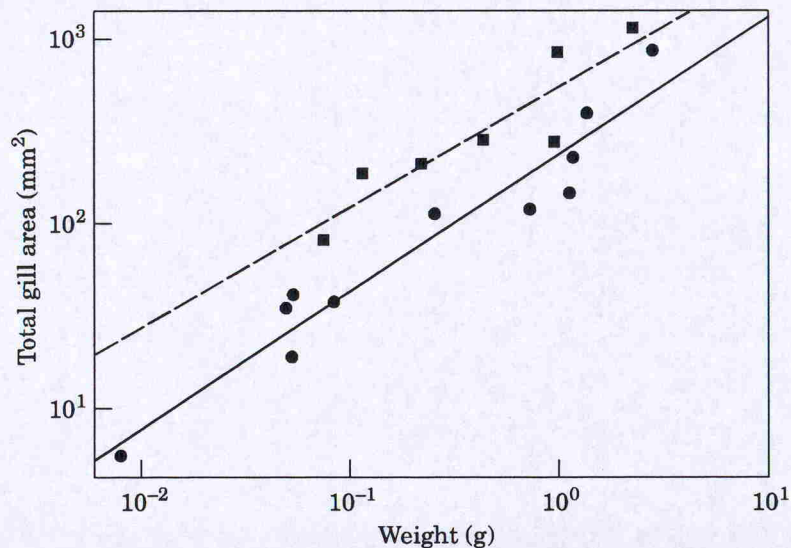


FIG. 4. Regressions of estimates of total gill areas of individual fish, plotted against body weight. Estimates from Al-Kadhomy (1985; ■) are compared with those from this study (●).

concluded that this would lead to a proportionate shrinkage of the interlamellar spaces. It was assumed that shrinkage-induced changes in these two parameters would effectively cancel each other out. Our measurements demonstrate that

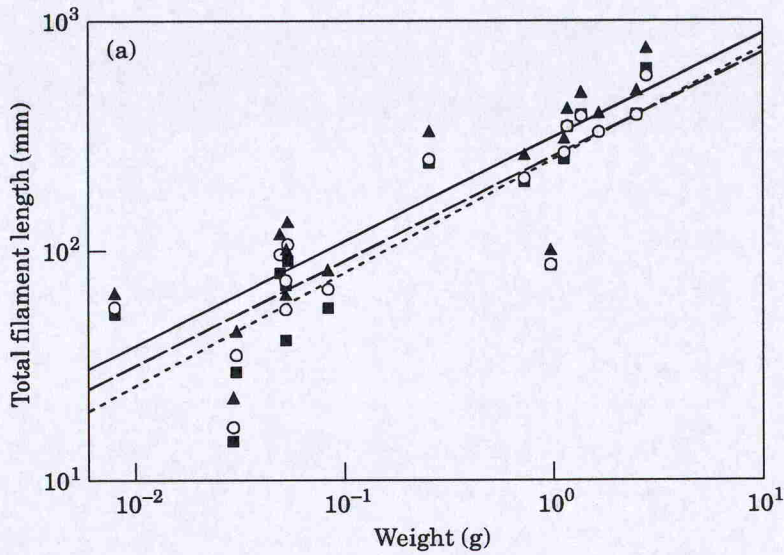


FIG. 5. Regressions of estimates of total filament lengths from individual fish plotted against body weight, calculated according to the methods of Gray (1954; ■) and Al-Kadhomi (1985; ○) compared with the direct measurement of every filament (this study; ▲).

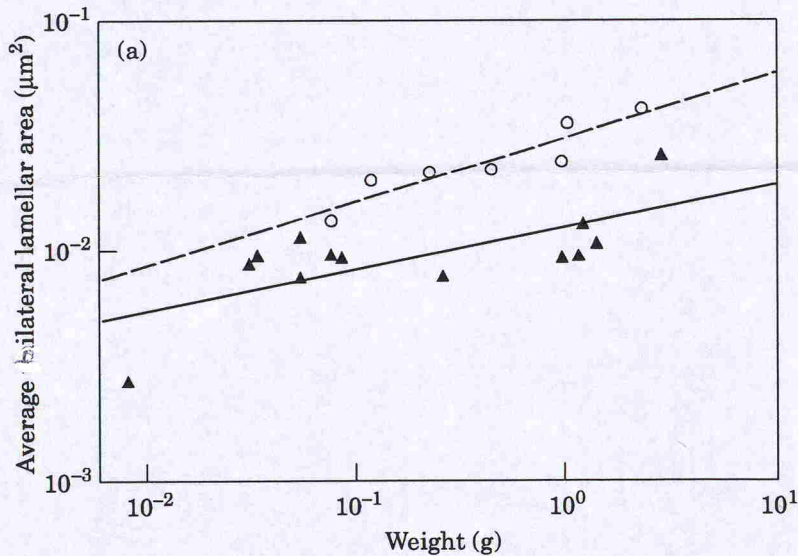


FIG. 6. Average bilateral lamellar areas from individual fish, plotted against body weight. Data from Al-Kadhomi (1985; ○) are compared with measurements from this study (▲).

although the shrinkage in both parameters is significant, the average inter-lamellar spacing shrank less than the average filament length (Table III). However, this had no significant ($P > 0.05$) effect on the frequency of lamellae (mm^{-1}). Hughes (1984) noted that it was not possible to obtain reliable shrinkage estimates for individual lamellae with the equipment available to him. Here, the combination of established techniques with digital image analysis seems especially beneficial and revealed a significant shrinkage (16%; $P < 0.05$) of unilateral lamellar area during fixation and storage. A further advantage in using digital image analysis of video pictures of the gills is that lamellar area on

a large number of filaments can be measured relatively quickly, compared with the time-consuming process of using an eye-piece graticule that, of necessity, forced workers to limit their sub-sampling to a practical minimum.

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Reduced Blood Osmolality in Freshwater-adapted European O-group Flounder, *Platichthys flesus* (L.), Exposed to Environmental Levels of Sediment-associated Tributyl- and Triphenyltin

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Following the partial bans on the use of organotin-based anti-fouling paints on boats and maritime equipment in most industrialized countries, water concentrations of tributyltin (TBT) have dropped dramatically, albeit with hotspots remaining in areas of intense shipping activity (Waite *et al.*, 1991, 1996; Stewart, 1996). However, there is increasing evidence to show that organotin species are persistent in marine and freshwater sediments, that act as both reservoirs of the element and sources for the secondary introduction of organotins to the environment (Valkirs and Seligman, 1986; Langston *et al.*, 1987; Waldock *et al.*, 1990; Langston and Burt, 1991; Steur-Lauridsen and Dahl, 1994; Watanabe *et al.*, 1995, 1997; Harris *et al.*, 1996). Despite this wealth of data, there have been few studies on the effects on benthic organisms exposed to environmental concentrations of organotin in sediments; the

most recent of these being the studies by Krone *et al.* (1996), Rouleau *et al.* (1998) and Werner *et al.* (1998). In particular, there is a lack of information on the disruption of osmoregulatory functions in benthic euryhaline species by organotins leaching from sediments; this, despite the pioneering work by Aldridge (1976) who showed that there was an inhibitory effect of TBT in vitro on the osmoregulatory enzyme Na⁺/K⁺-dependent ATPase. Subsequent in vivo experiments conducted to evaluate their potential effect on osmoregulation have focused on organotins in aqueous suspension, rather than sedimentary sources. In the latter studies, no changes in blood osmolalities were found in freshwater adapted rainbow trout (*Oncorhynchus mykiss*) exposed to acutely toxic concentrations of tributyltin oxide (Chliamovitch and Kuhn, 1977). A similar observation was made by Pinkney *et al.* (1989) for juvenile striped bass (*Morone saxatilis*) adapted to 50% seawater and exposed to sublethal concentrations of tributyltin oxide; these authors also found a significantly enhanced Na⁺/K⁺-ATPase activity.

The aim of the present study was to evaluate the effects of organotin-contaminated sediments on aspects of the osmoregulation of a benthic euryhaline fish, the European flounder *Platichthys flesus*. O-group flounders (0.02–1.3 g wet weight) were collected in the Itchen River estuary just below Woodmill, Southampton, UK. Stock populations of flounders were kept in an outdoor 3500 l glass fibre fish farming tank, shielded from direct sunlight and rain by a roof, but subject to natural temperature fluctuations and light/dark cycles. Prior to experimentation, the fish were selected from the stock populations and acclimated to tapwater at a temperature of 15°C and a light/dark regime of 12/12 h for at least 1 week. Fish were fed ad libitum on live brine shrimp (*Artemia salina*) during both acclimation and the 5-week experimental period, but starved for 24 h prior to sampling for analytical tests.

The exposure experiments were performed in 25 l polyethylene buckets (Carter *et al.*, 1989) containing organotin-contaminated fine-grained silica sand (horticultural 'Silver Sand'; grain size <1 mm). The water was continuously aerated and was changed once a week. To produce the required sediment concentrations of organotins, tributyltin chloride (TBTCl) or triphenyltin chloride (TPhTCl) in glacial acetic acid were first adsorbed to approximately 20 g of fine deep-sea mud, obtained from Porcupine abyssal plain (north-east Atlantic; organotin concentrations <1 ng g⁻¹) to reduce leaching. This mud was then mixed into the sand in separate buckets to produce final sediment concentrations of 150 ng g⁻¹ dry weight. This concentration was chosen as representative of local concentrations of organotins in sediment, as measured for TBT by Langston *et al.* (1994). Preliminary experiments showed that the calculated half-life for sediment-associated TBT was significantly higher than that for TPhT ($p < 0.05$). TBT and TPhT concentrations declined from 150 to

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121 ng g⁻¹ and 151–107 ng g⁻¹, respectively, during a 42-day incubation. Extrapolation of these values indicated a half-life of 95 days for TBT and 85 days for TPhT. The organic degradation products of TBT, dibutyltin (DBT) and monobutyltin (MBT), peaked at concentrations of 8.9 and 8.3 ng g⁻¹, respectively. The diphenyltin (DPhT) concentration peaked at 48 ng g⁻¹, while phenyltin (MPhT) was not detected. Sediment samples for organotin analysis were taken immediately after dosing and again at the end of the 5-week experiment, and were stored at -20°C. Organotin analysis was performed by gas chromatography with flame photometric detection (GC-FPD) after hydride generation, according to the method of Waldock *et al.* (1989). The osmotic concentrations of blood samples and the surrounding medium were measured by the cryoscopic method of Ramsay and Brown (1955). Statistical analysis of the data was carried out using a one-way ANOVA, and linear regressions were used to examine the dependency of osmolality on fish size.

Freshwater-adapted O-group flounders normally maintain a blood osmolality between 310 and 315 mOsm kg⁻¹ (Hutchinson and Hawkins, 1990). Fig. 1 shows that, following the 5-week exposure to sediment contaminated with TBTCI or TPhTCI, the experimental groups had significantly lower mean blood osmolalities ($p < 0.05$) when compared to a control group. There was no significant difference between the blood osmolalities of the fish in the two organotin groups ($p > 0.05$). The values for blood osmolalities in all three groups were independent of fish size ($r = 0.17, 0.22$ and 0.21 , respectively). Throughout the experimental period, the fish were in constant contact with the contaminated sediment, being either on top of the substrate or partially buried. This experiment is believed to represent a realistic pattern of how a benthic species interacts with sediment-associated organotins and of some of the consequent effects on osmoregulation.

It is considered that benthic fish which are in contact with contaminated sediments are more likely to suffer adverse effects to their osmoregulatory system than

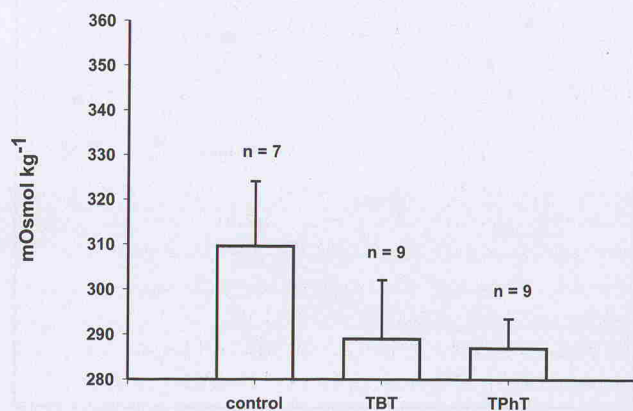


Fig. 1 Blood osmolality (mean values \pm S.D.) of O-group European flounder (*Platichthys flesus* L.) after chronic (5-week) exposure to 150 ng g⁻¹ (dry weight) TBTCI or TPhTCI.

pelagic species. This source of exposure may be more important than organotins in the water column, especially as far higher concentrations in water seemed to have little effect on blood osmolality in previous studies (Chliamovitch and Kuhn, 1977; Pinkney *et al.*, 1989). The results presented here show that tributyltin- and triphenyltin chlorides in sediments are capable of significantly disrupting the osmoregulatory functions of an estuarine fish, at concentrations currently found in local sediments.

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