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Organometallic complexes in ascidian embryonic development: II. Effects on different stages and larvae

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The effects of the organometallic compounds Bu_2Sn-D-(−)sorbitol, Bu_2Sn-D-(+)glucose, Bu_2Sn-D-(−)fructose and Bu_2Sn-D-(+)glyceraldehyde were tested *in vivo* on different stages of Ascidian development, larval movement and metamorphosis. Organotin(IV) complexes are organometallic compounds widely used as industrial biocides, antifouling agents and agricultural fungicides and are toxic to a range of organisms. Two-cell stage embryos, if incubated for one hour in the organotin (IV) solutions, stopped the cleavage, which was restored when they were transferred into normal sea water. The gastrula stage was seriously affected in 10^{-4} mol dm^{-3} solutions of the above-mentioned complexes: 85% of the embryos were anomalous neurulae with open neural folds, 5% were twisted larvae. The gastrulae, when incubated for 1 h in 10^{-5} mol dm^{-3} solutions, developed twisted larvae in ovular envelopes and immobile larvae with twisted tails. Larvae treated with 10^{-4} mol dm^{-3} and 10^{-5} mol dm^{-3} Bu_2Sn-D-(−)sorbitol, Bu_2Sn-D-(+)glucose and Bu_2Sn-D-(+)glyceraldehyde solutions stopped swimming, did not metamorphose and afterwards underwent cytolysis. An initial hyperactivity of circular movements, followed by immobility, was observed in the larvae incubated in Bu_2Sn-D-(−)fructose.

Keywords: Organometallic complexes, development, metamorphosis, ascidians

INTRODUCTION

Organotin(IV) derivatives are used as active components of antifouling paints to prevent the settling of algae and benthic invertebrate organisms on surfaces immersed in fresh and marine water. Because of their stability in the aquatic ecosystem, however, they interfere with the reproductive cycle of other marine organism.

There are numerous literature reports on the toxicity of organotin(IV) compounds, especially those of triorganotin(IV), which are more toxic than diorganotin, mono-organotin and inorganic tin derivatives. The cytotoxicity of organotin(IV) derivatives to animals, their metabolism or their bioconcentration has been the topic of several investigations.[1–16] Even at low concentrations (0.2–1.0 μg dm^{-3}) a number of effects of organotins have been detected, viz. high mortality of larvae,[17] decrease in body weight,[18] biochemical changes in the haemoglobin content of blood and hyperplasia of liver cells.[19]

Furthermore, organotin(IV) derivatives have been detected for example in aqueous ecosystems[20,21] and in marine plant and animal tissues.[22–24]

A new class of diorganotin derivatives has been synthesized by Donaldson *et al.*[25] and their activities on ascidian gametes before and after fertilization have been tested.[26,27]

Bu_2Sn-D-(−)sorbitol, Bu_2Sn-D-(+)glucose, Bu_2Sn-D-(−)fructose and Bu_2Sn-D-(+)glyceraldehyde at 10^{-4} mol dm^{-3} concentration prevent the fertilization and the cleavage of fertilized eggs.

Data on the effects of organotin compounds on embryonic development are scarce. Their possible influence needs, however, to be assessed in terms of egg development, larval motility, rates of

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growth and metamorphosis in laboratory cultures.

This paper reports results obtained by using these compounds on different stages of development up to the larva stage and on the metamorphosis of *Ciona intestinalis* and *Ascidia malaca*, and discusses the sensitivity of embryonic development to the different compounds and the reason for their toxicity at the cellular level. Development of ascidians proceeds as follows: the ascidians release gametes into the seawater, where development occurs. After fertilization the egg segments into two, four, eight cells, etc., up to gastrula, then to neurula, coiled larva, swimming larva and finally metamorphosed larva.

EXPERIMENTAL

The investigation has been carried out on different stages of embryonic development of *Ciona intestinalis* and *Ascidia malaca* from Palermo gulf and Termini harbor (Palermo).

Embryo batches were treated at the two-cell stage, gastrula, mid and late neurula, for one hour, with organometallic solutions in seawater and then maintained in seawater at 20 °C. Eggs of the same treated embryo batch were fertilized in seawater and allowed to develop up to the larva stage as controls. Fourteen larvae batches were incubated in the organometallic solutions in order to observe their movement and metamorphosis process. The experiments were reported whose controls developed up to larva in more than 90% of cases. All the observations were made by use of the light microscope.

Solutions (10^{-4} and 10^{-5} mol dm^{-3}) of the organometallic complexes were prepared in Millipore-filtered seawater giving high concentrations with respect to other toxicants occurring naturally because of the apparently lower toxicities of dibutyltin(IV) complexes compared with tributyltin(IV) derivatives. However, we observed[27] that, even at 10^{-9} and 10^{-11} mol dm^{-3} concentrations, the danger caused by dibutyltin(IV) was comparable with that of tributyltin(IV) derivatives when the exposure time was longer.

The pH ranged from 7.25 to 8.2 in all the solutions.

The diorganotin(IV) dichlorides were a kind gift from Schering (Bergkamen), while the carbohydrates and the polyalcohol (sorbitol) were Baker-analysed reagents (Deventer).

Bu_2Sn-D-(−)sorbitol (AG1), Bu_2Sn-D-(+)glucose (AG2), Bu_2Sn-D-(−)fructose (AG3) and Bu_2Sn-D-(+)glyceraldehyde (AG7) complexes were prepared according to literature reports.[25]

Carbohydrates, polyalcohol (sorbitol) and organometallic complex effects were previously tested.[26,27]

In order to elucidate the effects of the different compounds at different concentrations and different exposure times simultaneously on the two ascidian species, data from 14 experiments, for every stage, were treated by correspondence multivariate analysis.[28]

RESULTS

Two-cell stage

The results [Table 1 and Fig. 1(a)] show that only 10^{-4} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7) solution affects two-cell stage of embryo development. Incubated for one hour, they cleave more slowly than the controls, and 70% of them arrest at the neurula stage with open neural folds [Fig. 1(a), ①], while the remainder are larvae without pigment spots and with twisted tails [Fig. 1(a), ②]. In the other solutions the embryos develop up to the larval stage similarly to the controls [Fig. 1(a), ④]. In 10^{-4} and 10^{-5} mol dm^{-3} Bu_2Sn-D-(−)fructose (AG3) and 10^{-5} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7), development was delayed by three to four hours [Fig. 1(a), ③].

Early gastrula

The embryos were incubated with the toxicants, for one hour, when typical movements of gastrulation initiated. The results, summarized in Table 1 and Fig. 1(b), show that deleterious changes in the development occurs in 10^{-4} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7), Bu_2Sn-D-(−)-sorbitol (AG1) and Bu_2Sn-D-(+)glucose (AG2) solutions. This is due to the arrest of embryo development and production of anomalous gastrulae or neurulae with open neural folds [Fig. 1(b), ①, ②; Figs 2, 3]. A small percentage of *Ciona intestinalis* embryos (5% or 10%) reaching the larval stage had no adhesive organs, and twisted and short tails, in part covered by the ovular envelope and without movement (Fig. 3; Figs 4 and 5 are the controls).

Table 1 Development of ascidian embryos after incubation in solutions of organometallic complexes in sea water for a limited time (1 h) and after transferring to normal seawater[a]

Species	Compound	Concentration (mol/l)	Development stage				
			Two cells	Gastrula	Neurula	Late neurula	Larva
Ciona intestinalis	AG1	10^{-4}	Larvae (90)	Anomalous embryos(85) Twisted larvae(5)	Delayed larvae(90)	Delayed larvae(90)	Immobility
Ascidia malaca	AG1	10^{-4}	Larvae(90)	Anomalous embryos(85) Twisted larvae(5)	Delayed larvae(90)	Delayed larvae(90)	Immobility
Ciona intestinalis	AG1	10^{-5}	Larvae(90)	Delayed, twisted larvae(90)	Delayed larvae(90)	Larvae(90)	Immobility
Ascidia malaca	AG1	10^{-5}	Larvae(90)	Delayed, twisted larvae(90)	Delayed larvae(90)	Larvae(90)	Immobility
Ciona intestinalis	AG2	10^{-4}	Larvae(90)	Anomalous embryos(85) Twisted larvae(5)	Larvae(90)	Twisted larvae(60) Immobile larvae(10) Anomalous larvae(20)	Immobility
Ascidia malaca	AG2	10^{-4}	Larvae(90)	Anomalous embryos(60) Delayed larvae(30)	Larvae(90)	Twisted larvae(90)	Immobility
Ciona intestinalis	AG2	10^{-5}	Larvae(90)	Delayed larvae(90)	Larvae(90)	Larvae(90)	
Ascidia malaca	AG2	10^{-5}	Larvae(90)	Delayed larvae(90)	Larvae(90)	Larvae(90)	
Ciona intestinalis	AG3	10^{-4}	Delayed larvae(90)	Delayed, immobile twisted larvae(90)	Larvae(90)	Larvae(90)	Immobility
Ascidia malaca	AG3	10^{-4}	Delayed larvae(90)	Delayed, immobile, twisted larvae(90)	Larvae(80) Anomalous embryos(10)	Larvae(80) Twisted larvae(10)	Immobility
Ciona intestinalis	AG3	10^{-5}	Delayed larvae(90)	Delayed, twisted larvae(90)	Larvae(90)	Larvae(90)	
Ascidia malaca	AG3	10^{-5}	Delayed larvae(90)	Delayed, twisted larvae(90)	Larvae(90)	Larvae(80) Twisted larvae(10)	
Ciona intestinalis	AG7	10^{-4}	Anomalous neurulae(80) Twisted larvae(10)	Anomalous embryos(80) Twisted larvae(10)	Anomalous larvae(80) Immobile larvae(10)	Twisted larvae(60) Anomalous larvae(20) Immobile larvae(10)	Immobility
Ascidia malaca	AG7	10^{-4}	Anomalous neurulae(70) Twisted larvae(20)	Anomalous embryos(80) Twisted larvae(10)	Anomalous larvae(80) Immobile larvae(10)	Twisted larvae(90)	Immobility Immobility
Ciona intestinalis	AG7	10^{-5}	Delayed larvae(90)	Delayed, twisted larve(90)	Delayed larvae(90)	Delayed larvae(90)	Immobility
Ascidia malaca	AG7	10^{-5}	Delayed larvae(90)	Delayed, twisted larvae(90)	Delayed larvae(90)	Delayed larvae(90)	Immobility

[a] Data refer to 14 experiments and show the percentage of developed or arrested embryos in parentheses. Controls developed >90% of swimming larvae.

[b] AG1, Bu_2Sn-D-(−)sorbitol; AG2, Bu_2Sn-D-(+)glucose; AG3, Bu_2Sn-D-(−)fructose; AG7, Bu_2Sn-D-(+)glyceraldehyde.

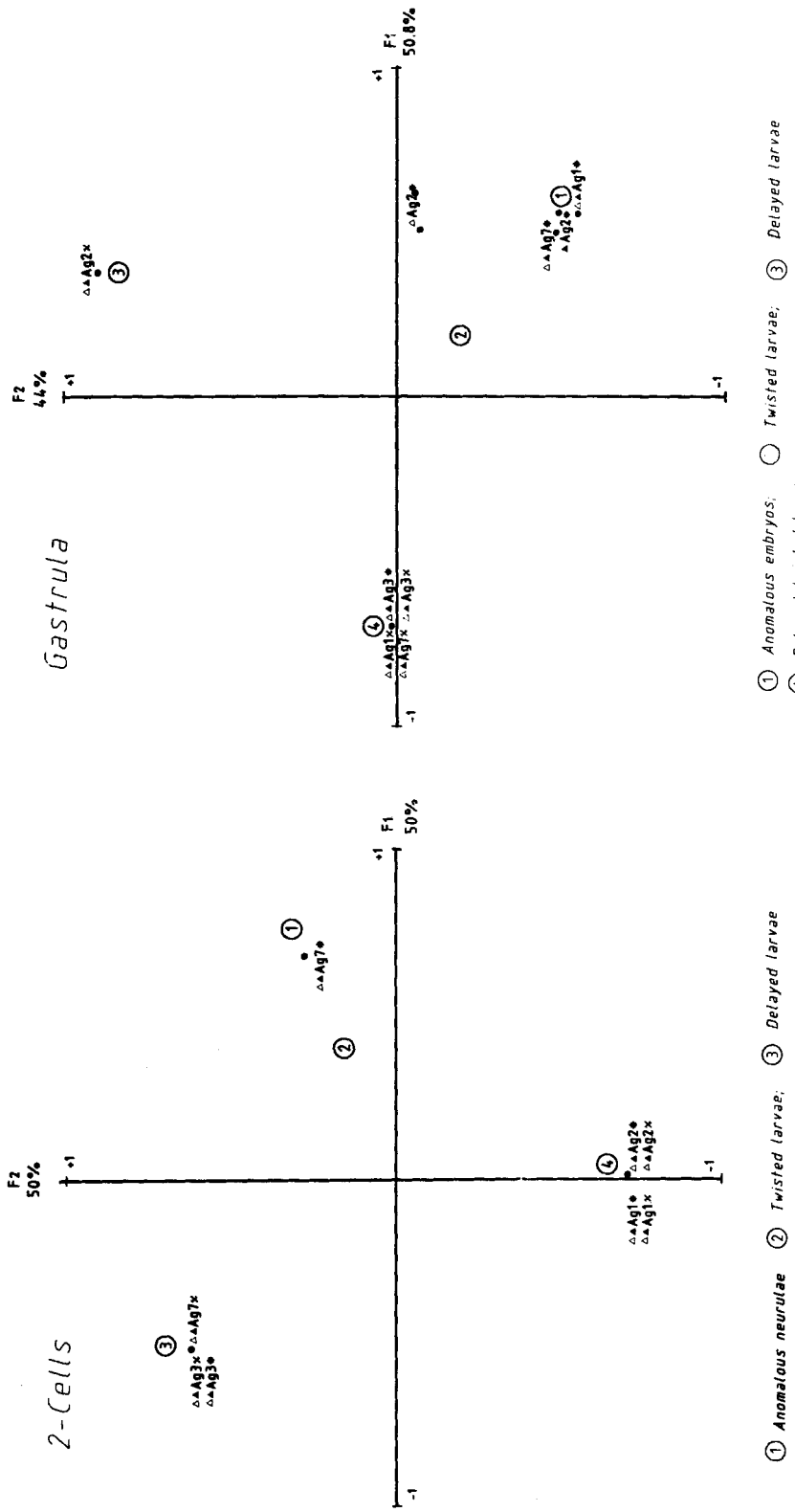

Figure 1a

Figure 1b

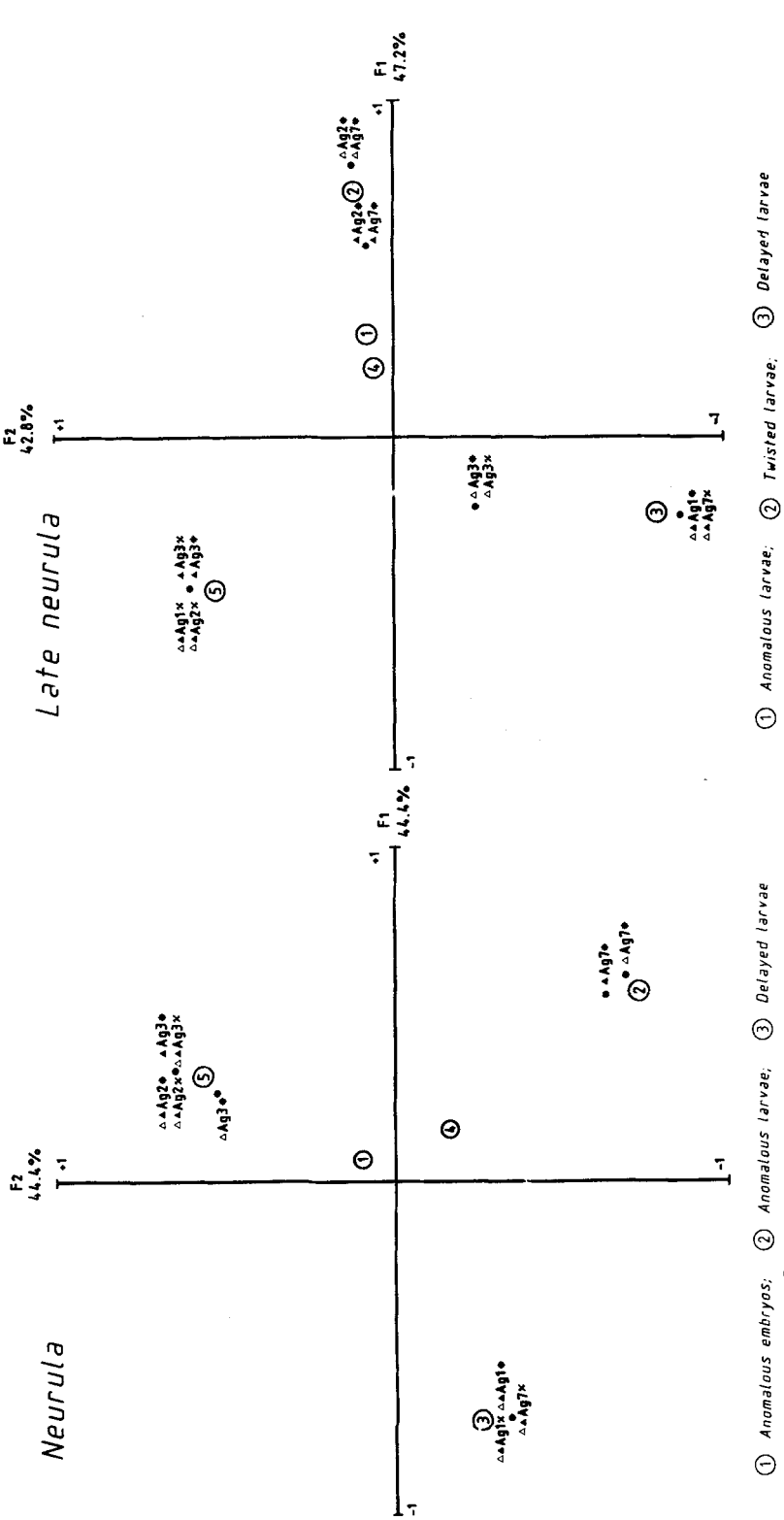

Figure 1c

Figure 1d

Figure 1 Results of embryo development at two-cell, gastrula, mid neurula and late neurula stages. Conditions: incubation in seawater of organometallic complex solutions during 1 h; subsequent transfer to normal seawater. ▲, *Ciona intestinalis*; △, *Ascidia malaca*; *, 10^{-4} mol dm^{-3}; ×, 10^{-5} mol dm^{-3}; Ag1, Ag2, Ag3, Ag4 are identical to AG1, AG2, AG3 and AG7, respectively which are defined in the footnote to Table 1.

Notes: As is evident from Fig. 1, a two-cell stage of two species is affected by 10^{-4} mol dm^{-3} AG7. In Fig. 1(b) gastrula are more sensitive to AG1, AG2 and AG7. In Fig. 1(c) only 10 mol dm^{-3} AG7 affects neurulae which develop up to anomalous larvae. In Fig. 1(d) late neuralae incubated in 10^{-4} mol dm^{-3} AG2 and AG7 originate larvae with twisted tails. % Values are variance of the analyses. F is the factorial axis.

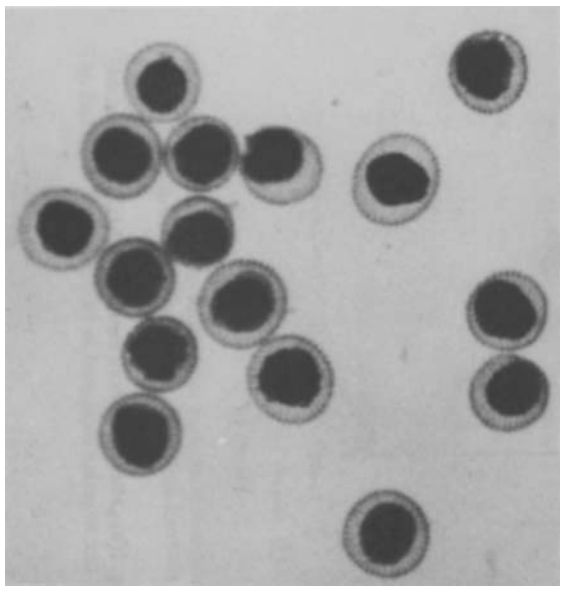

Figure 2 *Ascidia malaca* gastrulae incubated for 1 h in 10^{-4} mol dm^{-3} Bu_2Sn-D-(−)sorbitol (AG1) solution. The embryos are anomalous gastrulae and neurulae with open neural folds (magnification ×56).

Ascidia malaca embryos are more resistant to 10^{-4} mol dm^{-3} Bu_2Sn-D-(+)glucose toxicant (AG2), giving 60% anomalous embryos and 30% twisted larvae. The embryos incubated in 10^{-5} mol dm^{-3} Bu_2Sn-D-(−)sorbitol (AG1) [Figure 6(a) and (b)], 10^{-5} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7) and 10^{-4} and 10^{-5} mol dm^{-3} Bu_2Sn-D-(+)fructose (AG3) solutions [Fig. 1(b), ④] develop to twisted larvae, covered by ovular envelopes, while the controls are swimming larvae. Only the embryos incubated in 10^{-5} mol dm^{-3} Bu_2Sn-D-(+)glucose (AG2) develop to normal larvae but these show delayed hatching in comparison with the controls [Fig. 1(b), ③].

Mid neurula

The results (Table 1) show an improvement of development in comparison with the gastrula stage. After treatment with 10^{-4} and 10^{-5} mol dm^{-3} Bu_2Sn-D-(+)glucose (AG2) (Fig. 7) and Bu_2Sn-D-(−)fructose (AG3) (Fig. 8), *Ciona intestinalis* and *Ascidia malaca* neurulae develop similarly to the controls [Table 1; Fig. 1(c), ⑤]. Only the embryos treated with 10^{-4} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7) solution develop into anomalous larvae with twisted tails (80%) and immobile larvae (10%) [Figs 9, 10; Fig. 1(c), ②, ④)]. After treatment with 10^{-5} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7) and 10^{-4} and 10^{-5} mol dm^{-3} Bu_2Sn-D-(−)sorbitol (AG1) solutions, the larvae are delayed by three to four hours in comparison with the controls [Fig. 1(c), ③].

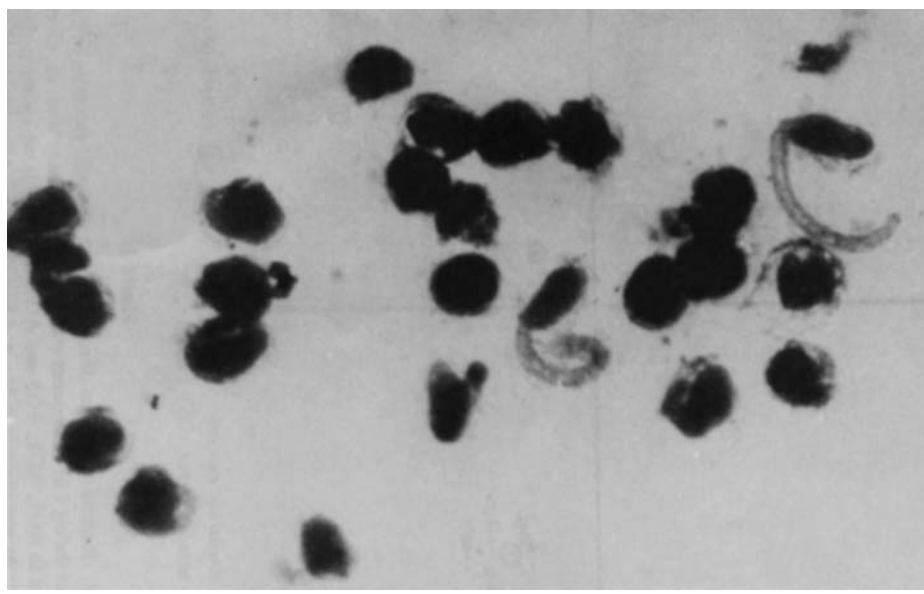

Figure 3 *Ciona intestinalis* gastrulae incubated for 1 n in 10^{-4} mol dm^{-3} Bu_2Sn-D-(−)sorbitol (AG1) solution. Anomalous early arrested embryos, larvae without adhesive organs with twisted tails, and anomalous larvae with short tails are present (magnification ×56).

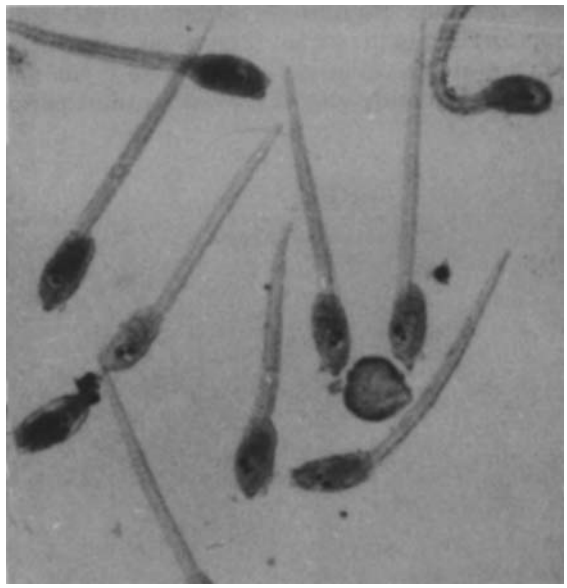

Figure 4 *Ascidia malaca* control larvae (magnification ×56).

Late neurula

The results are summarized in Table 1 and Fig. 1(d). *Ascidia malaca* and *Ciona intestinalis* late neurulae treated for one hour in 10^{-4} mol dm^{-3} Bu_2Sn-D-(+)glucose (AG2) and Bu_2Sn-D-(+)glyceraldehyde (AG7) solutions develop into larvae with twisted tails (60%) [Fig. 1(d), ②], anomalous larvae within ovular envelopes (20%) [Fig. 1(d), ①], and normal larvae without movement (10%) [Fig. 1(d), ④]. The two embryo species treated with 10^{-5} mol dm^{-3} Bu_2Sn-D-(+)sorbitol (AG1) and Bu_2Sn-D-(+)glucose (AG2) and *Ciona intestinalis* embryos incubated in 10^{-4} and 10^{-5} mol dm^{-3} Bu_2Sn-D-(−)fructose (AG3) solutions develop up to the larval stage in the same way as the controls [Fig. 1(d), ⑤]. The results of incubation of late neurulae of *Ciona intestinalis* and *Ascidia malaca* with 10^{-4} mol dm^{-3} Bu_2Sn-D-(−)sorbitol (AG1) and 10^{-5} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7) are reported in Fig. 1(d). A cluster ③ is shown which develops into larvae but is delayed about four hours in comparison with the controls. Late neurulae of *Ascidia malaca* incubated in 10^{-4} and 10^{-5} mol dm^{-3} Bu_2Sn-D-(−)fructose (AG3) solutions developed 80% larvae and 10% twisted larvae [Fig. 1(d), ⑤].

Swimming larva

During the experiments the control larvae actively moved in the seawater before they settled to metamorphose. When larvae were incubated in 10^{-4} mol dm^{-3} AG1 or AG2 solutions, they stopped moving and attached to the bottom of the syracuse dish and, after some hours, they underwent cytolysis.

The larvae incubated in 10^{-4} mol dm^{-3} Bu_2Sn-D-(−)fructose (AG3) solution at first showed circular and stressed movements and finally stopped moving.

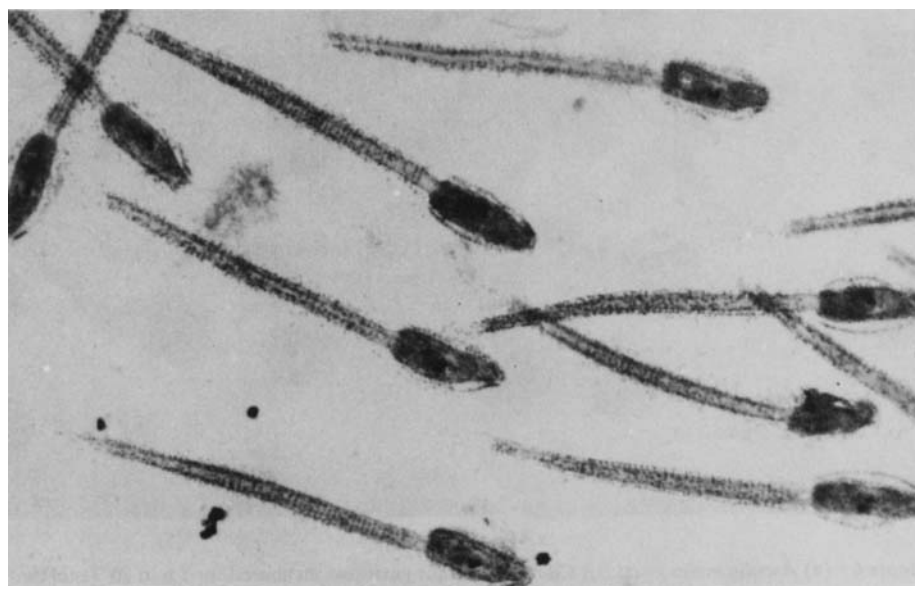

Figure 5 *Ciona intestinalis* control larvae (magnification ×56).

All the larvae treated with the toxicant solutions failed to metamorphose. [Fig. 12(a) and (b); Fig. 11 refers to *Ciona intestinalis* metamorphosed controls]. The larvae incubated in 10^{-4} mol dm^{-3} Bu_2Sn-D-(−)sorbitol (AG1) solution for 30 min presented some weak movements when transferred to normal seawater, but they did not metamorphose. Even with a short period

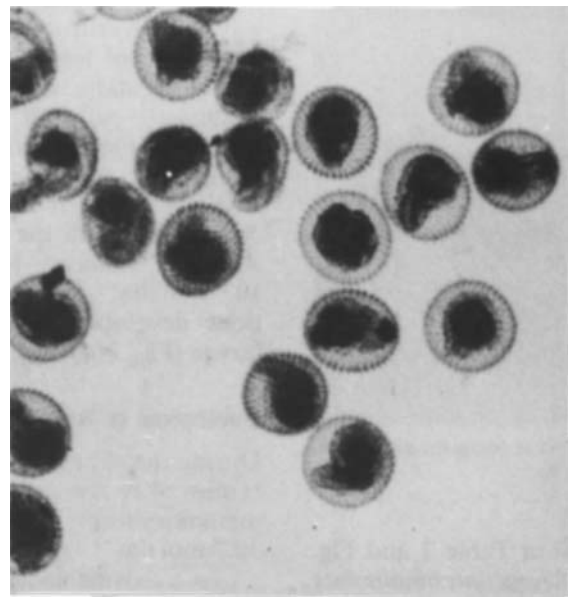

Figure 6a

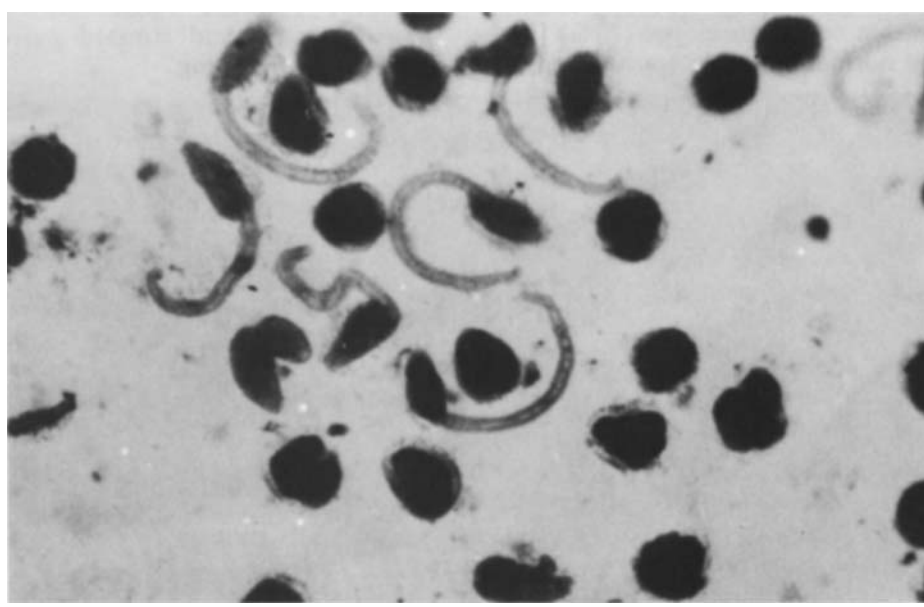

Figure 6b

Figure 6 (a) *Ascidia malaca* and (b) *Ciona intestinalis* gastrulae incubated for 1 h in 10^{-5} mol dm^{-3} Bu_2Sn-D-(−)sorbitol (AG1) solution. The embryos developed into twisted larvae in ovular envelopes and immobile larvae with twisted tails (magnification ×56).

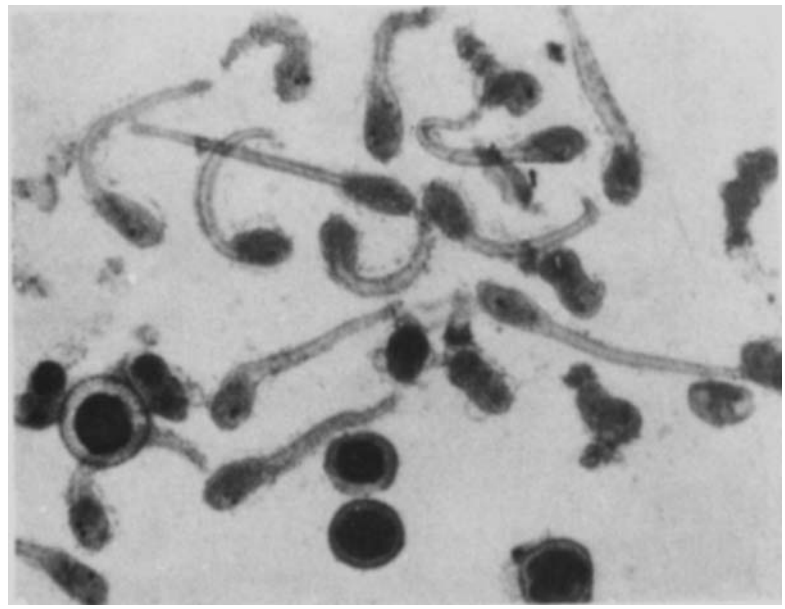

Figure 7 *Ascidia malaca* larvae from neurulae incubated for 1 h in 10^{-4} mol dm^{-3} Bu_2Sn-D-(−)glucose(AG2) solution (magnification ×56).

of incubation, the toxicant action is irreversible. The larvae incubated in 10^{-5} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7) and Bu_2Sn-D-(−)sorbitol (AG1) solutions showed no movement and did not metamorphose.

DISCUSSION

The work reported here shows that the organotin(IV) complexes affect some stages of ascidian development and that they are most sensitive at

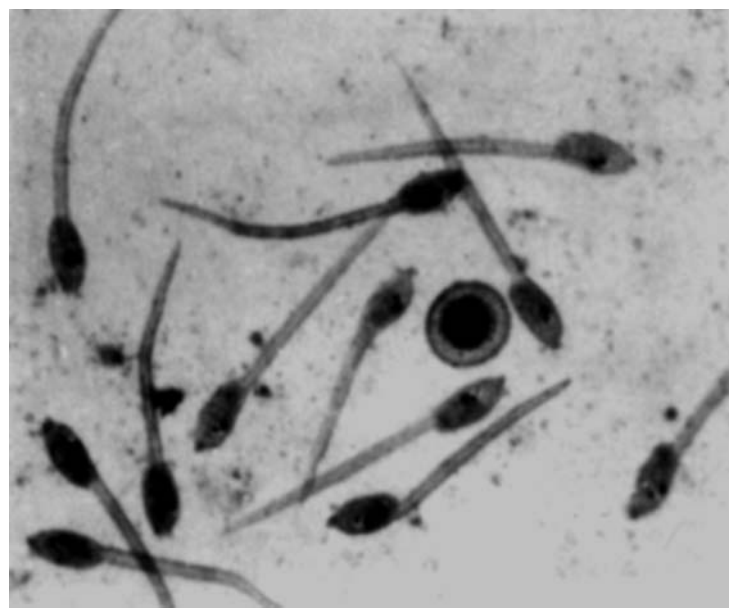

Figure 8 *Ascidia malaca* larvae from neurulae incubated for 1 h in 10^{-4} mol dm^{-3} Bu_2Sn-D-(−)fructose (AG3) solution (magnification ×56).

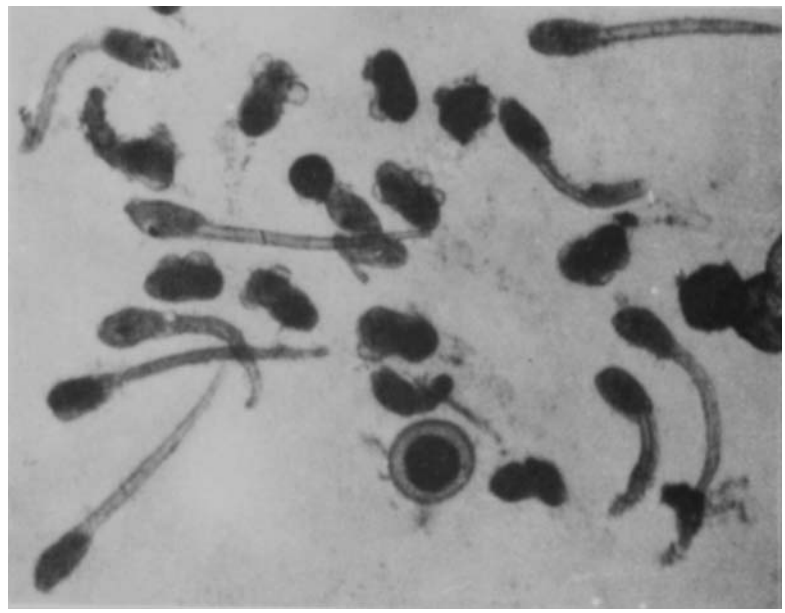

Figure 9 *Ascidia malaca* neurulae incubated for 1 h in 10^{-4} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7) solution. Most of the larvae are immobile and with twisted tails (magnification ×56).

the gastrula and larval stages. Development can be seen as follows:

Two cell→Early gastrula→Mid neurula
→Late neurula→Swimming larva.

In previous research it was observed that the toxicity of diorganotin(IV) complexes was related to the incubation time of the gametes and embryos; it has been shown that two-cell stage embryos stop developing at an anomalous 4–16

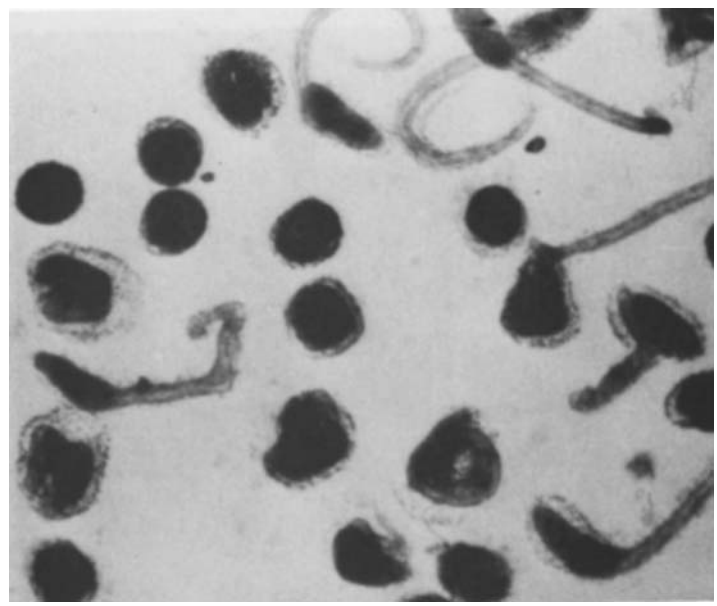

Figure 10 *Ciona intestinalis* neurulae incubated for 1 h in 10^{-4} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7) solution. The larvae are immobile and most have twisted tails (magnification ×56).

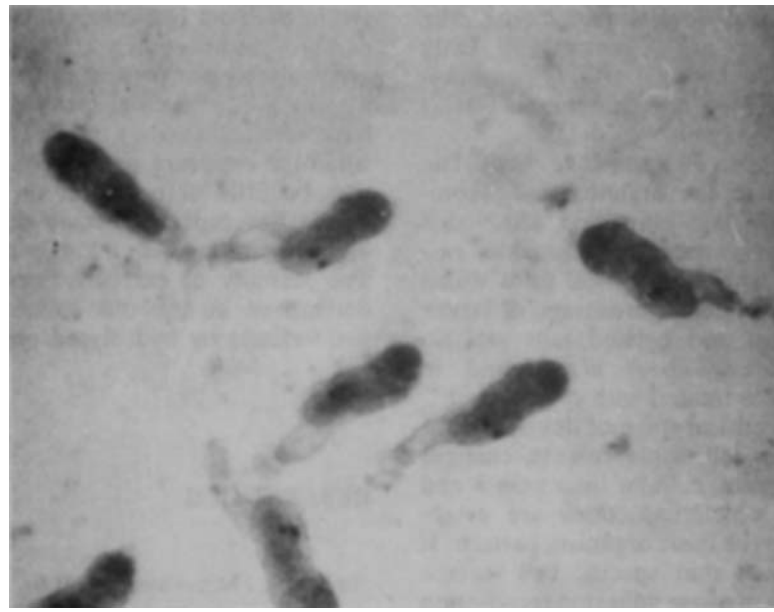

Figure 11 *Ciona intestinalis* metamorphosed control larvae (magnification ×56).

cell stage.[26,27] If the incubation of two-cell stage embryos with toxicants such as Bu_2Sn-D-(−)sorbitol (AG1), Bu_2Sn-D-(+)glucose (AG2) and Bu_2Sn-D-(−)fructose (AG3) is limited to one hour, and afterwards embryos are transferred to normal seawater, they develop into larva with a delay of one to two hours, except for Bu_2Sn-D-(+)glyceraldehyde (AG7).

A recovery is also observed when mid neurulae are incubated for one hour in Bu_2Sn-D-(−)sorbitol (AG1), Bu_2Sn-D-(+)glucose (AG2) and Bu_2Sn-D-(−)fructose (AG3) toxi-

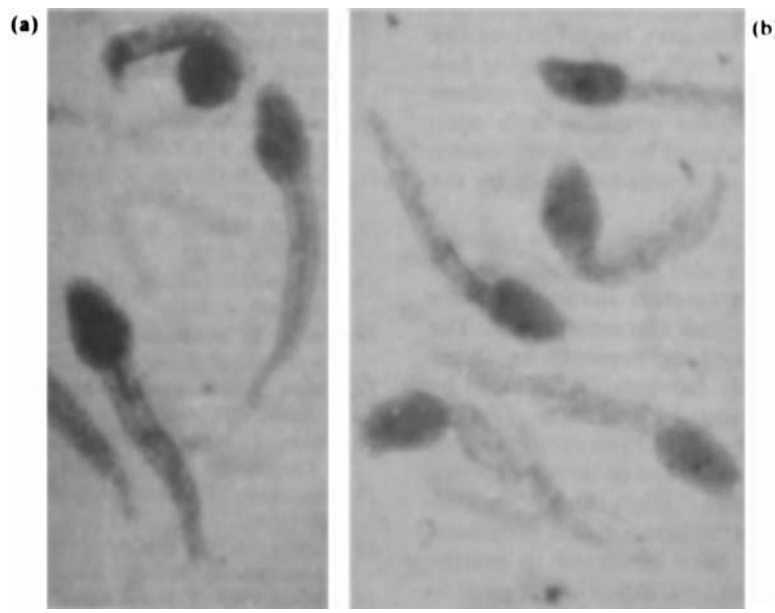

Figure 12 *Ciona intestinalis* larvae incubated in (a) 10^{-4} mol dm^{-3} Bu_2Sn-D-(+)glyceraldehyde (AG7) and (b) 10^{-4} mol dm^{-3} Bu_2Sn-D-(−)fructose (AG3) solutions do not metamorphose (magnification ×56).

cants; except for a small developmental delay, the larvae obtained are normal. Only Bu_2Sn-D-(+)glyceraldehyde (AG7) affects embryos, which develop into anomalous (80%) and immobile (10%) larvae.

The gastrula seems to be a sensitive stage. The embryos incubated in the organometallic complexes solutions for one hour and afterwards transfered to normal seawater are anomalous gastrulae or neurulae with open neural folds which stop developing. The small percentage of larvae obtained have short and twisted tails and no movement, as Pérez-Coll *et al.*[29] found in *Amphibians* gastrulae treated with cadmium.

The gastrula is a critical stage of development: the process involves cell displacements, changes in cellular adhesiveness, cellular interactions and recognition, after which inductions are established which lead to the basic organism pattern. It is generally accepted that specific cell surface molecules primarily mediate adhesion recognition events,[30,31] where the cytoskeleton could play a central role.[32]

At the present stage of the work, it is difficult to suggest the possible mechanism by which embryonic development is altered by the toxicants. We suggest that the organometallic complexes could cause the first perturbation on the surface molecules and/or on the cytoskeleton. On the other hand, the impairment of cytoskeletal function which blocks the mitosis may be inhibiting the polymerization of tubuline,[33,34] and reducing gastrula cell adhesion, giving rise to anomalous embryos. Furthermore, the neurulae with open neural folds could be caused by the involvement of the microfilaments of neural-fold cells by the toxicants.

Dramatic effects, due to exposure to organometallic complexes, are also observed in the larval stage. The two parameters investigated are the swimming activity and metamorphosis. The former is either blocked or presents an initial hyperactivity of circular movements followed by immobility in Bu_2Sn-D-(−)fructose (AG3). Moreover, the larvae begin tail resorption but do not metamorphose as observed in larvae exposed to copper[35] and three oils.[36] High rates of mortality, altered movement behaviour and structural irregularities have all been observed in marine invertebrate larvae sensitive to toxicants such as organotins,[17] heavy metals,[37–42] crude oil[36,43–45] or some detergents.[46,47]

However, the data presented all support the conclusion that some of the organotin derivatives synthesized act like other organotins on ascidian embryos, as heavy stressors at concentrations that are probably not present in the seawater; but, as we observed,[27] even at lower concentrations they have similar toxicity to tributyltin derivatives when the exposure time is enhanced. Thus there may be little or no effect on ascidian embryos whose development occurs during 24 h, unlike other organisms with a longer development time. The stability of carbohydrates and polyalcohol derivatives in aqueous solution would exclude toxic effects by hydrolysed organotin(IV) moieties.

REFERENCES

1. Barnes, J M and Stoner, H B *Br. J. Ind. Med.*, 1958, 15: 15
2. Elsea, I R and Paynter, O E *AMA Archs Ind. Health*, 1958, 18: 214
3. Stoner, H B *Br. J. Ind. Med.*, 1966, 23: 222
4. Knowles, C O *Envir. Hlth Perspect.*, 1976, 14: 93
5. Winec, C L, Marks, M J, Shanor, S P and Davis, E R *Clin. Toxicol.*, 1978, 13: 281
6. Pelikan, Z *Br. J. Ind. Med.*, 1969, 26: 165
7. Jonson, J H, Younger, R L, Witzel, D A and Radeleff, R D *Toxicol. Appl. Pharmac.*, 1975, 31: 66
8. Casida, J E, Kimmel, E C, Holm, B and Widmark, G *Acta Chem. Scand.*, 1971, 25: 1497
9. Freitag, K D and Bock, R *Z. Analyt. Chem.*, 1974, 270: 337
10. Brown, R A, Mazario, C M, Tirado, R S, Castrillon, J and Agard, E T *Envir. Res.*, 1977, 13: 56
11. Kimmel, E C, Fish, R H and Casida, J E *J. Agric. Fd Chem.*, 1977, 25: 1
12. Iwai, H, Manabe, M, Ono, T and Wada, O *J. Toxicol. Sci.*, 1979, 4: 285
13. Manabe, M, Iwai, H, Ono, T and Wada, O *J. Toxicol. Sci.*, 1979, 4: 284
14. Tsuda, T, Nakanishi, H, Aoki, S and Takebayashi, J *Carp. Toxicol. Envir. Chem.*, 1986, 12: 137
15. Tsuda, T, Nakanishi, H, Morita, T and Takebayashi, J *J. Assoc. Off. Analyt. Chem.*, 1986, 69: 981
16. Tsuda, T, Nakanishi, H, Aoki, S and Takebayashi, J *J. Chromatogr.*, 1987, 387: 361
17. Beaumont, A R and Budd, M D *Mar. Poll. Bull.*, 1984, 15(11): 402
18. Valkirs, A O, Davidson, B M and Seligman, P F *Chemosphere*, 1987, 16: 201
19. Chliamovitch, Y P and Kuhn, C *J. Fish. Biol.*, 1977, 10: 575
20. Vitturi, R, Mansueto, C, Pellerito, L, Girasolo, M A and Catalano, E *Appl. Organomet. Chem.*, 1992, 6: 525
21. Waldock, M J, Thain, J and Miller, D *Int. Council Exp.*

Sea, 1983, CM1983/E: 52
22. Tugrul, S, BalKas, T I and Goldberg, E D *Mar. Poll. Bull.*, 1983, 14(8): 297
23. Seidei, S L, Hodge, V F and Goldberg, E D *Thalassia Jugoslavia*, 1980, 16(2–4): 209
24. Ishu, T *Bull. Japan Soc. Sci. Fish.*, 1982, 48(11): 1609
25. Donaldson, J D, Grimes, S M, Pellerito, L, Girasolo, M A, Cambria, A and Famà, M *Polyhedron*, 1987, 6: 383
26. Mansueto, C, Pellerito, L and Girasolo, M A *Acta Embryol. Morphol. Exper.*, 1985, n.s., 6(3): 267
27. Mansueto, C, Pellerito, L and Girasolo, M A *Acta Embryol. Morphol. Exper.*, 1989, n.s., 10(3): 237
28. Benzecri, J P *L'Analyse des Données*, 2 vols, Dunod, Paris, 1973
29. Pérez-Coll, C S, Herkovits, J and Salibàn, A *Experientia*, 1986, 42: 1174
30. Monroy, A and Moscona, A A *Introductory Concepts in Developmental Biology*, University of Chicago Press, Chicago, 1979
31. Edelman, G M *Proc. Natl. Acad. Sci. USA*, 1984, 81: 1460
32. Chong, A S F, Parish, C R and Coombe, D R *Immun. Cell. Biol.*, 1987, 65(1): 85
33. Sager, P R, Doharty, R A and Holmsted, J B *Exp. Cell. Res.*, 1983, 146: 127
34. Faulstich, H, Stournaros, C, Doenges, K H and Zimmermann, H P *Fed. Eur. Biochem. Soc. Lett.*, 1984, 174: 128
35. Pryterch, H F, *Ecol. Monographs.*, 1934, 4: 49
36. Renzoni, A *Mar. Poll. Bull.*, 1975, 6(8): 125
37. Wisely, B and Blick, R A P *Aust. J. Mar. Freshwater Res.*, 1967, 18
38. Connor, P M *Mar. Pollut. Bull.*, 1972, 3: 190
39. Brereton, A, Lord, H and Webb, J S *Mar. Biol.*, 1973, 19: 96
40. Shealy, M H Jr. and Sandifer, P A *Mar. Biol.*, 1975, 33: 7
41. Calabrese, A, MacInnes, J R, Nelson, D A and Miller, J E *Mar. Biol.*, 1977, 41: 179
42. Weis, P and Weis, J S *Teratology*, 1977, 16: 317
43. Linden, O *Ann. Zool. Fennic.*, 1974, 11: 141
44. Linden, O *Ambio*, 1975, 4: 130
45. Wells, P C and Sprague, J B *J. Fish. Res. Bd Canada*, 1976, 33: 1604
46. Renzoni, A *Rev. Int. Oceanograph. Méd.*, 1971, 24: 50
47. Renzoni, A *Arch. Oceanogr. Limnol.*, 1974, 18: 99