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Arsenic Transformations in Short Marine Food Chains studied by HPLC–ICP MS

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The chemical forms of arsenic in some herbivorous or mainly herbivorous marine animals and, in some cases, the algae on which they feed were determined by HPLC–ICP MS. In most cases arsenobetaine was present in the animals as well as arsenosugars consumed directly from the algae. However in the case of copepods *Gladioferens imparipes* fed only on the diatom *Chaetoceros concavicornis* which had been grown in axenic culture, arsenobetaine was absent. Arsenobetaine was also absent from the muscle of the silver drummer *Kyphosus sydneyanus*, although trimethylarsine oxide was present. This is the first reported case of the absence of arsenobetaine in a marine teleost fish and may be related to its fermentative faculty for digesting the macroalgae that it consumes.

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INTRODUCTION

It is now well established[1–3] that marine organisms naturally contain higher concentrations of arsenic than their terrestrial counterparts, and that marine animals of almost all classifications contain the bulk of this arsenic in most cases as arsenobetaine (Fig. 1). Marine algae, on the other hand, while containing similar overall concentra-

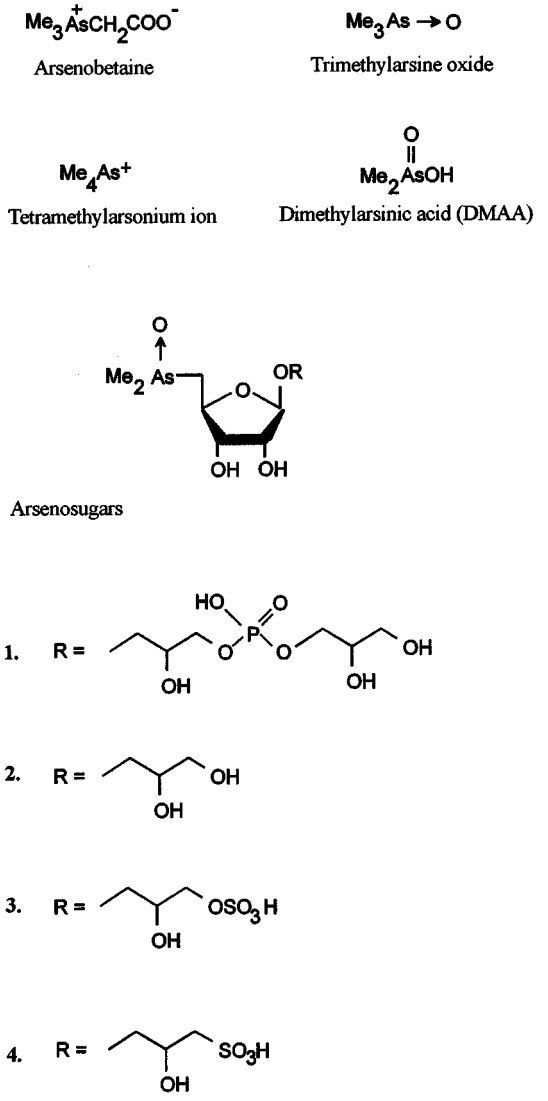

Figure 1 Arsenic compounds found in the various samples and referred to in the text.

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tions of arsenic to marine animals, do not contain arsenobetaine at all but in most instances contain their arsenic in the form of relatively complex carbohydrate derivatives[4] (arsenosugars; Fig. 1).

Where animals have been shown to contain arsenic in the form of arsenosugars, the source of the compounds has been assumed to be recently consumed algae.[4–6] We have proposed[7] schemes for the biogenesis of arsenobetaine that depend upon the degradation of algal arsenic compounds. Support for such schemes has come from experiments showing that arsenosugars can be microbially degraded to compounds that could easily be seen as precursors to arsenobetaine.[8] However, complete conversion of arsenosugars to arsenobetaine under natural conditions has not been shown to occur.[9]

A major aspect of the overall cycling of arsenic in the marine environment and of elucidating the origin of arsenobetaine (and indeed whether it is derived from arsenosugars or has a separate origin) concerns whether animals can directly convert arsenosugars into arsenobetaine. Of course, if they cannot, the compounds must be accumulated separately in those animals that contain both. Animals that eat algae obviously consume arsenosugars. Limited experimental evidence[10] indicated that the American lobster *Homarus americanus* was unable to convert algal arsenic compounds into arsenobetaine and this observation provided support for the hypothesis that a microbially mediated stage was necessary for the transformation to occur.[8] However, a number of observations (for example, the high concentrations of arsenobetaine in the pelagic puerulus larvae of the western rock lobster (K. A. Francesconi and J. S. Edmonds, unpublished results) and the demonstration of the presence of arsenobetaine in strictly herbivorous animals such as the abalone[5]) highlighted problems about the sites of the transformations and raised the possibility that arsenobetaine might be accumulated from ambient water by animals other than bivalve molluscs.[11, 12]

In the experiments reported here we have analysed the components of short food chains—generally where animals eat algae, either as their sole source of food or as a major component of their diet. In some cases both the animal and the plant material comprising the diet were analysed; in other cases only the animal was analysed. The experiments were designed to provide information on the ability of marine animals to convert arsenosugars to arsenobetaine, and whether absorption from water is the source of arsenobetaine in herbivorous animals.

MATERIALS AND METHODS

Samples

The following samples were examined.

Copepods *Gladioferens imparipes* fed on the diatom *Chaetoceros concavicornis* cultured under axenic conditions.
The amphipod *Allorchestes compressa*.
Antarctic krill *Euphausia superba*.
Foot muscle, digestive gland, fore-and hindgut contents and food (algae) of the abalone *Haliotis roeii*.
Muscle and gut contents of the teleost fish silver drummer *Kyphosus sydneyanus*.

The diatom *Chaetoceros concavicornis* was cultured under axenic conditions in 5 l flasks under three different conditions of arsenic exposure: (a) normal seawater (with arsenic mainly as arsenate at a concentration of about 2 μg/l[13, 14]), (b) normal seawater containing an additional 1 mg/l of arsenic as arsenate and (c) artificial seawater[15] constructed of chemicals with arsenic concentrations as low as were available. However, despite all efforts to reduce the arsenic concentration of this medium, it contained arsenic at approximately half the level of normal seawater. Presumably this was also in the form of arsenate. Algal cultures were maintained at 20°C (in a constant-temperature room), were aerated with oil-free, submicron-filtered air, and harvested towards the end of their log-growth phase (four days).

There was thus the potential for the three separate cultures of *Chaetoceros* (the only food of the copepods) to contain different concentrations of arsenic and also a different range or different proportions of arsenic compounds. Three separate cultures of copepods were raised, each supplied with *Chaetoceros* from each of the three water treatments. Three samples of *Chaetoceros* and three of *Gladioferens* were thus extracted and examined.

Algal cells were collected by filtration (Whatman GF/A glass microfibre) for analysis. Those cells raised in seawater with an elevated arsenic concentration were washed, when on the filter pad, with normal seawater to remove arsenate contained in the experimental seawater that still

surrounded the algal cells (i.e. to replace seawater containing elevated arsenic with normal seawater).

Robertson and Lucas showed[16] that the amphipod *Allorchestes compressa* preferred, in laboratory experiments, to feed on the brown kelp *Ecklonia radiata. Allorchestes* for extraction were harvested by agitating fronds of freshly collected *Ecklonia* (from a site adjacent to the laboratory, 20 km north of Fremantle, Western Australia) in buckets of seawater and subsequently collecting dislodged *Allorchestes* with forceps. Despite their preference[16] for *Ecklonia, Allorchestes* were more abundant on branched red algae, and we assumed that by actually harvesting individuals from *Ecklonia* we would ensure that it was overwhelmingly the major food source of the animals. *Ecklonia radiata* was previously shown[17, 18] to contain more than 80% of its arsenic burden as the arsenosugars **1, 2** and **4**.

A single freeze-dried individual male Antarctic krill *Euphausia superba* collected in March, 1993 was extracted (see HPLC–ICP MS examination of extracts, below). Although known to eat primarily phytoplankton, *Euphausia* also consumes zooplankton.[19]

A single individual of abalone *Haliotis roeii* and the algae on which it was obviously feeding were collected from a site adjacent to the laboratory. The animal was dissected to provide foot muscle, digestive gland and the contents of the fore- and hind-guts, as well as the algal food, for extraction and analysis. Previous examination[5] of a whole abalone had shown arsenobetaine and arsenosugars to be present.

The gut of an adult silver drummer *Kyphosus sydneyanus* (total length 495 mm), caught in a shark net off Rottnest Island, Western Australia, was carefully removed from the fish. The total length (1980 mm) of the gut tract was cut into 12 sections of approximately equal length and the contents of each section were extruded and stored frozen in separate containers. The first section of the gut, the stomach, was full of algal fragments (144 g) and consisted of pieces of *Ecklonia radiata* (about 90%) and red algae (about 10%). Much of the *Ecklonia* was in the form of circular pieces about 20 mm in diameter, presumably corresponding to the bite of the fish. This was consistent with the observations of Rimmer and Wiebe[20] that rather large fragments of algae enter the digestive process and are not further mechanically degraded. The algae in the stomach both looked and smelled fresh. The stomach contents and the contents of two further gut sections were examined; one taken from halfway along the tract (gut 2) and the other from near the anus (gut 3). Samples gut 2 and 3 consisted of brown pastes, with the consistency of gut 3 being rather more liquid than that of gut 2.

HPLC–ICP MS examination of extracts

Fresh samples (or freeze-dried material in the case of Antarctic krill), including those consisting of gut contents, were extracted with methanol and the methanol was removed from the extracts by evaporation. The residues were then dissolved/suspended in water and partitioned between water and diethyl ether. Analyses for total arsenic showed the bulk of the arsenic (>95%) in all cases to be in the aqueous layer, and the ether layers were discarded. The aqueous layers were evaporated to dryness and stored at −20°C prior to analysis. For analysis by HPLC–ICP MS, material was redissolved in water and filtered (Sartorius Minisart 165 55K, pore size 0.45μm) and the volume adjusted to give a total arsenic concentration of between 100 ng and 1 μg/ml.

The HPLC–ICP MS technique and conditions used to examine samples in this study were as previously reported[21, 22] and involved the analysis of samples under three separate chromatographic conditions [gel permeation (GS 220 or GS 220 HQ column) at pH 6.8, and ion-pair (ODS reversed-phase column) chromatography at pH 3.0 and 6.8]. Silver drummer and abalone extracts were analysed by two chromatographic procedures (GS 220 HQ at pH 6.8 and ODS reversed-phase at pH 6.8).

Standards used were also as previously described[21–23] and were either derived from natural sources and identified principally by NMR spectroscopy or were obtained synthetically. If a compound was detected with retention times that did not correspond to those of available standard materials it was recorded in Table 1 as an unknown compound.

RESULTS AND DISCUSSION

Arsenic compounds detected in all samples are shown in Table 1.

Antarctic krill contained a substantial proportion of its arsenic as arsenobetaine, with the bulk of the balance as arsenosugars, suggesting that phytoplankton are a major part of its food. However the observation[19] that zooplankton contribute to the diet of krill means that it cannot be assumed that the arsenobetaine it contains has its direct origin in seawater or that krill possess the facility to convert arsenosugars to arsenobetaine.

Despite its very different location of collection and reported feeding habit, the pattern of arsenic compounds in the amphipod *Allorchestes compressa* was very similar to that for Antarctic krill; even the same arsenosugars were accumulated to approximately the same proportions. In the case of *Allorchestes*, though, the food would appear to be both more restricted and better understood and to consist, in the area from which the animals in this experiment were taken, of fragments of *Ecklonia radiata*.[16] *Ecklonia* has been shown to contain more than 80% of its arsenic (present at about 10 mg/kg wet weight) as arsenosugars **1**, **2** and **4** (Fig. 1), and arsenobetaine was absent.[17, 18] The presence of arsenobetaine in *Allorchestes* is thus not likely to result from the consumption of animal material and must either be synthesized from the algal arsenic compounds or have its origin in the ambient seawater. However, Robertson and Lucas reported[16] that in laboratory

Table 1 Arsenic compounds detected in the various samples and the approximate percentage that each compound contributed to the total arsenic in each organism

Sample	Arsenic compounds detected[a]
Chaetoceros in normal seawater	Arsenosugar **1** (2%), **3** (90%), unknown compounds (5%)
Chaetoceros with elevated arsenic	Arsenosugar **3** (>99%)
Chaetoceros in artificial seawater (reduced arsenic)	Arsenosugar **1** (2%), **3** (60%), unknown compounds (30%)
Copepods fed *Chaetoceros* from normal seawater	Arsenosugar **3** (70%), trimethylarsine oxide (10%), unknown compounds (>15%)
Copepods fed *Chaetoceros* with elevated arsenic	As(V) (40%), trimethylarsine oxide (25%), arsenosugar **3** (20%), unknown compounds(>10%)
Copepods fed *Chaetoceros* from artificial seawater	Trimethylarsine oxide (70%), arsenosugar **3** (20%), unknown compounds (>5%)
Amphipods	Arsenobetaine (60%), arsenosugar **1** (5%), **2** (7%), **3** (5%), **4** (1%)
Antarctic krill	Arsenobetaine (60%), arsenosugar **1** (5%), **2** (1%), **4** (1%), DMAA[b] (20%)
Silver drummer muscle	Trimethylarsine oxide (>95%), tetramethylarsonium ion (3%), arsenosugars **1** and **3** (1%)
Silver drummer gut 1 (stomach)	Trimethylarsine oxide (65%), arsenosugar **1** (5%), **2** (2%), **3** (<1%), **4** (10%)
Silver drummer gut 2	Trimethylarsine oxide (50%), arsenosugar **1** (10%), **2** (15%), **3** (<1%), **4** (15%)
Silver drummer gut 3	Trimethylarsine oxide (90%), arsenosugar **1** (2%), **2** (2%), **4** (2%)
Abalone foot muscle	Arsenobetaine (90%), tetramethylarsonium ion (1%), arsenosugar **1** (3%), **2** (2%), **3** (1%)
Abalone digestive gland	Arsenobetaine (40%), tetramethylarsonium ion (1%), arsenosugar **1** (10%), **2** (35%), **3** (<1%), **4** (10%)
Abalone fore-gut contents	Unknown compounds (60%), arsenosugar **1** (5%), **2** (30%), **4** (2%)
Abalone hind-gut contents (faeces)	Unknown compounds (35%), arsenosugar **1** (5%), **2** (50%), **4** (5%)
Abalone food (algae)	Unknown compounds (30%), arsenosugar **1** (25%), **2**(10%), **3** (5%), **4** (25%)

[a] Approx. percentages in parentheses.
[b] DMAA, dimethylarsinic acid (Fig. 1).

experiments the main food preference of *Allorchestes* was for decomposing *Ecklonia* and so the possible intervention of microbes in the decomposition of algal arsenosugars and the accumulation of arsenobetaine in the amphipods cannot be ruled out. Also unexpected was the observation that *Allorchestes* contained more of arsenosugar **3** than of **4**. Arsenosugar **3** was not detected in *Ecklonia*[17, 18] but was relatively abundant in *Sargassum lacerifolium*.[24] *Sargassum lacerifolium* is also found in the coastal waters and on the limestone reefs where silver drummer are found, but contributes much less of the biomass in shallow water (H. Kirkman, personal communication). The discovery of an arsenic compound in *Allorchestes* which was likely to have come from *Sargassum* despite the animals having been taken from fronds of *Ecklonia* suggests the possible value of the arsenic compounds as food-chain tracers.

The idea that arsenobetaine was not necessarily obtained by the consumption of animal material was reinforced by analysis of tissues of the abalone *Haliotis roeii*. The food (algae) and fore-gut and hind-gut contents (faeces) of *Haliotis* did not contain arsenobetaine, but the foot muscle and the digestive gland both contained substantial amounts of arsenobetaine. The foot muscle had more than 95% of its arsenic (about 1 mg/kg) as arsenobetaine and the digestive gland about 35%. Material comprising the digestive gland consisted of both the contents (food material passing through the gut) and the substance of the gland itself. Again, then, we are left with the possibilities that either *Haliotis* is converting arsenosugars to arsenobetaine or that water is its source. The observation that the foot muscle contained the bulk of its arsenic as arsenobetaine with very small quantities of arsenosugars present might also indicate, if the assumption is made that the animal is exposed to much greater quantities of the latter than the former (whatever its source), that arsenobetaine is retained with high selectivity.

The only system in this study in which the food (the diatom *Chaetoceros concavicornis*) of the animal (the copepod *Gladioferens imparipes*) was completely controlled by its being cultured under defined axenic conditions produced no arsenobetaine. No arsenobetaine was accumulated in the copepods. If arsenobetaine had been accumulated in *Gladioferens* at concentrations proportional to their level of exposure to the arsenic-containing carbohydrates contained in their algal food supply, this would have provided good evidence that animals have the ability to convert arsenosugars to arsenobetaine. No such evidence was obtained.

Chaetoceros accumulated the arsenosugar **3** to a concentration dependent on its degree of exposure to inorganic arsenate in its ambient seawater; this might indicate that the conversion is a detoxification process.[4] A high concentration of arsenate was detected in extracts of copepods fed with algal cells that had been grown in water containing elevated (1 mg/kg) arsenate. Probably, arsenate was transferred to the cultures of copepods within the water containing their food, was adsorbed to external surfaces and remained adhering to the animals. Presumably it was not removed when they were harvested.

Silver drummer muscle contained arsenic at about 1 mg/kg (quite normal for a teleost fish[3]) but, alone of all fish, both teleost and elasmobranch, that have been examined to date, this arsenic was not present as arsenobetaine. More than 85% of the arsenic burden of the silver drummer consisted of trimethylarsine oxide, with the balance being arsenosugars. Not only is the silver drummer unique among reported fin-fish in containing no arsenobetaine, but to our knowledge there has been only one published report of undetectable arsenobetaine concentrations in an animal when other arsenic compounds were evident. Arsenosugars but not arsenobetaine were found in the bivalve mollusc *Corbicula japonica* taken from estuarine waters.[6]

The presence of trimethylarsine oxide as the main arsenic compound in the silver drummer was also unexpected because this compound is no more obviously derived from its algal diet than is arsenobetaine. However, the most difficult observation to explain was the high proportion of trimethylarsine oxide in the stomach of the fish. An extract of the stomach contents contained 65% of its arsenic as trimethylarsine oxide, yet an equivalent amount of uneaten algae would have yielded only arsenosugars and no trimethylarsine oxide. The algal material in the stomach looked and smelled fresh and there was no evidence of the extent of microbial activity that might be invoked to explain such a drastic transformation. The presence of so much trimethylarsine oxide in the gut tract (it was also found in the other two gut sections examined) of the silver drummer must be considered unexplained at this time. Obviously further study is necessary.

Trimethylarsine oxide has previously been found in fish. Norin *et al*. reported[25] it in fish from the Baltic Sea and considered there to be higher levels in material that had been stored frozen longest and that it was therefore derived from breakdown of arsenobetaine, the major form of arsenic in all fish that were examined. Edmonds and Francesconi found trimethylarsine oxide to be a minor (relative to arsenobetaine) natural component of the estuary catfish *Cnidoglanis macrocephalus* but that the proportion of trimethylarsine oxide could be increased by feeding the fish arsenate as part of their diet.[26] Trimethylarsine oxide was also accumulated in the school whiting *Sillago bassensis* fed with arsenate, although for this species only arsenobetaine was found in fish that did not have their diet supplemented with arsenate. It was assumed that bacterial activity in the gut tract of the fish was converting the inorganic arsenic into trimethylarsine oxide which was then absorbed and retained in the fish muscle. The degree of retention of trimethylarsine oxide relative to administered arsenate was low (about 1%).[26]

If arsenobetaine, or a precursor, was being absorbed from ambient water, there is no obvious reason why the copepods or the silver drummer should not contain at least some of their arsenic load in such a form. Does this then suggest that marine animals have the ability to convert arsenosugars to arsenobetaine but that such a facility is lacking in these two very different species? In the case of the silver drummer this might be the case. Although alone of all teleost (and elasmobranch) fish examined so far, the silver drummer does not contain arsenobetaine, its unusual fermentative method of digesting its macroalgal diet[20] might account for this absence in some way. It is far from obvious, though, why arsenobetaine should be absent from the copepod *Gladioferens* but present (as about 60% of the total arsenic burden) in the amphipod *Allorchestes*. Although the *Chaetoceros* on which the copepods were fed was cultured under axenic conditions, the algae were given to the copepods growing in normal seawater and it is difficult to see what relevance this might have to the transformation of arsenosugars in the algal cells. Possibly, as suggested above, microorganisms associated with the macroalgal fragments eaten by the amphipods are in some way involved with the production of arsenobetaine.[16]

In both cases where arsenobetaine was absent it seems to have been replaced by trimethylarsine oxide. Trimethylarsine oxide has been reported[27] as a product of the microbial breakdown of arsenobetaine under a variety of conditions. In the case of the silver drummer such a breakdown of any arsenobetaine in the gut tract might be possible when the high level of apparently unusual microbial activity is considered (although it was far from obvious in the stomach of the individual examined here). Such an explanation would be unlikely to apply to the case of the copepods, however. Possibly trimethylarsine oxide is a precursor of arsenobetaine and for these two species the biosynthetic process has been arrested at the earlier stage. This would of course require the transfer of acetate to arsenic and a mechanism for this conversion is not currently apparent. Neither of these explanations (breakdown of arsenobetaine or failure to complete its biosynthesis) seems to apply to both copepods and silver drummer and clearly more work is necessary for a complete understanding of these unusual results and to gain some insight into how they might aid our understanding of overall arsenic transformations in the sea. Perhaps a development of the studies involving radiolabelled compounds carried out by Cullen *et al*.,[28, 29] and involving species other than the mussels used by those workers, would be helpful.

REFERENCES

1. W. R. Cullen and K. J. Reimer, *Chem. Rev.* **89**, 713 (1989).
2. Y. Shibata, M. Morita and K. Fuwa, *Adv. Biophys.* **28**, 31 (1992).
3. K. A. Francesconi and J. S. Edmonds, *Oceanog. Mar. Biol. Ann. Rev.* **31**, 111 (1993).
4. J. S. Edmonds, K. A. Francesconi and R. V. Stick, *Nat. Prod. Rep.* **10**, 421 (1993).
5. M. Morita and Y. Shibata, *Anal. Sci.* **3**, 575 (1987).
6. Y. Shibata and M. Morita, *Appl. Organomet. Chem.* **6**, 343 (1992).
7. J. S. Edmonds and K. A. Francesconi, *Experientia* **43**, 553 (1987).
8. J. S. Edmonds, K. A. Francesconi and J. A. Hansen, *Experientia* **38**, 643 (1982).
9. J. S. Edmonds and K. A. Francesconi, *Appl. Organomet. Chem.* **2**, 297 (1988).
10. R. V. Cooney and A. A. Benson, *Chemosphere* **9**, 335 (1980).
11. K. A. Francesconi and J. S. Edmonds, Accumulation of arsenobetaine from seawater by the mussel (*Mytilus*

edulis). In: *Heavy Metals in the Environment*, Vol. II, Lindberg, S. E. and Hutchinson, T. C. (eds), CEP Consultants, Edinburgh, 1987, pp. 71–73.
12. J. Gailer, K. A. Francesconi, J. S. Edmonds and K. J. Irgolic, *Appl. Organomet. Chem.* **9**, 341 (1995).
13. F. A. J. Armstrong and H. W. Harvey, *J. Mar. Biol. Assoc. UK* **29**, 145 (1950).
14. M. O. Andreae, *Limnol. Oceanogr.* **24**, 440 (1979).
15. J. R. Stein (ed.), *Handbook of Phycological Methods, Culture Methods and Growth Measurements*, Cambridge University Press, Cambridge, 1973.
16. A. I. Robertson and J. S. Lucas, *J. Exp. Mar. Biol. Ecol.* **72**, 99 (1983).
17. J. S. Edmonds and K. A. Francesconi, *Nature (London)* **289**, 602 (1981).
18. J. S. Edmonds and K. A. Francesconi, *J. Chem. Soc., Perkin Trans. 1*, 2375 (1983).
19. Y. Nishino and A. Kawamura, *Bull. Fac. Bioresources Mie Univ.* **12**, 249 (1994).
20. D. W. Rimmer and W. J. Wiebe, *J. Fish. Biol.* **31**, 229 (1987).
21. Y. Shibata and M. Morita, *Anal. Chem.* **61**, 2116 (1989).
22. Y. Shibata and M. Morita, *Anal. Sci.* **5**, 107 (1989).
23. J. S. Edmonds, Y. Shibata, K. A. Francesconi, J. Yoshinaga and M. Morita, *Sci. Total Env.* **122**, 321 (1992).
24. K. A. Francesconi, J. S. Edmonds, R. V. Stick, B. W. Skelton and A. H. White, *J. Chem. Soc., Perkin Trans. 1*, 2702 (1991).
25. H. Norin, A. Christakopoulos, M. Sandström and R. Ryhage, *Chemosphere* **14**, 313 (1985).
26. J. S. Edmonds and K. A. Francesconi, *Sci. Total Env.* **64**, 317 (1987).
27. K. Hanaoka, S. Tagawa and T. Kaise, *Appl. Organomet. Chem.* **6**, 139 (1992).
28. W. R. Cullen and J. C. Nelson, *Appl. Organomet. Chem.* **7**, 319 (1993).
29. W. R. Cullen and S. A. Pergantis, *Appl. Organomet. Chem.* **7**, 329 (1993).

