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Biotransformation of [^{3}H]methylarsonic acid in a static seawater system containing *Mytilus californianus*

William R Cullen* and Spiros A Pergantis
Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, B.C., Canada V6T 1Z1

Mytius californianus **exposed for 9 days to a seawater system containing [^{3}H]methylarsonic acid, was found to contain [^{3}H]methylarsonic acid along with [^{3}H]arsenobetaine and two unknown ^{3}H-labeled compounds in the tissue parts of the mussel. A linear increase with time in the specific activity present in the flesh of *Mytilus californianus* was also observed. The highest specific activity was found in the visceral mass and the gills of the mussel.**

Keywords: Organoarsenic, methylation, marine environment, algae, mussel, bioaccumulation, ^{3}H label, methylarsonic acid, arsenobetaine

INTRODUCTION

A great number of organoarsenical compounds found in the marine environment have been isolated and identified. Arsenobetaine (AsB) is present in almost all marine animals so far examined.[1,2] Arsenocholine (AsC),[3] the tetramethylarsonium ion ($TMAs^+$)[4,5] and trimethylarsine oxide[6] have also been identified. Marine algae have been found to contain arsenosugars[7] and recently an arsenic-containing nucleoside has been isolated from the Giant clam *Tridacna maxima*.[8]

A number of accumulation and biotransformation experiments have provided results that have contributed to our limited knowledge of arsenic cycling in the marine environment. AsC has been found to be converted into AsB, glycerylphosphorylarsenocholine and phosphatidylarsenocholine in yellow-eye mullet following oral administration.[9] Accumulation experiments with mussels (*Mytilus edulis*) suggested that AsB is readily taken up from seawater by these animals.[10] In other radiotracer experiments the effects of certain environmental (concentration, temperature, salinity) and biological (tissue parts) variables on arsenate accumulation and elimination processes in the mussel *Mytilus galloprovincialis* have been studied.[11] Similar studies have supported the observation that arsenic is not biomagnified up the food chain.[12]

In order to further understand the biotransformation of arsenic in the marine environment, an experiment was designed to study the accumulation and transformation of ^{3}H-labeled methylarsonic acid ([^{3}H]MMAA) by *Mytilus californianus* (Californian mussel). In a similar experiment,[13] water-soluble ^{3}H-labeled arsenic compounds were phenol-extracted from mussels (*Mytilus edulis*) and seawater after exposure to [^{3}H]MMAA and [^{3}H]dimethylarsinate. [^{3}H]AsB was found in both the mussels and the seawater. The results of this experiment indicate that AsB is biosynthesized by microscopic organisms, probably primary producers, in the seawater and that it is bioaccumulated quite rapidly by mussels.

EXPERIMENTAL

Instrumentation

A Packard Tri-carb® 1900 TR liquid counter was used to measure the activity of ^{3}H-labeled compounds in all experiments.

The HPLC system consisted of a Waters M45 pump, a Waters U6K injector, the appropriate column and an automated fraction collector. Separations were achieved by using two different columns, a Protein Pak DEAE 5PW column [7.5 mm (i.d.) × 7.5 cm; Waters] and a Bondclone 10 C18 column [3.9 mm (i.d.) × 30 cm; Phenomenex].

A Varian Techtron Model AA 1275 atomic

* To whom correspondence should be addressed.

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absorption spectrometer, equipped with a Varian GTA-95 accessory, was used to determine arsenic by using graphite furnace atomic absorption spectrometry (GF AA). For these determinations a chemical modifier consisting of 200 ppm palladium in 2% citric acid was used.

A rotary evaporator was used for evaporation of solvents.

Chemicals

AsB,[14] AsC,[15] $TMAs^+$,[16] and [^{3}H]MMAA[17] were synthesized by literature methods; all other chemicals were commercially available.

Procedures for the speciation of ^{3}H-labeled compounds extracted from *Mytilus californianus*

Mussels (*Mytilus californianus*) collected from Quatsino Sound, B.C., were stored in holding tanks at the University of British Columbia/Federal Department of Fisheries and Oceans, West Vancouver Laboratory, for four months prior to the experiment. These facilities are capable of providing a continuous flow of seawater which is subsequently aerated in the holding tank. For the experiment 18 mussels were selected randomly and were placed in a static but aerated tank containing 15 liters of seawater and 34 μCi [^{3}H]MMAA (1.5 ppm As). The mussel shells varied in length from 7 to 16 cm.

After nine days, nine mussels were removed, providing a total of 200 g of wet tissue. The tissue was homogenized and 500 ml of methanol was added; the mixture was then placed on a shaker for two days. This extraction step was repeated with another 500 ml of methanol. The extracts were combined and evaporated to dryness. The residue was dissolved in water and extracted with portions of diethyl ether until the ether portion was colorless. The water fraction was evaporated down to a volume of 25 ml, applied to a gel-permeation column (Sephadex LH/20, 2.5 cm × 30 cm), and then eluted with water. Fractions of 9.5 ml were collected, and the ^{3}H activity in each was determined by withdrawing a 500 μl aliquot, which was mixed with 5 ml of scintillation liquid in a counting vial before being transferred to a scintillation counter where disintegrations per minute (dpm) were measured. The ^{3}H-containing fractions were combined and evaporated down to a volume of 10 ml and then applied to a strong cation-exchange column (Dowex 50W × 8 (H^+), 200–400 mesh, 2.5 cm × 30 cm column). The following mobile phases were used to elute the ^{3}H-containing compounds: 200 ml water, 200 ml 5% ammonium hydroxide, 40 ml water and 150 ml 2M HCl. Fractions of 9.5 ml were collected and analyzed for ^{3}H activity. The ^{3}H-containing fractions were bulked into four main fractions, each of which corresponded to a peak eluting from the Dowex 50W × 8 (H^+) column. These fractions were then evaporated to dryness and redissolved in a minimum volume of water.

Each of these four solutions was chromatographed on an anion-exchange Protein Pak DEAE column. Two mobile phases were used; the first consisted of 5 mM sodium acetate adjusted to pH 4 with acetic acid and the second consisted of 5 mM ammonium acetate, pH 6.8. A Bondclone 10 C18 reversed-phase column was also used for ion-pair reversed-phase liquid chromatography, with water–methanol 95:5 as the mobile phase and 5 mM tetrabutylammonium nitrate as the ion pair. The flow rate in all cases was 1 ml min^{-1}. In order to establish retention times for identification purposes a number of standard arsenicals [AsB, methylarsonic acid (MMAA), dimethylarsinic acid (DMAA), and glycerylarsenocholine (GPAC)] were also chromatographed. The standards were detected by using GF AA, with 15-μl aliquots from 0.5-ml fractions. The sample extracts were monitored by counting aliquots of 1 ml that were mixed with 5 ml of scintillation liquid.

Determination of total ^{3}H in mussel tissue

On the third, sixth and ninth days, four mussels (*M. californianus*) were removed from the tank and dissected into six parts: gills, adductor muscle, foot, mantle, muscle tissue and visceral mass. The same parts from each of the four mussels were bulked together. The byssal threads and the shells were also set aside for total ^{3}H determinations. All of the tissue parts were freeze-dried and then ground into a fine powder.

The Oxygen Flask combustion method was used to prepare the mussel tissue for ^{3}H liquid-scintillation counting. This method has been used in a wide range of analytical applications as well as for the determination of ^{3}H and ^{14}C activities in biological samples.[18,19]

The apparatus consisted of a 3 l round-bottomed flask, a stopper and a platinum basket.

The freeze-dried sample (100 mg) was wrapped in a paper sample wrapper and placed in the platinum basket. The flask was filled with oxygen and then the tip of the sample wrapper was ignited and the stopper and basket were inserted into the flask. Once combustion had ceased, 5 ml of absolute ethanol was added to absorb the ^3H_2O produced. A 2-ml aliquot of the alcohol solution was transferred to a counting vial and 4 ml of scintillation liquid was added before counting. This procedure was performed in triplicate for each tissue sample.

Shells were washed with deionized water, air-dried, crushed and ground to a fine powder in a mortar. The powder (2.0 g) was placed in a 250 ml beaker and 10 ml of 2 M hydrochloric acid was added. After dissolution the solution was filtered to remove the insoluble residue, then 500-μl portions were withdrawn and mixed with 5 ml of scintillation liquid and counted.

RESULTS AND DISCUSSION

Speciation of the ^3H-labeled compounds extracted from *M. californianus*

Mussels were exposed for nine days to seawater containing [^3H]MMAA. The preliminary methanol treatment of their tissue extracted 74% of the ^3H-labeled compounds. The extraction efficiency was determined by measuring the ^3H activity of the mussel flesh before and after the extraction. The methanol extract was evaporated to dryness and redissolved in water. Diethyl ether extracted approximately 9% of the counts from this solution; this is probably a measure of the amount of lipid-like arsenicals in the original methanol extract.

The water-soluble ^3H-labeled compounds eluted from Sephadex LH-20 between 100 and 140 ml. Standard arsenobetaine eluted within the same retention volume. This is an indication that ^3H label has been incorporated into water-soluble compounds that exhibit similar physical properties (size and/or adsorption characteristics) to those of AsB, on this particular column.

A strong cation-exchange resin has been commonly employed to accomplish a preliminary separation of arsenicals into fractions eluting with water, ammonium hydroxide and hydrochloric acid.[4,5,20,21] This procedure was used in the present study. The radioactivity in the fractions eluting from the Dowex 50W × 8 (H^+) was measured

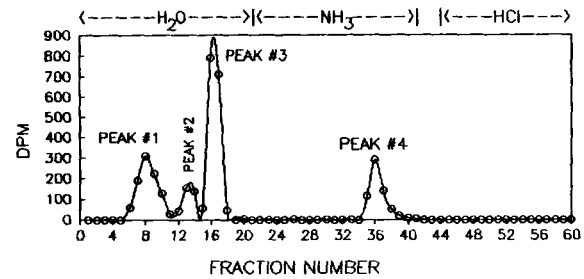

Figure 1 Dowex 50W × 8 (H^+) liquid-scintillation chromatogram; mobile phase 200 ml water, 200 ml 5% ammonium hydroxide, 40 ml water and 150 ml 2M HCl; 9.5-ml fractions collected; 0.5 ml of each fraction mixed with 5 ml scintillate and counted.

and the resulting chromatogram is presented in Fig. 1. Four peaks containing ^3H-labeled compounds eluted from this column. The first peak contained a compound (which will be referred to as Peak 1) that essentially showed no interaction with the strong cation-exchange resin. The compounds comprising the second (Peak 2) and third (Peak 3) peaks were weakly retained by the resin, and were eluted by using water. Finally the compound in the fourth peak (Peak 4) was eluted by a 5% ammonium hydroxide solution. Methylarsonic acid elutes from this column with water, whilst DMAA and AsB elute with ammonium hydroxide. AsC and TMA^+I^- elute with 2M HCl. No ^3H activity was detected in any of the fractions when 2M HCl was used as the eluant for the mussel extracts.

After this preliminary information about the properties of the ^3H-labeled compounds had been obtained, HPLC was used for further identification.

The HPLC Protein Pak DEAE chromatograms (5 mM sodium acetate, pH 4, mobile phase), of Peaks 3 and 4, and of a mixture of standards AsB, DMAA and MMAA, are presented in Fig. 2. Standard MMAA and Peak 3 exhibit the same retention time. Peak 4 also has the same retention time as standard AsB and DMAA, which are not separated under these conditions. However, these two standards are readily separated on the Protein Pak column by using 5 mM ammonium acetate (pH 6.8) as the mobile phase.[16] This is presented in Fig. 3 along with the elution profile of Peak 4. Peak 4 exhibits the same retention time as standard AsB.

Ion-pair reversed-phase liquid chromatography was also used for these identifications. Figure 4 presents the elution profile of standard AsB, DMAA, MMAA and that of Peaks 3 and 4.

Again Peak 4 is identified as AsB and Peak 3 as MMAA.

All these chromatographic results are summarized in Table 1.

Peaks 1 and 2 have also been chromatographed under all the above conditions. The resulting retention times do not correspond with any of the standard arsenicals available to us. Glycerylphosphorylarsenocholine, a compound that has been found to accumulate in yellow-eye mullet following oral administration of arsenocholine, was also tested.[9] The reported chromatographic behavior of this compound seemed to be similar to those of Peaks 1 and 2. However, the retention times acquired for this compound on the Protein Pak column did not match those of Peaks 1 and 2.

Determination of total ^3H in mussel tissue

After three, six, and nine days of exposure to [^{3}H]-MMAA-containing seawater the specific activity detected in the *M. californianus* mussel

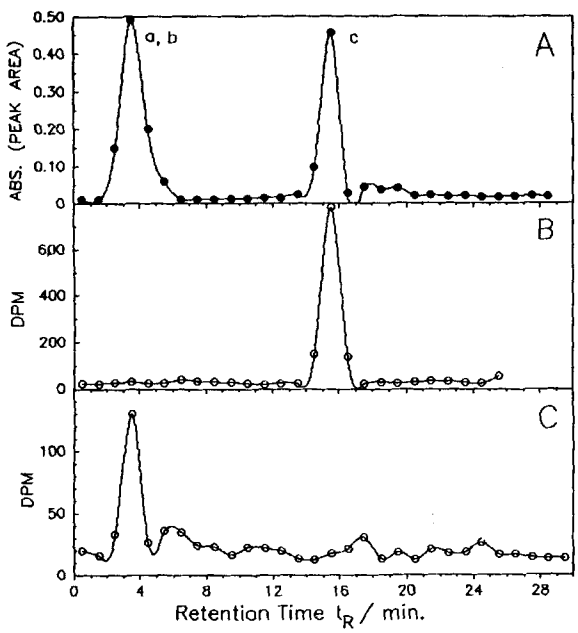

Figure 2 Waters Protein Pak DEAE column; mobile phase 5 mM sodium acetate, pH adjusted to 4 with acetic acid; flow rate 1 ml min^{-1}; fractions collected every 1 min. (A) HPLC–GF AA chromatogram of standards: a, arsenobetaine (250 ng As); b, dimethylarsinic acid (500 ng As); c, methylarsonic acid (500 ng As). (B) HPLC liquid-scintillation chromatogram of Peak 3; 1 ml fraction mixed with 5 ml scintillate and counted. (C) HPLC liquid-scintillation chromatogram of Peak 4; 1 ml fraction mixed with 5 ml scintillate and counted.

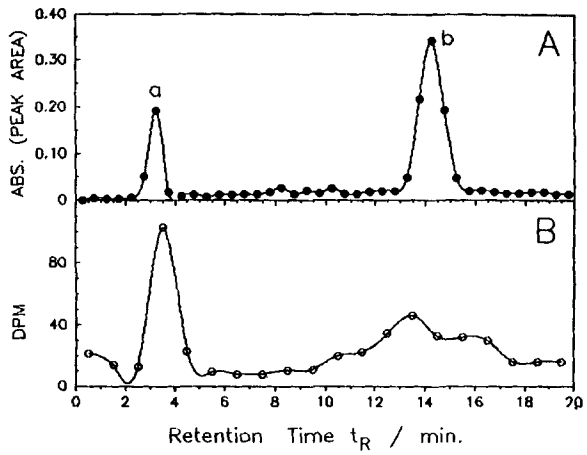

Figure 3 Waters Protein Pak DEAE column; mobile phase 5 mM ammonium acetate, pH 6.8; flow rate 1 mL min^{-1}. (A) HPLC GF AA chromatogram of standards: a, arsenobetaine (250 ng As); b, dimethylarsinic acid (250 ng As); fractions collected every 30 s. (B) HPLC liquid-scintillation chromatogram of Peak 4; 1 ml fraction mixed with 5 ml scintillate and counted.

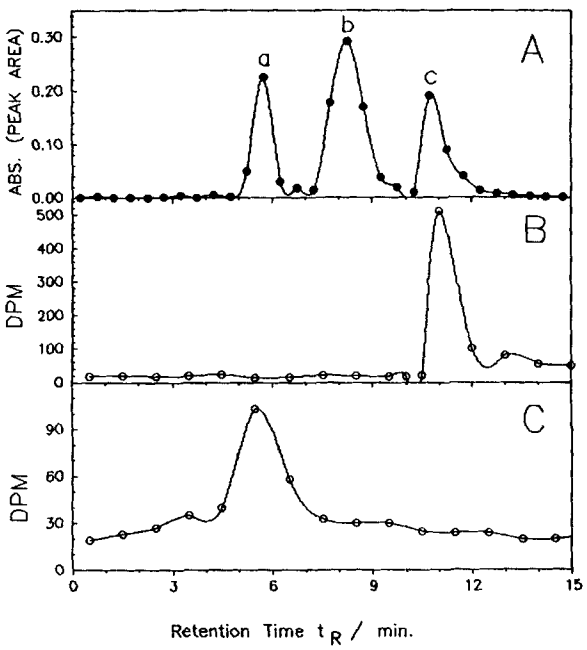

Figure 4 Bondclone 10 C18 reversed-phase column; mobile phase water–methanol (95:5, v/v); ion-pair reagent 5 mM tetrabutylammonium nitrate; flow rate 1 ml min^{-1}. (A) HPLC GF AA chromatogram of standards: a, arsenobetaine; b, dimethylarsinic acid; c, methylarsonic acid; fractions collected every 30 s. (B) HPLC liquid-scintillation chromatogram of Peak 3; 1 ml fraction mixed with 5 ml scintillate and counted. (C) HPLC liquid-scintillation chromatogram of Peak 4; 1 ml fraction mixed with 5 ml scintillate and counted.

Table 1 Summary of chromatographic experiments

	Dowex H^+ (eluant)	HPLC anion-exchange, 5 mM sodium acetate, pH 4: t_R (min)	HPLC anion-exchange, 5 mM ammonium acetate, pH 6.8: t_R (min)	HPLC C-18 Ion-pair, 5 mM tetrabutylammonium nitrate, pH 6.8: t_R (min)
Compound proposed to be MMAA (Peak 3)	H_2O	15.5±0.5	nd[a]	10.75±0.25
Standard MMAA	H_2O	15.5±0.5	nd	11.0±0.5
Compound proposed to be AsB (Peak 4)	5% NH_4OH	3.5±0.5	3.5±0.5	5.5±0.5
Standard AsB	5% NH_4OH	3.5±0.5	3.25±0.25	5.75±0.25
Standard DMAA	5% NH_4OH	3.5±0.5	14.25±0.25	8.25±0.25
Peak 2	H_2O	8.0±0.5	3.5±0.5	nd

[a] nd, not determined.

tissue was 27, 42, and 65 dpm mg^{-1} respectively. The distribution of ^3H activity within the mussel was also determined. After the mussels had been exposed for a nine-day period, the highest specific activity was found in the visceral mass and the gills of the mussel (Fig. 5). Similar distribution of the activity within the tissue parts was observed on days 3 and 6. No ^3H activity was detected in the shells after using the sample preparation procedure described, thus indicating that physical processes such as surface sorption play a minor role in the uptake of arsenic compounds by the mussel shells.

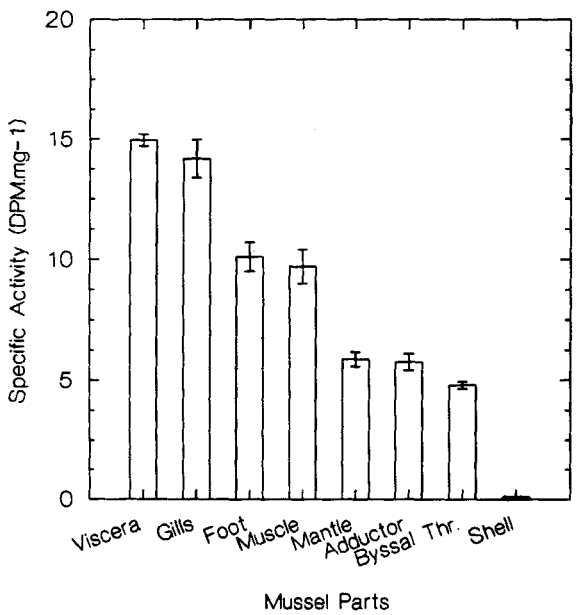

Figure 5 Distribution of ^3H activity within mussel parts, sampled on day 9.

CONCLUSION

When *Mytilus californianus* is exposed to [^{3}H]MMAA in a static seawater system, [^{3}H]MMAA, [^{3}H]AsB, and two unknown ^3H-labeled compounds accumulated in the tissue parts of the mussel.

AsB has been found to occur naturally in *Mytilus californianus*, whilst MMAA has not been detected (Cullen, W R and Pergantis, S, unpublished results). Thus the ratio of [^{3}H]MMAA to [^{3}H]AsB found in the mussel flesh following exposure to [^{3}H]MMAA does not reflect the natural ratio. It should be pointed out, however, that the high arsenic concentration (1.5 ppm As) in the static seawater system may have caused overloading of the mussels, thus not allowing them to function in a natural way. Therefore we can only speculate that the conversion of [^{3}H]MMAA into [^{3}H]AsB does not take place within the mussel itself. This is based on the fact that if [^{3}H]MMAA was readily converted to [^{3}H]AsB within the mussel tissue, then the measured content of [^{3}H]MMAA would be much lower. If, however, the conversion is a slow process we would expect to find naturally occurring MMAA within this mussel, which (as indicated above) is not the case.

These results indicate that [^{3}H]AsB is either accumulated from water and/or food, or may be synthesized from arsenic compounds other than

MMAA within the mussel itself. Cullen and Nelson[13] reached similar conclusions from studies with *Mytilus edulis*, a bivalve that readily takes up AsB from seawater.[10] Wrench *et al.*[22] studied a three-step food chain consisting of an autotroph, a grazer and a carnivore. They concluded that the muscle tissue of the carnivorous shrimp could not itself form 'organic arsenic', which is believed to be synthesized by primary producers.

Questions remain about the identity of the two compounds that were not identified. Only recently has it been established that arsenosugars are present in bivalves, although *Mytilus californianus* was not examined.[23] Although Peaks 1 and 2 may contain arsenoribose derivatives, these may not necessarily be those originally extracted from the mussel flesh, since such derivatives are sensitive to extremes of pH and may not survive passage down the strong cation-exchange column.[24]

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REFERENCES

1. Cullen, W R and Reimer, K J *Chem. Rev.*, 1989, 89: 713
2. Maher, W A *Comp. Biochem. Physiol.*, 1985, 80C(1): 199
3. Lau, B Y, Michalik, P, Porter, C J and Krolik, S *Biomed. Envir. Mass Spectr.*, 1987, 14: 723
4. Cullen, W R and Dodd, M *Appl. Organomet. Chem.*, 1989, 3: 79
5. Shiomi, K, Kakehashi, Y, Yamanaka, H and Kikuchi, T *Appl. Organomet. Chem.*, 1987, 1: 177
6. Norin, H, Christakopoulos, A, Sandstrom, M and Ryhage, R *Chemosphere*, 1985, 14(3/4): 313
7. Edmonds, J S and Francesconi, K A *Nature (London)* 1981, 289: 602
8. Francesconi, K A, Edmonds, J S and Stick, R V *J. Chem. Soc., Perkin Trans. 1*, 1992, 1349
9. Francesconi, K A, Stick, R V and Edmonds, J S *Experientia*, 1990, 46: 464
10. Francesconi, K A PhD Thesis, University of Western Australia, 1991
11. Unlu, M Y and Fowler, S W *Mar. Biol.*, 1979, 51: 209
12. Klumpp, D W *Mar. Biol.*, 1980, 58: 265
13. Cullen, W R and Nelson, J C *Appl. Organomet. Chem.*, 1993, 7: 319
14. Edmonds, J S, Francesconi, K A, Cannon, J R, Raston, C L, Skeleton, B W and White, A *Tetrahedron Lett.*, 1977, 18: 1543
15. Irgolic, K J, Junk, T, Kos, C, McShane, W S and Pappalardo, C C *Appl. Organomet. Chem.*, 1987, 1: 403
16. Cullen, W R and Dodd, M *Appl. Organomet. Chem.*, 1989, 3: 401
17. Cullen, W R, McBride, B C, Pickett, A W and Hasseini, M *Appl. Organomet. Chem.*, 1988, 3: 71
18. Lisk, D J *Agric. Fd Chem.*, 1960, 8: 2
19. Ober, R E, Hansen, A R, Mourer, D, Baukema, J and Gwynn, G W *Int. J. Appl. Rad. Isotopes*, 1969, 20: 703
20. Pacey, G E and Ford, J A *Talanta*, 1981, 28: 935
21. Francesconi, K A and Edmonds, J S *Comp. Biochem. Physiol.*, 1987, 87C(2): 345
22. Wrench, J, Fowler, S W and Unlu, M Y *Mar. Pollut. Bull.*, 1979, 10: 18
23. Shibata, Y and Morita, M *Appl. Organomet. Chem.*, 1992, 6: 343
24. Edmonds, J S and Francesconi, K A *J. Chem. Soc., Perkin Trans. 1*, 1983, 2383