Journal of Chromatography, 643 (1993) 189–195
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2738

Comparison of the abundance of the fecal sterol coprostanol and fecal bacterial groups in inner-shelf waters and sediments near Sydney, Australia

Peter D. Nichols*, Rhys Leeming, Mark S. Rayner and Val Latham

CSIRO Division of Oceanography, GPO Box 1538, Hobart, Tasmania 7001 (Australia)

Nick J. Ashbolt and Christine Turner

Australian Water Technologies - Science & Environment, Water Board Sydney, PO Box 73, West Ryde, NSW 2114 (Australia)

ABSTRACT

Concurrent measurement of the sewage tracer coprostanol and fecal indicator bacteria were made for water and sediments collected in January 1992 from coastal waters off Sydney, Australia. The coprostanol results were compared with data from an earlier survey conducted in 1989 before the commisioning of Sydney's deepwater ocean outfalls in 1990 and 1991. Good correlations were observed for both water and sediment samples between coprostanol and the two fecal indicator organisms, fecal coliforms and *Clostridium perfringens* spores, thereby validating the use of coprostanol as a sewage signature in this environment. For sediments, most inner-shelf sites (1–10 km offshore) showed an increase in the concentration of coprostanol between the two surveys. The areas of highest concentration have been shifted further off-shore, to zones adjacent to the diffusers.

INTRODUCTION

Sewage contamination has traditionally been determined by enumeration of fecal coliform bacteria. However, the reliability of coliforms as an adequate indicator of sewage contamination has been questioned [1–3]. This is mainly due to the extreme variability found for fecal coliform survival under varying environmental conditions [4], as expected with coastal outfall systems, and a poor correlation with specific pathogens [5].

As alternatives to microbial indicators, a range of sewage specific organic compounds have been proposed. Such compounds can be readily analysed by capillary GC. One of these, coprostanol, which is produced in the digestive tract of higher animals by microbial degradation of cholesterol [6], has proven a successful and sensitive indicator of sewage pollution [7–9]. Chemical signatures also overcome many of the shortcomings of classical microbiological indicators of sewage pollution [10]. Readman *et al.* [11] proposed an analytical protocol including analysis of coprostanol to quantify sewage, oil and polycyclic aromatic hydrocarbon pollution in estuarine and coastal environments. The use of these techniques shows much potential. To date, however, only limited studies, apart from various analyses of hydrocarbons, have been undertaken in Australia.

In 1989, before the three deepwater ocean outfalls built for the disposal of the majority of Sydney's sewage were completed, a survey (twenty-six sites) was undertaken of the area deemed likely to be affected by the sewage plumes. The daily flow from the nearshore Malabar outfall, the largest of the outfalls, is approximately 640 Ml/day [12]. The Malabar discharge includes the bulk of Sydney's industrial waste that enters the sewerage sys-

* Corresponding author.

0021-9673/93/$06.00 © 1993 Elsevier Science Publishers B.V. All rights reserved

tem. The effluent is released after primary treatment together with sludge which included a median discharge of 22 tonnes day^{-1} of grease [13].

The discharge contains many industrial pollutants including heavy metals, polycyclic aromatic hydrocarbons and organochlorines [12]. The deep-water ocean outfalls were commissioned between September 1990 and August 1991 [13]. In January 1992, sites studied in the 1989 survey were reanalysed as well as additional sites bringing the total to 96.

The major objective of the program was to determine the distribution and fate of Sydney's sewage effluent using combined chemical, microbiological and physical oceanographic data. In this study, comparative water column and sedimentary results are presented for coprostanol, measured using modern capillary GC methods, and common fecal bacteria indicator groups. Results are presented for samples collected from 9 stations along a 35-km transect commencing 1 km off-shore from the old Malabar cliff-face outfall. These data were obtained to aid scientists and managers who are examining the impact of the deep ocean outfalls.

EXPERIMENTAL

Sample collection

Sampling was conducted aboard RV Franklin during November 1989 (cruise FR 13/89) and January 1992 (cruise FR1/92). Station locations are shown in Table I. The Malabar deep water ocean outfall is located immediately south of stations 29 and 30. Sediments were collected to water depths in excess of 1000 m using a Smith-McIntyre grab. Surface sediment (0–2 cm) was removed from the grab using a stainless steel or a sterile plastic spoon. Water samples were collected using a Neil Brown CTD fitted with a rosette of 10-l Niskin bottles. Water was filtered at sea through glass fibre filters (15 cm, Schleicher and Schuell No. 8, nominal pore size 0.5 μm) to obtain particulate matter samples. All samples for chemical analysis were stored immediately at −20°C and were transported to the CSIRO Marine Laboratories in Hobart for subsequent analyses.

Reagants

Nanograde solvents and reagants (Mallinkrodt) were used in the lipid procedures. All glassware was prerinsed with nanograde solvent prior to use. Concurrent analyses of laboratory blanks were undertaken during sample analysis.

Lipid extraction and fractionation

Samples (30–80 g, wet weight) were extracted quantitatively by the modified one-phase $CHCl_3$-MeOH Bligh and Dyer method [14,15]. After phase separation, the lipids were recovered in the lower $CHCl_3$ layer (solvents were removed *in vacuo*) and were made up to a known volume and stored sealed under nitrogen at −20°C. Total lipid sterols were obtained following alkaline saponification of an aliquot (10%) of the total lipids [16,17]. Products were extracted into hexane–$CHCl_3$ (4:1, v/v) and stored at −20°C. Sterols were converted to their corresponding trimethylsilyl (TMSi) ethers by treatment with bis(trimethylsilyl)trifluoroacetamide (50 μl, 60°C, 60 min).

Gas chromatography (GC) and GC-mass spectrometry (GC–MS)

GC analyses were performed with a Hewlett-Packard 5890 GC equipped with a 50 m × 0.32 mm I.D. cross-linked methyl silicone (0.17 μm film thickness) fused-silica capillary column (Hewlett-Packard), a flame ionization detector (FID) and a split/splitless injector. After addition of methyltricosanoate internal standard, samples were injected (2 μl out of typically 50–1000 μl of sample) in the splitless mode at 50°C and after 1 min the oven was raised to 150°C at 30°C min^{-1}, then to 250°C at 2°C min^{-1}, and finally to 300°C at 5°C min^{-1}. Hydrogen was used as the carrier gas (inlet pressure 70 kPa, 1 ml min^{-1}). Peak areas were quantified using chromatography software (DAPA Scientific software) operated using an IBM-XT personal computor. Component identification was based on comparison of retention time data with that obtained for authentic and laboratory standards. Coprostanone eluted after both coprostanol and epicoprostanol under the GC conditions used in this study.

The FID response was found to be linear in the practical concentration range (0.5–150 ng of individual components injected) used in this study. Samples were routinely analysed by GC within 1–2 weeks of extraction. Prior to instrumental analysis samples were stored in solvent and were not allowed

TABLE I

STATION LOCATIONS, WATER COLUMN COPROSTANOL CONCENTRATIONS AND INDICATOR ORGANISM DATA

Station number	Position		Distance from shore (km)	Water depth (m)	Sampling depth (m)	Coprostanol (ng/l)	Indicator organisms (CFU/100 ml)[a]		
	Latitude	Longitude					FC	FS	CP
27	33 58.27	151 16.61	0.9	49	5	9.4	150	ND	ND
					40	19	45	ND	ND
28	33 58.14	151 17.25	1.9	70	5	5.4	3	1800	0.45
					60	625	9900	2.3×10^4	1.7
29	33 58.00	151 18.13	3.7	78	5	2.5	25	ND	ND
					70	1660	1.6×10^4	ND	ND
30	33 58.07	151 18.19	3.7	78	5	9.2	50	520	0.4
					65	3810	2.5×10^5	400	161
31	33 58.31	151 19.50	5.6	88	5	ND	2.5	39.5	2.8
					60	1500	1.4×10^4	240	53
32	33 58.23	151 20.74	7.4	91	5	2.4	<1	ND	ND
					85	55	34	ND	ND
33	33 58.03	151 22.02	9.2	95	5	2	<0.5	24.5	2
					90	11	3.5	4.6	1.2
34	33 57.90	151 27.88	18.5	141	5	0.05	1.5	41.5	0.35
					133	4	<0.5	9.8	0.15
35	33 58.18	151 33.74	27.8	169	5	0.05	1.3	320	0.85
					165	ND	<0.5	95	0.45
36	33 58.83	151 49.26		1031	–	–	ND	ND	ND

[a] CFU = Colony forming units. FC = fecal coliforms; FS = fecal streptococci; CP = *Clostridium perfringens* spores. ND = not determined.

to go to dryness. During a typical column lifetime (6–12 months), repeat analysis indicated that minimal loss of coprostanol occurred as long as routine injector maintenance was performed. Although synthetic coprostanol was not available, analysis of replicate samples indicated good agreement for within day, day to day, and longer term variation samples. The relative standard deviation for replicate samples was generally <5% or better. Duplicate GC assays of the same sample showed 1–10% standard deviation over the concentration range used.

Verification of identifications was by GC–MS analysis of samples performed on a HP 5890 GC and 5970 Mass Selective Detector fitted with a direct capillary inlet and a split/splitless injector. Data were acquired and processed on an HP 59970C Workstation operated in scan aquisition mode. Operating conditions are described in detail elsewhere [17–19]. The non-polar column was similar to that described above. The mass spectra of coprostanol and coprostanone were distinguishable.

Microbiology

Fecal coliforms, fecal streptococci and spores of *Clostridium perfringens* were enumerated by membrane filtration (0.45 μm) using standard methods [20], and m-FC (Difco No. 0883-01), *m*-enterococcus (Difco No. 0746-01) and perfringens (Oxold CM543) agars, respectively. Sediments (10 g) were dispersed by sonication (180 W, 30 s) in 100 ml sterile seawater prior to filtering. Selected colonies were confirmed by standard methods and counts are reported as colony forming units (CFU) on a 100 ml or 100 g basis. Relative standard deviations for sediments were: fecal coliforms, 0.5–20%; fecal streptococci, 0.2–5%; *Clostridium perfringens* spores, <0.5%. Standard deviations were generally an order of magnitude greater for water samples.

Sedimentary organic matter

Sedimentary organic carbon content was determined by measuring weight loss on ignition (550°C, overnight).

RESULTS AND DISCUSSION

Representative gas chromatograms illustrating sterol profiles for water column particulate matter and sediments from the Malabar transect are shown in Fig. 1. A high-resolution 50-m column was chosen for the analyses in order to ensure that separation was achieved of coprostanol and epicoprostanol, in addition to other sterols and non-sterol components. Under the GC conditions employed, sterols eluted between approximately 55 and 65 min and base-line separation was observed for coprostanol and epicoprostanol. The GC program was designed to resolve other components (*e.g.* hydrocarbons, linear alkyl benzenes, alcohols; data not shown) found in the non-saponifiable neutral lipid fraction. Many of these components provide useful information on other sources of organic matter in environmental samples. If information is only being sought on coprostanol alone, then the GC analysis time can be shortened considerably through the use of a faster rate of oven temperature increase. Similarly, scope exists to further streamline the procedure through direct saponification of either filter (water column) or sediment samples, thereby eliminating the solvent extraction step used used in this study.

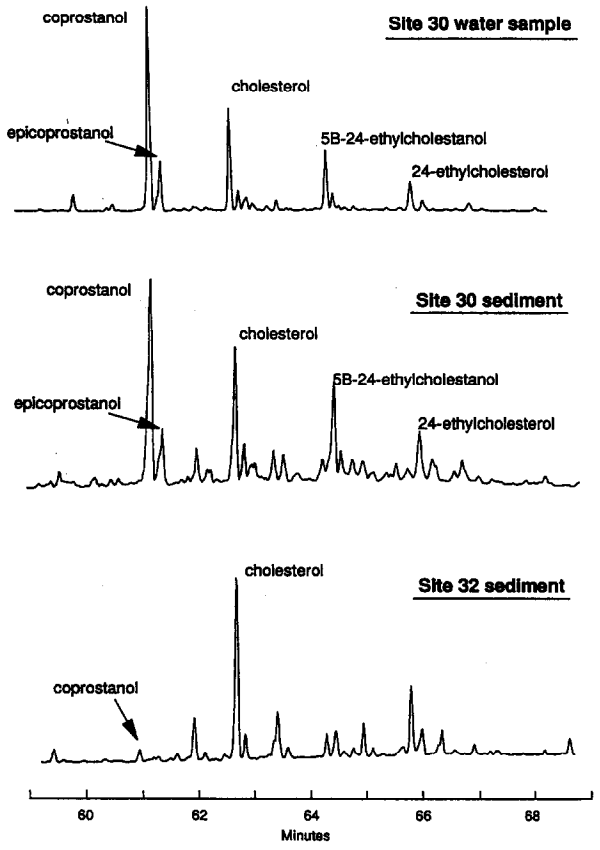

Fig. 1. Partial gas chromatograms of sterols (as TMSi ethers) in particulate matter (station 30, 65 m water sampling depth) and sediments (stations 30 and 32). Station locations refer to Table I. HP1 fused-silica capillary column.

The theoretical limit of detection for coprostanol in this study was 1 ng/l for water column particulate matter (typically 4 l analysed) and 1 ng/g for sediments (from 5–10 g). Effluent and sludge from sewage treatment plants in the Sydney region typically contain around $3 \cdot 10^5$ ng/l and $1 \cdot 10^6$ ng/g of coprostanol, respectively (unpublished data). Therefore measurement of coprostanol in field samples can theoretically estimate dilution factors approaching 10^5 and 10^6 for effluent and sludge, respectively. These detection limits could be improved through extraction of more material, performing quantitation by SIM GC–MS or other specific detectors after appropriate derivatization. Using coprostanol, in related studies we have measured dilution factors of 10^4 for sewerage effluent at distances of 100 km from the Malabar outfall. The dilution factors were verified using other chemical and microbiological parameters.

In coastal regions typical of those around Sydney and other Australian coastal cities and towns, we believe a background concentration of coprostanol in aerobic surface sediments is no greater than 5–10 ng/g. This value is based on analyses of sediments collected from pristine locations in Australian coastal waters, in central Bass Strait and near Jervis Bay. Coprostanol was below detection (*i.e.* < 1 ng/l) in all water samples from pristine locations, indicating that the background concentration in water is at least an order of magnitude lower than for sediments. Coprostanol was not present in routinely performed laboratory blank assays.

Coprostanol in marine waters

The concentrations of coprostanol in surface water samples along the Malabar transect were generally very low. Concentrations ranged from below detection at the off-shore sites (stations 34 and 35)

to 10 ng/l adjacent to the coast. In contrast, coprostanol in bottom water samples ranged from 20–3800 ng/l at stations between 1 and 7 km from the coast. Beyond 7 km from the shore, coprostanol levels were similar to those of the surface waters. The highest concentrations were at sites (stations 29 and 30) immediately north of the Malabar deep-water ocean outfall located 4 km from shore. An effluent coprostanol concentration of $330 \cdot 10^3$ ng/l was determined for effluent collected concurrently with the shipboard sampling. Using this data, effluent dilution factors of between 1/80–1/220 can be calculated for bottom waters at stations 29 to 31.

Oceanographic measurements at the time of sampling showed that the water column was well-stratified, with the bottom waters around 8°C cooler than surface waters. Although strong north to south currents can prevail for much of the year in these waters, the nearshore current at the time of this study was northward, possibly due to the passage of a coastly trapped wave [21]. An intrusion of continental slope water with a temperature of < 14°C reached in across the floor of the shelf to the 85 m contour and then withdrew. The results observed for coprostanol in waters along the Malabar transect are consistent with the oceanographic currents at time of sampling.

Coprostanol in marine sediments

The sediments reflect the cumulative effects of the different current types encountered in the Sydney region, while the water column data provides only a picture of effluent dispersion based on prevailing currents at the time of sampling. From the 1992 survey, concentrations ranged from 40–2800 ng/g in the sediments collected along the Malabar transect as shown in Table II. For 1989, coprostanol concentrations for inner shelf sediments ranged from 20–530 ng/g. The higher coprostanol concentrations (> 500 ng/g) are in the range associated with significant sewage pollution [11,22].

Nearly all inner shelf sites (1–10 km) showed large increases in the amount of coprostanol present after commissioning of the deep ocean outfalls. The increase in coprostanol concentration at most sites (1989 to 1992) ranged from 10 to 900%, with many sites showing over 100% increase. Only one site, at 1 km from the old Malabar cliff-face outfall, showed a significant decrease. The coprostanol concentration had fallen by 58%.

This study did not investigate the influence of the new outfalls on the beaches, as this has been undertaken by a number of state authorities. The results for sediments at the innermost stations and data for water at the sites closest to shore are, however, con-

TABLE II

SEDIMENT COPROSTANOL CONCENTRATIONS, INDICATOR ORGANISM DATA AND ORGANIC CARBON CONTENT

Station number	Organic carbon (mg/g)	Coprostanol (ng/g)		Indicator organisms[a] (CFU/100 g)		
		1989	1992	FC	FS	CP
27	11.6	254	106	2000	2200	$1.2 \cdot 10^4$
28	15.3	329	560	8200	2600	$3.6 \cdot 10^4$
29	26.7	531	1850	$2.4 \cdot 10^4$	3000	$1.7 \cdot 10^5$
30	42.8	531	2870	$6.4 \cdot 10^4$	10^4	$3.4 \cdot 10^5$
31	56.1	126	1260	$1.2 \cdot 10^4$	1500	$8.3 \cdot 10^4$
32	18.9	26	62	350	300	8500
33	24.9	19	38	100	< 200	5000
34	52.2	23	26	1000	< 100	3500
35	56.3	19	12	< 200	< 200	1500
36	109.9	–	13	< 200	< 200	< 1000

[a] FC = fecal coliforms; FS = fecal streptococci; CP = *Clostridium perfringens* spores.

sistent with the early results for beaches which show a general improvement in water quality [23,24].

Relationship of coprostanol to indicator organisms

Previous reports show variability in the relationship of coprostanol to various indicator groups in water samples [1,25–27]. Some of these differences relate to the use of different indicators and their variability in die-off. Results for the three indicator organisms (fecal coliforms, fecal streptococci and *Clostridium perfringens* spores) are provided in Tables I and II). Spores of *C. perfringens* were included in the present study to largely remove the effect of variability in the die-off of the other two more commonly used indicator organisms. Nevertheless, in this study in Australian marine waters, we observed a strong linear correlation between the water column particulate matter concentration of coprostanol and faecal coliforms (r^2 = 0.80); it was even higher for *Clostridium perfringens* (r^2 = 0.97). Light is an important factor in die-off of faecal bacteria. The water depth and associated lower light penetration for the bottom water samples has, we believe, reduced fecal coliform die-off, resulting in the good correlations observed.

Good correlations were observed with all three indicator groups for sediments. We believe this is the first report for marine sediments of parallel coprostanol and standard bacterial counts (Table II). The good correlations noted in this study for sediments (coprostanol-fecal coliforms, r^2 = 0.91; coprostanol-fecal streptococci, r^2 = 0.79; coprostanol-*Clostridium perfringens* spores, r^2 = 0.96) emphasize the validity of using coprostanol as a tracer for sewage in the marine environment, including sediments.

Several of the coprostanol concentrations from samples near the deepwater ocean outfall were generally as high or higher than values reported in the literature, indicating that those samples are significantly polluted by sewage. Several studies have also attempted to relate coprostanol and fecal coliform concentrations [1,25–27]. For primary contact recreational waters (Canada), a water standard of 500 ng/l was proposed with secondary contact recreational water an order of magnitude higher *i.e.* 5000 ng/l.

Results from this and other studies in Sydney coastal waters and in the Derwent Estuary, Tasmania indicate that the primary contact limit used in many Australian states (median 150 fecal coliforms per 100 ml) corresponds to around 100 ng/l coprostanol.

A new method for rapid detection of sewage contamination in natural waters has been recently reported [28]. The technique involves instrumental-based fluorometric assay of β-D-galactosidase enzyme activity and the method has been found to correlate well with fecal coliform abundances enumerated by standard methods. Coprostanol concentrations were also found to correlate with enzyme assay for both surface and bottom water samples (r^2 = 0.88).

CONCLUSIONS

A survey of Sydney's coastal environment was conducted during January, 1992 after the commissioning of the new deepwater ocean outfalls. The study repeated sampling conducted in 1989, before the commissioning. Results for the sewage tracer coprostanol show that it has accumulated in sediments of the inner shelf (1–10 km offshore). The area of highest coprostanol concentrations has been shifted further offshore from adjacent to the old cliff face outfalls to around the zones of the deep water ocean outfalls. The dispersion of sewage appears to be confined to the inner shelf, *i.e.* no further than 10 km from the coast. Dispersion further offshore (east) appears to be minimal, rather sewage-derived material may be moving in a north-south direction.

ACKNOWLEDGEMENT

We extend our sincere thanks to the master and crew of RV Franklin and to our fellow CSIRO colleagues, in particular George Cresswell and Jan Peterson, and external collaborators for their cooperation and assistance throughout the project. George Cresswell and John Volkman and two anonomous reviewers are thanked for their comments on an earlier version of the manuscript.

REFERENCES

1 B. J. Dutka, A. S. Y. Chau and J. Coburn, *Water Res.*, 8 (1974) 1047.
2 E. F. Landry, J. M. Vaughn, T. J. Vicale and R. Mann, *Appl. Environ. Microbiol.*, 45 (1983) 238.

3 G. McFetters, J. S. Kippin and M. W. LeChevallier, *Appl. Environ. Microbiol.*, 51 (1986) 1.
4 M. W. Rhodes and H. Kator, *Appl. Environ. Microbiol.*, 54 (1988) 2902.
5 J. C. Geldenhyts and P. D. Pretorius, *Water Sci. and Technol.*, 21 (1989) 105.
6 R. S. Rosenfield and T. F. Gallagher, *Steroids*, 4 (1964) 515.
7 P. G. Hatcher and P. A. Mcgillivary, *Environ. Sci. and Technol.*, 13 (1979) 1225.
8 I. M. Venkatesan and I. R. Kaplan, *Environ. Sci. Technol.*, 24 (1990) 208.
9 L. A. LeBlanc, J. S. Latimer, J. T. Ellis and J. G. Quinn, *Esttuarine Coastal Shelf Sci.*, 34 (1992) 439.
10 P. D. Nichols and R. Leeming, *Chem. Aust.*, 58 (1991) 274.
11 J. W. Readman, M. R. Preston and R. F. C. Mantoura, *Mar. Pollut. Bull.*, 17 (1986) 298.
12 M. W. Whyte, in *Marine treatment of sewage and sludge*, Thomas Telford, London, 1988, pp. 79–83.
13 *SPCC licence compliance report*, Sydney Water Board, Sydney, 1990.
14 E. G. Bligh, and W. M. Dyer, *Can. J. Biochem. Physiol.*, 35 (1959) 911.
15 D. C. White, R. J. Bobbie, J. S. Herron, J. D. King and S. J. Morrison, in J. W. Costerton and R. R. Colwell (Editors), *Native Aquatic Bacteria: Enumeration, Activity and Ecology ASTM STP 695*, American Society of Testing Materials, Philadelphia, PA, 1979, pp. 69–81.
16 G. J. Perry, J. K. Volkman, R. B. Johns and H. J. Bavor, *Geochim. Cosmochim. Acta*, 43 (1979) 1715.
17 P. D. Nichols, D. W. Klumpp and R. B. Johns, *Phytochem.*, 21 (1982) 1613.
18 P. D. Nichols, J. K. Volkman, A. C. Palmisano, G. A. Smith and D. C. White, *J. Phycol.*, 24 (1988) 90.
19 P. D. Nichols, D. G. Holdsworth, J. K. Volkman, M. Daintith and S. Allanson, *Aust. J. Mar. Freshwater Res.*, 40 (1989) 645.
20 *Standard Methods for the Examination of Water and Wastewater*, American Public Health Association, Washington, D.C., 16th ed., 1985.
21 A. Huyer, R. L. Smith, P. J. Stabeno, J. A. Church and N. J. White, *Aust. J. Mar. Freshwater Res.*, 39 (1988) 245.
22 P. D. Nichols and Q. Espey, *Aust. J. Mar. Freshwater Res.*, 42 (1991) 327.
23 Beachwatch, *Winter season report*, Sydney, 1991.
24 *Sydney Deepwater Outfalls Environmental Monitoring Program, Monitoring Sydney's deepwater outfalls*, Environmental Protection Authority, Sydney, 1992.
25 R. M. Goodfellow, J. Cardosa, G. Eglinton, J. P. Dawson and G. A. Best, *Mar. Pollut. Bull.*, 8 (1977) 272.
26 B. J. Dutka and A. El-Shaarawi, *Can. J. Microbiol.*, 21 (1975) 1386.
27 L. M. Churchland, G. Kan and A. Ages, *Can. J. Microbiol.*, 28 (1982) 239.
28 S. C. Apte and G. E. Batley, *Chem. Aust.*, 59 (1992) 397.