J. Mar. Biol. Ass. U.K. (2000), **80**, 311–320
Printed in the United Kingdom

Comparisons of fatty acid and stable isotope ratios in symbiotic and non-symbiotic brittlestars from Oban Bay, Scotland

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The bed-forming brittlestars *Ophiothrix fragilis*, *Ophiocomina nigra* and *Amphiura chiajei* from Oban Bay, Scotland were studied using methods previously employed to study chemoautotrophic symbioses. *Ophiothrix fragilis* and *A. chiajei* both contain symbiotic bacteria (SCB) while *Ophiocomina nigra* is non-symbiotic. Samples were taken of *Ophiothrix fragilis* at approximately two-week intervals for one year. Symbiotic bacteria numbers were determined by direct counting of homogenates of the arms of 50 individual brittlestars. Water samples were analysed for chlorophyll content. Stable isotope ratios for carbon and nitrogen were determined for each homogenate sample. Regular SCB counts were made on the infaunal brittlestar *A. chiajei*. Homogenate samples of *Ophiothrix fragilis*, *A. chiajei* and the non-symbiotic *Ophiocomina nigra* were analysed to produce fatty acid profiles for each species. Symbiotic bacteria count varied by up to one order of magnitude in both *Ophiothrix fragilis* and *A. chiajei* with no evidence of seasonality in this variation. Symbiotic bacteria number was inversely correlated with δ^{15}N but no relationship was established with δ^{13}C. 16:1ω7 and 18:1.ω7 fatty acids were used as putative bacterial markers. Both symbiotic species had higher percentages of 16:1ω7 than the non-symbiotic *Ophiocomina nigra*. However, only *Ophiothrix fragilis* appeared to receive appreciable quantities of 18:1ω7 from its SCB. The SCB are heterotrophic and may contribute to the nitrogen budget of the host. The two symbiotic species studied here derive the bulk of their nutrition from conventional feeding but SCB make significant, additional contributions.

INTRODUCTION

Many echinoderm species harbour symbiotic bacteria: the subcuticular bacteria or SCB (Holland & Nealson, 1978), including three of the four principal bed-forming brittlestar species found in European waters (McKenzie & Kelly, 1994). Dense beds of epifaunal brittlestars (often $>1000\,m^{-2}$) are a common feature of the continental shelf seas of the Atlantic coast of Europe. These beds are a striking benthic community and may have important roles in nutrient fluxes (Davoult et al., 1991) and in reducing phytoplankton densities (Hily, 1991). Equally common in European waters are dense beds of infaunal amphiurid brittlestars. These have been suggested as important food sources for benthic fish (Duineveld & Van Noort, 1986) and are often the dominant component of the macrofauna in soft-bottomed habitats (Buchanan, 1964). An obvious question is whether SCB contribute significantly to the biology of their host. The bacteria are estimated to form up to 1% of the ash-free dry weight biomass of brittlestar arms (McKenzie & Kelly, 1994). They are thus potentially important in the energy balance of the host, given that echinoderms have a very low mass specific metabolic rate compared to most bacteria (Siebers, 1979). Indeed, if SCB were respiring at similar rates to *Eshericha coli* (Neidhart et al., 1990), then the symbiont population in an arm would exceed quoted figures for oxygen uptake by brittlestar arms by one to two orders of magnitude (Pentreath, 1971). Symbiotic bacteria are frequently seen being phagocytosed by their hosts (e.g. McKenzie & Kelly, 1994) giving a possible route for symbiont–host energy flow. Reliance on symbionts for part of their nutrition may make the hosts more susceptible to certain types of ecological stress, such as pollution, if the stressors have a direct or indirect effect on the SCB. The presence of SCB also has profound implications for studies of many aspects of echinoderm biology, particularly those of the role of dissolved organics in nutrition (Siebers, 1979).

The nutritional role (if any) of SCB is unclear. All of their hosts have a normal gut and actively feed. This is in contrast to many of the described chemoautotrophic symbioses between bacteria and a variety of marine invertebrates where the role of the symbionts in host nutrition is usually self-evident (Cavanaugh et al., 1981). It is important not to judge the likely importance of this

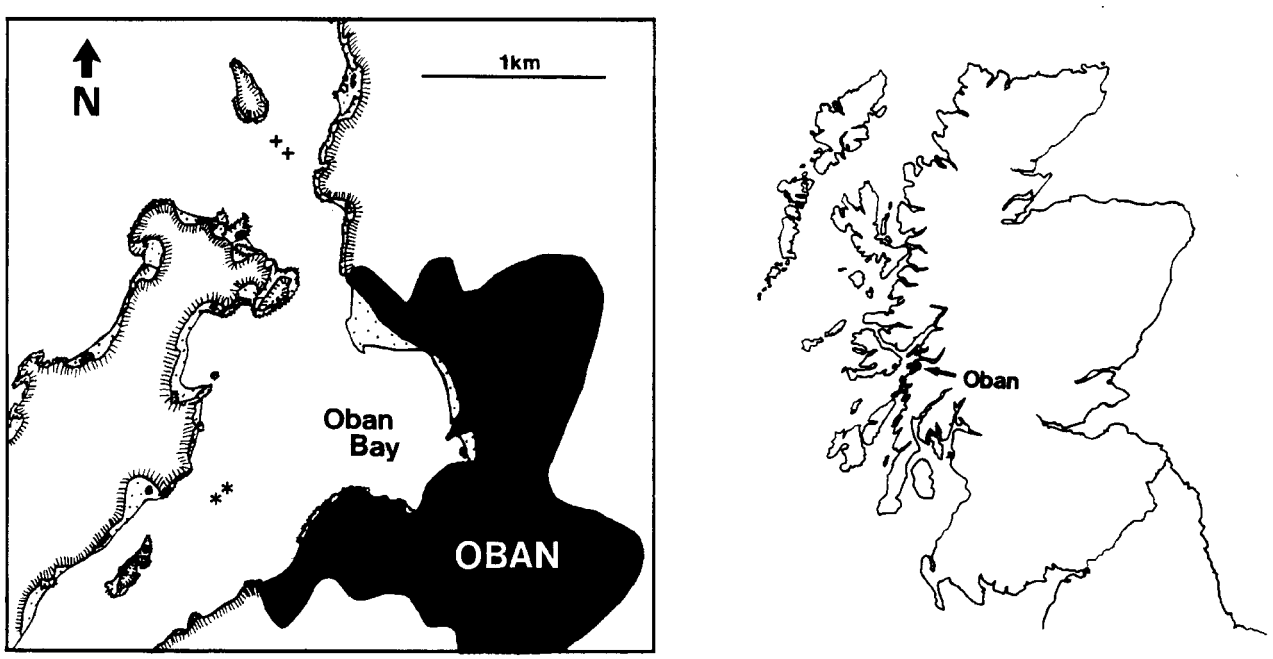

Figure 1. Maps showing Oban Bay and the location of Oban within Scotland. Sampling sites for *Ophiothrix fragilis* and *Ophiocomina nigra* indicated by + and *Amphiura chiajei* by *.

symbiosis against what we know of the chemoautotrophic ones. In the latter there is generally a bulk transfer of energy from symbiont to host and the symbionts are usually the most significant, though not always the sole, contributor to the nutrition of the host (e.g. Spiro et al., 1986; Conway & Capuzzo, 1990; Johnson et al., 1994; Pond et al., 1997). In contrast, many other symbioses are nutritional but not primarily energetic. The intracellular bacterial symbionts of blood-sucking insects, which provide their hosts with vitamins otherwise absent in their diet, are a good example of this (Douglas, 1994). Any role of the SCB in echinoderm nutrition is likely to be a smaller than those symbioses where the hosts feed feebly, if at all.

Symbiotic bacteria, like most marine symbiotic bacteria, have not been convincingly cultured (Kelly et al., 1995), preventing the use of most conventional microbiological methods for investigating their biology. Indirect methods of assessing the bacterial contribution to the energy balances of the host have been successfully used in studies of chemoautotrophic symbioses, particularly the use of stable isotope ratios and biomarker molecules (Conway & Capuzzo, 1990; see references in Conway et al., 1994 for stable isotopes). However, if the SCB are heterotrophic, then identifying signals associated with the bacterial component in nutrition of the host may be difficult to detect against the background 'noise' of the conventional heterotrophic feeding. These methods may therefore not provide the clear-cut evidence found in chemoautotrophic associations but they could still provide useful information. Brittlestars in temperate waters experience a seasonally variable diet (Warner, 1982), with phytoplankton much more common in the summer months than in winter. It may be possible to detect perturbations caused by changes in food sources and any influence of the SCB.

A detailed, year-long study has been made of the epifaunal, bed-forming brittlestar *Ophiothrix fragilis* to monitor variations in SCB load and relate these to carbon and nitrogen stable isotope fluctuations. To put these results into context, stable isotope and fatty acid profile studies have also been made of other brittlestar species, including *Ophiocomina nigra* which lacks SCB (McKenzie & Kelly, 1994). The results provide evidence of bacterial contribution to the nutrition of *Ophiothrix fragilis* that is probably derived from the SCB.

MATERIALS AND METHODS

Species studied

All of the species were collected from Oban Bay. *Ophiothrix fragilis* is a large, epifaunal, symbiotic brittlestar that lives in dense aggregations. It is a microphagous suspension-feeder, although it will also scavenge (Warner, 1982). *Ophiocomina nigra* is a non-symbiotic species of a similar size and habit to *Ophiothrix fragilis*. It is also primarily a microphagous suspension-feeder but has more developed carnivorous behaviour (Warner, 1982). The symbiotic *Amphiura chiajei* is a more fragile, surface deposit-feeding, infaunal animal. *Ophiura albida* is a small, epifuanal symbiotic species and little is known about its feeding habits.

Stable isotope and seasonality studies

Specimens of *Ophiothrix fragilis* and *A. chiajei* were collected approximately every two weeks between February 1992 and February 1993 by dredging between 30 and 40 m in depth at two sites in Oban Bay (see Figure 1). Due to the strong currents and very heterogeneous seabed at this site, other collection methods (such as grabbing) were impractical. Collection of *A. chiajei* did not start until

https://doi.org/10.1017/S0025315499001885 Published online by Cambridge University Press

March 1992. Any specimens of *Amphiura filiformis* in the samples were discarded. Animals were transferred to the Dunstaffnage Marine Laboratory as quickly as possible and kept in running seawater. The arms of 50 specimens of *Ophiothrix fragilis* were used to avoid any problems of contamination from non-assimilated material that might be present in the guts. Excess water was shaken off the arms and the weight recorded. Filtered seawater (<2 μm) was added at a ratio of 2:1 v:w; this generally resulted in a homogenate volume of 150–170 ml. The tissue was then homogenized using a heavy-duty blender. The calcite was allowed to settle out and the supernatant then removed. Samples of the supernatant were removed for immediate counting of bacteria using acridine orange and fluorescence microscopy (Kelly et al., 1995).

Symbiotic bacteria from the species of symbiotic brittlestars have a characteristic morphology making it easy to separate them from other bacteria in the homogenates. In fact bacteria obviously different from SCB were only rarely seen and previous studies have shown that the SCB contribute the vast majority of the bacterial total in the symbiotic species (McKenzie & Kelly, 1994).

Stable isotope analysis was by continuous flow isotope ratio mass spectrometry (CF-IRMS). This was carried out using a Europa Scientific Tracermass with a Roboprep-CN combustion sample converter, or a Europa Scientific ANCA-NT 20-20 Stable Isotope Analyser with ANCA-NT Solid/Liquid Preparation Module. These were operated in the dual isotope mode, allowing δ^{15}N and δ^{13}C to be measured on the same sample. For the Tracermass system, the analytical precision (SD, N=5) for invertebrate material analysed using the dual isotope mode was ±0.7 per ml for δ^{15}N and ±0.4 per ml δ^{13}C, as estimated from standards analysed along with the samples. With the NT system, the precision improved to ±0.2 per ml for both nitrogen and carbon. Working standards were 1 mg leucine prepared by freeze-drying 50 ml of a 20 mg/ml stock solution into the tin cups, and calibrated against 'Europa flour' and International Atomic Energy Agency standards N1 and N2.

Specimens of *O. fragilis*, *Ophiocomina nigra*, *Amphiura chiajei* and *Ophiura albida* were collected from Oban Bay in May 1991 (*O. albida* from same site as *Ophiothrix fragilis* and *Ophiocomina nigra*) and separate samples of arms only, discs only and whole individuals homogenized in filtered seawater. The non-soluble material was collected on a Whatman GF/F filter, scraped off the filter and dried at 40°C. The material was then ground to a paste using a pestle and mortar and analysed for δ^{15}N and δ^{13}C using the Tracermass. It was difficult to obtain consistent results from these samples, largely due to intrasample physical heterogeneity. This was possibly due to a high content of cohesive lipids making it difficult to grind the material down into sufficiently fine particles. Samples of *Ophiothrix fragilis* for stable isotope analysis taken as part of the seasonal study were processed (after an aliquot had been removed for SCB counting) into a 'non-lipid' fraction and a 'lipid' fraction in an attempt to avoid this problem of sample heterogeneity. Chloroform (200 ml) and methanol (400 ml) were added and the mixture shaken vigorously for 5 min. A further 200 ml of chloroform was added and shaken for 2 min then 200 ml of distilled water was added to the mixture and shaken for a further 2 min. The mixture was then allowed to settle, after which the chloroform-extracted lipid-fraction was drawn off. The precipitated non-lipid and methanol extract were filtered and 1M HCl was added to remove any remaining calcite (indicated by no further bubbling on the addition of fresh HCl). The samples were then refiltered and dried at 40°C ('non-lipid fraction'). Non-lipid fraction samples were finely ground in a ball mill and 1 mg samples, weighed into 6×4 mm tin cups for stable isotope analysis. Lipid-fraction samples were taken up in dichloromethane and 10–50 μl aliquots put into tin cups containing solvent-washed Chromosorb AW. The solvent was removed by gentle warming before closing the cups. Sample size was adjusted to give ~1 mg of lipid per cup. Samples of *Ophiocomina nigra* and *Ophiothrix fragilis* were taken in August 1994 (arms from 15 individuals of each species; 15.81 g and 32.21 g wwt respectively) and analysed as 'whole' arms (i.e. not treated to extracted lipids) as well as 'non-lipid' fraction and 'lipid' fraction. These results were compared to determine what change in δ value resulted from the fractionation treatment. A homogenate of *A. chiajei* arms (100 animals, 16.63 g wet weight, ww) was processed to give a 'non-lipid' fraction.

Water samples were taken from above the *O. fragilis* bed at 5 and 35 m using an inverted water bottle. On

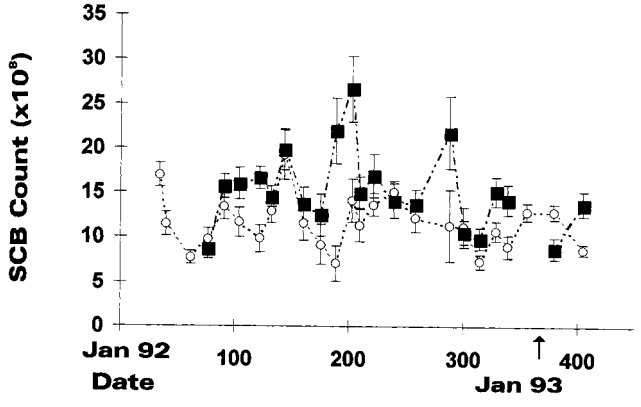

Figure 2. Temporal fluctuations in symbiotic bacteria (SCB) counts (per gram tissue ash-free dry weight—see McKenzie & Kelly, 1994 for conversion factors) for *Ophiothrix fragilis* (○) and *Amphiura chiajei* (■) from Oban Bay. Error bars are 95% confidence limits. However, note that the error bars relate to internal variation within each homogenate and not to population variation.

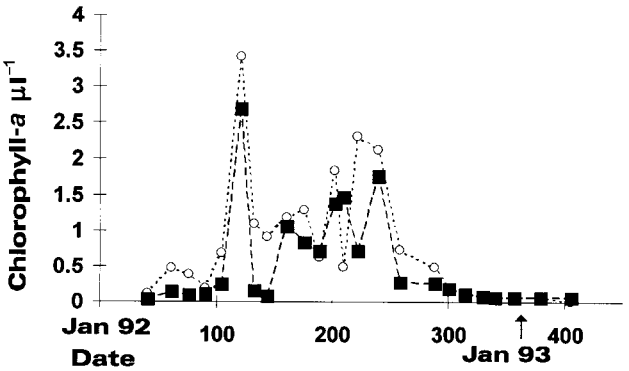

Figure 3. Seasonal variation in chlorophyll from Oban Bay. Chlorophyll concentrations at in water samples taken at 5 m (○) and 36 m (■).

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Table 1. *Isotopic signatures associated with whole and portions of brittlestars (±SE)*

	Ophiothrix fragilis	*Amphiura chiajei*	*Ophiocomina nigra*	*Ophiura albida*
δ^{13}C whole			−17.0	−21.45 ±0.76
δ^{13}C arms	−18.56 ±0.25	−18.94 ±1.77	−16.58 ±0.62	
δ^{13}C discs	−18.37 ±0.55	−19.47 ±1.37	−17.78 ±0.01	
δ^{15}N whole			12.76	11.77 ±0.64
δ^{15}N arms	12.40 ±1.25	12.14 ±1.73	14.7	
δ^{15}N discs	12.52 ±1.58	12.35 ±2.18	12.67 ±0.09	

returning to the laboratory the water samples (<0.2 μm) were filtered onto a glass fibre filter (Whatman GF/F). The filter was then folded in half, placed in a sample tube and frozen at −20°C prior to analysis. Neutralized acetone (8 ml) was added to each tube and the samples left for 24 h at approximately 4°C. After the 24 h, the tubes were inverted several times then spun in a centrifuge for 5 min at 3000 rpm. Analyses of chlorophyll content was made using a Turner Designs Mk10 fluorimeter (Jones et al., 1984). All the chlorophyll samples were analysed at the same time and corrected for phaeophytin content.

Fatty acid analyses

Samples were taken in November 1994, February 1996 and November 1996 from Oban Bay (see Figure 1). For the first two sampling dates, arms from approximately 100 *Amphiura chiajei*, 50 *Ophiocomina nigra* and 50 *Ophiothrix fragilis* were severed and total lipid extracted by the Folch-Lee method (Folch et al., 1957). On the third date, ten individuals of each species were similarly processed but analysed separately to give an estimate of individual variation. Arms only were used for the fatty-acid studies both to maintain consistency with the SCB and stable isotope studies but also to minimise the contribution of non-polar, storage lipids to the analyses by excluding gonad tissues. Fatty acid methyl esters (FAMES) were prepared according to the acid catalysed method of Christie (1989). The crude esters were purified by thin-layer chromatography (TLC) using heptane: diethyl ether: acetic acid (70:30:1.5) as eluent. The purified esters were taken up in heptane containing one drop of a methanolic solution of butylated hydroxytoluene (2%) and stored in the freezer under nitrogen. FAMES were separated, in triplicate, using Chrompack CPSil 88 and SGE FFAP WCOT fused silica columns by GC (Carlo Erba 4160). FAMES were identified by comparison of their retention times with those of standards and confirmed by GC-MS (Carlo Erba 8000 GC coupled to an MD800 MS). Reliable FAMES could not be obtained from individual *Amphiura chiajei* because of the low levels of lipid recovered from this small species.

Table 2. *Effects of lipid removal on isotope ratios of brittlestars.*

Treatment	*Ophiothrix fragilis*	*Amphiura chiajei*	*Ophiocomina nigra*
δ^{13}C whole	−17.71		−15.66
δ^{13}C processed	−15.30	−16.035	−14.59
δ^{15}N whole		11.48	13.49
δ^{15}N processed	11.785	12.70	13.65

RESULTS

Stable isotope and seasonality studies

Symbiotic bacteria counts for *Ophiothrix fragilis* and *Amphiura chiajei* are shown in Figure 2. The differences in counts indicate fluctuations in bacterial load of between 0.71×10^9 to 1.92×10^9 SCB g^{-1} ash-free dry weight (AFDW) for *O. fragilis* and 0.87×10^9 to 2.66×10^9 SCB g^{-1} AFDW for *A. chiajei*. Mean SCB loads were 1.16×10^9 and 1.51×10^9 SCB g^{-1} AFDW respectively. The two sets of data show no significant correlation with each other (Spearman's rank correlation), but there are similarities between them in direction of change (though not scale). The *A. chiajei* data are perhaps slightly advanced in trend from the *O. fragilis* data but exceptions exist. The fluctuations were not obviously related to season in either species nor were any significant correlations found between SCB count and chlororophyll concentrations. Chlorophyll content showed the expected pattern of chlorophyll

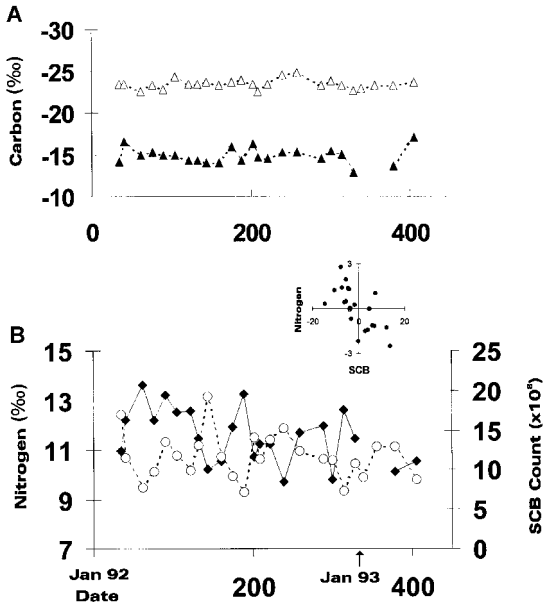

Figure 4 *Ophiothrix fragilis*. Temporal fluctuations in stable isotope ratios. (A) δ^{13}C for 'lipid fraction' (△) and 'non-lipid' (▲); (B) temporal variation in δ^{15}N (◆) and SCB count (○). Insert shows plot of change in δ^{15}N against change in SCB count between consecutive sampling periods.

https://doi.org/10.1017/S0025315499001885 Published online by Cambridge University Press

Table 3. *Recovery of total lipid from brittlestar limbs, November 1994.*

Species	Tissue wet weight (g)	Lipid recovered (mg)	%
Ophiothrix fragilis	54.0	944.0	1.75
Amphiura chiajei	18.0	114.5	0.64
Ophiocomina nigra	62.7	412.0	0.66

concentrations for a temperate, inshore locality, with winter minima, a sharp rise in the spring months then fluctuating levels in the summer and autumn followed by a decline again in the winter (Figure 3). Both 5 and 36 m water samples showed similar trends with the 36 m sample regularly having a slightly lower concentration of chlorophyll than the 5 m sample.

Results for the stable isotopes from simple, untreated homogenates of four brittlestar species are shown in Table 1. The variability of subsamples taken from each sample was high and the reproducibility of values from repeats of subsamples was poor indicating sample heterogeneity. This makes it difficult to meaningfully compare the differences between the species but the isotope values for carbon are in the expected range for heterotrophic sources feeding on phytoplankton.

The results for comparisons between whole arms and those processed into 'lipid' and 'non-lipid' fractions are shown in Table 2. The ball-milling has reduced the sample heterogeneity in the 'whole' samples, though the observed values for δ^{15}N and δ^{13}C were similar to the values

Table 4. *Mean (N=10) fatty acid profiles from brittlestars, November 1996.*

	Fatty acid	*O.f.*	SD	*O.n.*	SD	ANOVA
	14:0	6.30	1.17	5.30	0.87	NS
	i15:0	0.20	0.04	0.30	0.07	<0.01
	a15:0	0.20	0.05	0.20	0.04	NS
	15:0	0.20	0.04	0.40	0.08	<0.01
	i16:0	—	—	—	—	—
	16:0DMA	0.60	0.22	0.20	0.08	<0.01
	16:0	9.30	1.28	5.40	1.38	<0.01
	16:1ω7	4.40	0.58	1.10	0.22	<0.01
	16:2	1.00	0.14	1.10	0.19	<0.01
	i17:0	—	—	—	—	—
	17:0	—	—	—	—	—
	17:1	—	—	—	—	—
	18:0DMA	3.30	0.57	0.60	0.10	<0.01
	16:3	2.20	0.26	0.40	0.09	<0.01
	18:0	7.90	1.49	17.60	4.79	<0.01
	18:1DMA	3.30	0.57	0.60	0.10	<0.01
	18:1ω9	1.40	0.14	1.20	0.50	NS
	18:1ω7	6.80	0.61	1.70	0.37	<0.01
	16:4	2.70	0.64	—	—	—
	18:2ω6	0.50	0.08	0.70	0.27	NS
	20:1ω9	7.70	1.30	15.40	2.49	<0.01
	18:3ω3	1.40	0.14	0.10	0.07	<0.01
	18:4ω3	*	*	*	*	*
	20:2ω6	0.50	0.06	0.60	0.12	<0.01
	20:4ω6	1.80	0.22	4.70	0.79	<0.01
	22:1ω9	0.30	0.06	0.20	0.38	<0.01
	22:1ω11	0.30	0.08	0.10	0.14	<0.01
	20:5ω3	21.20	2.12	9.30	2.27	<0.01
	22:5ω3	0.10	0.04	0.30	0.10	<0.01
	22:6ω3	0.20	0.10	0.20	0.15	NS
	24:4ω6	—	—	0.21	0.10	—
	24:5ω6	0.80	0.18	0.50	0.21	<0.05
	24:5ω3	1.00	0.07	0.20	0.12	<0.01
	24:6ω3	3.70	0.70	6.60	2.40	<0.01
	26:5ω3	*	*	*	*	*
	26:6ω3	*	*	*	*	*
Totals		91.20		82.40		
Unidentified		8.80		17.60		
Saturates		29.60		37.10		
Monounsaturates		24.50		20.50		
ω6 polyunsaturates		0.54		6.60		
ω3 polyunsaturates		27.60		16.70		
ω6/ω3		0.02		0.40		

SD, standard deviation; DMA, dimethyl acetal; i, iso; a, anteiso. *O.f.*, *Ophiothrix fragilis*; *O.n.*, *Ophiocomina nigra*. 18:4:ω3 co-eluted with 19:0; 26:5ω3 and 26:6ω3 were beyond the length of the separation. ANOVA carried out on arc-sine transformed data.

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Table 5. *Fatty acid profiles from pooled samples of brittlestars.*

	Fatty acid	*O.f.*94	*O.f.*96	*A.c.*94	*A.c.*96	*O.n.*94	*O.n.*96
	14:0	7.1	5.5	5.3	4.8	4.4	3.8
	i15:0	0.3	0.2	1.3	0.6	0.6	0.2
	a15:0	0.1	—	0.4	—	0.3	—
	15:0	tr	0.4	0.7	0.5	0.3	0.3
	i16:0	—	—	0.2	—	0.3	—
	16:0DMA	1.5	—	0.9	0.8	0.7	0.4
	16:0	9.5	12.4	4	4.4	3.4	3.4
	16:1ω7	5.3	5.5	4.3	3.1	1.3	1.3
	16:2	tr	tr	tr	0.4	tr	tr
	i17:0	tr	tr	tr	tr	tr	tr
	17:0	tr	tr	tr	tr	tr	tr
	17:1	tr	tr	tr	tr	tr	tr
	18:0DMA	2.7	2.4	9.4	10.3	11.5	12.4
	16:3	1.4	1.7	0.4	0.6	0.2	0.4
	18:0	5.8	4.7	9.8	7.2	8.3	7.0
	18:1DMA	4	—	0.8	—	0.8	—
	18:1ω9	1.4	9.2	4	2.4	1.7	1.9
	18:1ω7	7.7	4.8	2.8	2.5	1.8	2.1
	16:4	5.7	1.3	tr	—	0.8	—
	18:2ω6	1.6	2.2	2	1.3	0.7	0.2
	20:1ω9	5.7	5.4	12	12.5	11.8	13.4
	18:3ω3	0.2	0.6	0.4	0.3	0.2	—
	18:4ω3	2.3	2.0	0.4	0.5	0.2	0.6
	20:2ω6	0.4	0.4	1.6	0.8	1.2	0.7
	20:4ω6	1.5	1.2	7.6	8.1	6.1	6.1
	22:1ω9	tr	tr	tr	tr	tr	tr
	22:1ω11	tr	6.4	tr	1.1	tr	1.4
	20:5ω3	23	13.4	11.6	10.5	14.6	15.1
	22:5ω3	0.3	0.7	0.2	tr	0.2	—
	22:6ω3	0.2	3.4	—	0.6	0.2	0.5
	24:4ω6	—	0.5	0.2	0.7	tr	0.9
	24:5ω6	—	—	0.7	1.0	0.6	0.4
	24:5ω3	0.9	0.4	1.3	1.2	0.7	0.7
	24:6ω3	3.4	2.8	11.2	12.0	15.5	14.4
	26:5ω3	—	—	—	—	tr	—
	26:6ω3	—	—	—	0.4	3.0	2.1
Totals		91.9	89.1	93.6	88.8	91.6	77.0
Unidentified		8.1	10.9	6.4	11.2	8.4	23.0
Odd and branched		0.4	0.6	2.6	1.1	1.5	0.5
Saturates		27.0	25.6	32.0	28.6	29.8	27.5
Monounsaturates		24.1	31.3	23.9	21.6	17.4	20.1
ω6 polyunsaturates		3.5	4.3	12.1	11.9	8.6	8.3
ω3 polyunsaturates		30.3	23.3	25.1	25.5	34.6	33.4
ω6/ω3		0.12	0.18	0.48	0.47	0.25	0.25

SD, standard deviation; DMA, dimethyl acetal; i, iso; a, anteiso; tr, trace. *O.f.*, *Ophiothrix fragilis* (N=50); A.c., *Amphiura chiajei* (N~100); *O.n.*, *Ophiocomina nigra* (N=50). 94 and 96 refer to years sampled.

obtained for the untreated homogenates (Table 1). The extraction methods produce no discernible change in δ^{15}N values in any of the species. With δ^{13}C, there was an enrichment of ^{13}C relative to 'whole' tissues. In *O. fragilis* this was approximately 2.5 psu. In *Ophiocomina nigra* a smaller enrichment was observed (~1 psu). The results of this analysis appear to confirm those of the earlier, untreated samples, with *O. nigra* having a slightly higher δ^{15}N than the other species and a less pronounced depletion of δ^{13}C.

Both nitrogen and carbon isotope ratios showed sizeable fluctuations over the study period (Figure 4). Nitrogen ratios varied between 9.72 and 13.61 (mean ±SD=11.55 ±1.12). Lipid carbon ratios were more stable and consistently showed an increased depletion of ^{13}C (−22.52 to −24.82, mean=−23.45 ±0.55) relative to the methanol fraction carbon ratios (−12.93 to −17.07, mean=−14.894 ±0.95). There was no correlation between the lipid carbon and methanol fraction carbon ratios. There was no particularly obvious temporal pattern to the isotope fluctuations. However, a significant negative correlation ($P<0.05$) was found between SCB count and δ^{15}N (Figure 4B). Change in SCB count and change in δ^{15}N (Figure 4B) between consecutive sampling

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periods show a strong negative correlation ($P<0.001$). Three of the plot points do not follow the main pattern (those in the upper right quadrant and lower left quadrant). Two of these are consecutive and are followed by a point which is only just in the upper left quadrant. However, the remaining exceptional point is isolated as is the point which is only just in the lower right quadrant.

Fatty acid profiles

All three species contained a wide range of identifiable fatty acids (Tables 3, 4 & 5). Odd-chained fatty acids were detected but only 15 carbon-chain acids were present in greater than trace amounts. Notable features of the profiles were the presence of very long chain polyunsaturates (>24 carbon-chains) and a number of dimethyl acetals (DMA), three of which (16:0 DMA, 18:0 DMA and 18:1 DMA) were present in significant concentrations. Analysis of individual (N=10) *Ophiothrix fragilis* and (N=10) *Ophiocomina nigra* (individual *A. chiajei* did not have sufficient lipid for FAME analysis) found very little intraspecific variation between individuals of the same species but highly significant interspecific variation in FAME's (Table 4). This low level of individual variation suggests that comparisons between pooled samples are valid. *Amphiura chiajei* and *O. nigra* had very similar fatty acid profiles to each other but those of *Ophiothrix fragilis* were noticeably different. Neither *A. chiajei* nor *Ophiocomina nigra* showed any remarkable intraspecific differences in their samples. There were, however, obvious differences in the *Ophiothrix fragilis* profiles. At all three sampling dates there were large amounts of 20:5ω3 present compared with the other two species but this fatty acid was proportionally much more common in the November samples of *O. fragilis*. *Ophiothrix fragilis* had notably higher percentages (at least double in each case) of 16:0, 16:3, 18:1 DMA, 18:1ω7, 16:4, 18:4ω3 and 22:1ω11 (last February 1996 sample only) than the other species. In contrast, *A. chiajei* and *Ophiocomina nigra* usually had at least double the percentage concentration of 18:0 DMA, 20:1ω9, 20:4ω6, and 24:6ω3 than was found in the *Ophiothrix fragilis* samples. Obvious interspecific differences between *A. chiajei* and *Ophiocomina nigra* were more difficult to find. Although *A. chiajei* had more than twice the amount of 15:0i, 18:1ω9 and 18:2ω6 than *O. nigra*, 15:0i and 18:2ω6 occurred in rather low overall concentrations, making it difficult to judge if the differences between the species were valid. The only clear case where *A. chiajei* resembles *Ophiothrix fragilis* more than *Ophiocomina nigra*, is for 16:1ω7, where both of these species had over twice the amount of this fatty acid as is found in *O. nigra*. *Ophiocomina nigra* was the only species to have a significant amount of 26:6ω3.

DISCUSSION

Stable isotope and seasonality studies

Stable isotope ratios and fatty acid analyses are most powerful in researching simple systems (e.g. Ramsay & Hobson, 1991). As system complexity increases so the conclusions of such analyses are of necessity more cautious. However, even in highly structured food-webs, useful information can be obtained (e.g. Peterson & Howarth, 1987; Kharlamenko et al., 1995). The current study is the first to use these techniques to investigate the role of presumed heterotrophic symbionts of a marine invertebrate *in situ*. As such it is also instructive as to the possibilities and limitations of these methods in highly complex nutritive symbioses such as the echinoderm–SCB symbiosis.

Symbiotic bacteria number was variable within a single order of magnitude in both *Ophiothrix fragilis* and *Amphiura chiajei* but there was neither obvious seasonality nor any link to a single external factor, such as chlorophyll count. Ophiuroids have high levels of individual variation in SCB load (McKenzie & Kelly, 1994) but the large numbers of individuals used to produce the homogenates used in the current study would have eliminated this as a significant factor in the overall variability. As samples were not taken from exactly the same place each time, it could be argued that these fluctuations are due to spatial rather than temporal effects. However, just as the use of pooled homogenates will 'smear' individual variation so the use of dredged samples will tend to break up any microspatial variation as the individual animals will come from a larger area than would be the case with grab sampled animals. The fluctuations in SCB load are, however, likely to be genuine, temporal effects. This natural variation in SCB load has consequences for their use as *in situ* indicators of sublethal stress (Newton & McKenzie, 1995) particularly the need for good control sites when evaluating the effects of pollution on SCB load. Changes in SCB load could result from variation in bacterial replication and senescence rates or from changes in host cropping rate. The former would be caused by changes in nutrient supply, temperature or alteration of any host mechanisms controlling bacterial growth.

The δ^{13}C results from all four species are what would be expected of organisms forming part of a phytoplankton-dominated food chain. Phytoplankton in northern temperate waters generally have signatures around −22 psu (Boutton, 1991). Chemoautotrophic associations either have heavier tissue values (< −12 psu), as in the case of vestimentiferans (e.g. Rau, 1981) or are much lighter (usually > −25 psu), as in the various bivalve associations (cf. Kennicut et al., 1992). Isotopic signatures of carbon from marine invertebrates are usually enriched by only 1 psu of the dietary signature (Michener & Schell, 1994). The small but consistent enrichment of ^{13}C within *Ophiocomina nigra* compared with *Ophiothrix fragilis* and *A. chiajei* may reflect the greater importance of predation to its diet. Lipid stable isotope signatures are generally 7 psu lighter than the whole animal signature for δ^{13}C (Johnston et al., 1995) and this is the case here, with the non-lipid component showing a proportional small increase in δ^{13}C compared to the whole animal. Brittlestar arms were used preferentially to avoid any distortion of the signatures from non-assimilated gut contents. However, comparison of the signatures for arms versus discs or whole animal (Table 1) suggest that there are no significant differences between different parts of the animal.

The data for δ^{15}N showed little change in processed and unprocessed tissues (Table 2) and δ^{15}N values were similar regardless of which part of the body from which

https://doi.org/10.1017/S0025315499001885 Published online by Cambridge University Press

they were obtained. Similar $\delta^{15}N$ values have been found in other echinoderms (Van Dover & Fry, 1989). The source of the $\delta^{15}N$ in the brittlestars is uncertain. Marine invertebrates show a range of values for enrichment of $\delta^{15}N$ relative to their diet with +3 psu being considered the norm (Michener & Schell, 1994). However, the information available on typical marine $\delta^{15}N$ values is scanty compared to $\delta^{13}C$. In part this is because inshore sources of nitrogen tend to have very similar isotopic ratios (Michener & Schell, 1994) making it a less favoured choice compared with carbon in tracer studies. The values were considerably heavier than those of chemoautotrophic symbioses (Van Dover & Fry, 1989). The negative correlation between SCB load and $\delta^{15}N$ values suggest that the bacteria may be a significant source of nitrogen to their hosts. Alternatively, both SCB loadings and host $\delta^{15}N$ are being simultaneously altered by a unknown factor. The change in $\delta^{15}N$ is far in excess of what can be explained merely by the change in bacterial biomass. Either the SCB are releasing nitrogen compounds which are absorbed by the host or the correlation between $\delta^{15}N$ and SCB number reflects a deeper correlation with bacterial production and cropping rates. There is no matching correlation between SCB load and $\delta^{13}C$, so SCB either do not form a significant carbon source for their hosts or their $\delta^{13}C$ signature is indistinguishable from the other food sources (or both). Stable isotope tracer experiments would be the obvious way forward in confirming and quantifying the transfer of SCB-derived nitrogen to their hosts and measuring any carbon flux.

The lack of obvious seasonality in the isotope data for both $\delta^{13}C$ and $\delta^{15}N$ in the face of the distinct seasonality in chlorophyll content is surprising. *Ophiothrix fragilis* feeds mostly on phytoplankton and other suspended particles (Warner, 1982). This was supported by the fatty acid profiles (see below) and the stable isotope results. As stable isotope measurements usually reflect the recent dietary history of an animal (Michener & Schell, 1994), the lack of phytoplankton in the winter months might be expected to produce some perturbation in the isotope ratios in *O. fragilis*. Assimilation rates will also vary with temperature, producing changes in the isotope signature that might mask changes in the dietary signature. Arm tissues, though rapidly growing, may not turnover quickly which would decouple the relationship between isotope ratio and recent diet. Gonad tissue, which shows seasonal growth, may be better at reflecting recent diet than arm tissue. Any influence of the SCB may also be masking deeper seasonal variation in stable isotope ratios. Another factor is seasonal variation of stable isotope ratios in the phytoplankton which may offset any biomass related shifts in stable isotope. Without any direct measurements of the stable isotope ratios in the phytoplankton we cannot comment further. Johnson et al. (1994) found that $\delta^{13}C$ did vary over a year in the symbiont-containing bivalve *Loripes lucinalis*, with evidence of an increased bacterial contribution to host diet between January and May. The degree of variation in $\delta^{13}C$ was very similar to that observed in the present study. They did not measure fluctuations in symbiont numbers to see if the changes in $\delta^{13}C$ were correlated with loadings, nor did they measure chlorophyll variation.

Fatty acid studies

The fatty acid analyses were not intended to be an exhaustive lipid analysis but rather an initial attempt to discern any evidence of SCB involvement in host nutrition. The analyses of *O. fragilis*, *A. chiajei* and *Ophiocomina nigra* revealed a complex picture with numerous unusual features. *Ophiothrix fragilis* had fatty acid profiles that were distinctly different from the other two species (Tables 4 & 5). Most of the differences appear related to *O. fragilis* being more exclusively a suspension-feeder than either *A. chiajei* (which is a deposit-feeder with opportunistic scavenging tendencies) or *Ophiocomina nigra* (which does suspension-feed but is also an active carnivore and scavenger) (Warner, 1982). Although 16:0, 16:4, 18:4ω3, and 20:5ω3 can all arise from various dietary origins, in combination and in the levels found in *Ophiothrix fragilis*, they suggest a diet rich in phytoplankton, particularly diatoms (Volkman et al., 1989; Kharlamenko et al., 1995). This would fit with previous observations of the feeding behaviour of *O. fragilis* (Warner, 1982). While care has to be taken in ascribing reasons to temporal variations where only three sample periods are available, the lower amounts of 16:4 and 20:5ω3 seen in the February 1996 sample compared with the November 1994 and November 1996 samples for *O. fragilis* could be interpreted as a decline in importance of diatoms in the diet of *O. fragilis* in the later winter months. The smaller contribution of these fatty acids to the profiles of *A. chiajei* and *Ophiocomina nigra* would indicate that phytoplankton are less important in the diet of these two species regardless of season. The presence of significant amounts of 20:4ω6 in *A. chiajei* and *O. nigra* may indicate a macroalgal contribution to their diet (Yongmanitchai & Ward, 1989; Kharlamenko et al., 1995) though it could also be biosynthesized by the brittlestars. Although 20:1ω9 is a major component of calanoid copepods, the absence of significant amounts of 22:1ω11 in either *A. chiajei* or *O. nigra* suggests that the 20:1ω9 is being synthesised *de novo* (Sargent et al., 1983). The increase in the latter fatty acid in the February 1996 sample may, however, indicate the ingestion of some copepod material by *Ophiothrix fragilis*, possibly as particulate lipids (Sargent & Henderson 1986). An unusual feature is the low importance of 22:6ω3 in all three species despite the presence of other markers for phototrophic microplankton. The unusually high concentrations of 24:6ω3 may indicate that 22:6ω3 derived from dietary phytoplankton is being elongated to the former.

A major aim of the fatty acid analysis was to look for bacterial signals that might indicate contribution of SCB to their hosts' diet. Odd-chained and branched fatty acids are often associated with benthic, mostly anaerobic, marine bacteria (e.g. Perry et al., 1979; Kharlamenko et al., 1995). Such fatty acids were present in all three species but mostly in trace amounts (Table 2). Potentially more significant are the differing levels of 16:1ω7 (palmitoleic acid) and 18:1ω7 (*cis*-vaccenic acid). These are often considered good markers for bacteria (Perry et al., 1979; Sargent et al., 1983; Kharlamenko et al., 1995; Pond et al., 1997, 1998) and have been shown to occur at high levels in some symbioses, presumably of bacterial origin (Conway & Capuzzo, 1990; Fullarton et al., 1995; Pond

et al., 1998). 16:1ω7 is, however, present in many marine phytoplankton and invertebrates so it could have other origins. What is informative, however, is not the presence or even the quantity of 16:1ω7 present but the ratio between symbiotic and non-symbiotic species. As this fatty acid is present at much higher amounts in the two symbiotic species studied than in the non-symbiotic species, it may be providing evidence of SCB contribution to their hosts' fatty acid pool.

If the 18:1ω7 resulted from the ingestion of external bacteria, it should be proportionally higher in the deposit-feeding *A. chiajei* and present in equal, but small amounts in *O. fragilis* and *Ophiocomina nigra*. Instead, it was highest in *Ophiothrix fragilis*. This excess 18:1ω7 was probably largely obtained from SCB as *O. fragilis* has no obvious access to a significant external source of bacterial lipids. In a wider study of fatty acid profiles in echinoderms, we have found that symbiotic species consistently have higher levels of 18:1ω7 than non-symbiotic species (unpublished data). Takagi et al. (1986) found similar levels (6.3% total lipid) of 18:1ω7 in *Asteronyx loveni*, a predatory ophiuroid known to have SCB (McKenzie & Kelly, 1994). Interestingly, Sargent et al. (1983) found high levels of 18:1ω7 in the gonads of two asteroids, *Ctenodiscus crispatus* and *Pteraster militaris* compared to a third species, *Asterias linki*. This was suggested to be the result of differences in their diets. However, *C. crispatus* and *P. militaris* probably have SCB as all the other species of asteroid that have been examined in their respective orders have them while all the studied species of Forcipulatida (including the genus *Asterias*), lack them (McKenzie et al., 1998). The presence of unusual undersaturation associated with non-methylene interrupted unsaturated fatty acids derived from 18:1ω7 has also been suggested as an indicator of bacterial contribution (Fullerton et al., 1995). This may be a valuable approach for further studies.

CONCLUSIONS

Results from this study reinforce the view (McKenzie & Kelly, 1994) that the SCB–echinoderm symbiosis is heterotrophic. Chemoautotrophic associations have different fatty acid profiles and different stable isotope signatures. This is in addition to the differences in habitat, bacterial morphology and host adaptation to symbiont accommodation seen in many (though not all) of the chemoautotrophic symbioses. Enzyme studies and molecular probes for specific bacterial genes related to nutrition are probably the best way to further resolve the actual nutritive mode of SCB while *in situ*. Careful experimental manipulation of the symbiosis will be necessary to provide a definitive figure relating SCB activity to host fitness. The inverse correlation between δ^{15}N and SCB number suggests a possible role for SCB in the nitrogen budget of their hosts. The presence of significantly larger amounts of 16:1ω7 in both symbiotic species compared to the non-symbiotic *Ophiocomina nigra* and of 18:1ω7 in *Ophiothrix fragilis* also suggests a contribution to the nutrition of their host. These results could, however, be the consequence rather than the contribution of SCB to host nutrition. The vital contribution may involve trace substances rather than just bulk transfer of energy. The study shows that fatty acid profiles and stable isotope natural abundance studies provide interesting clues as to the role of heterotrophic symbionts. These techniques will prove useful tools during experimental studies of SCB–host interactions.

This work was funded by Mobil North Sea Ltd and by the Natural Environment Research Council (GR9 335). We would like to thank Dr John Leftley for useful information on algal fatty acids, the Director of the Dunstaffnage Marine Laboratory for facilities and the crew of the 'Seol Mara' for procuring samples. Special thanks to Rachel Carrie for additional fatty-acid analysis.

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Submitted 12 April 1999. Accepted 1 November 1999.