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- ☐ NEW PROPOSAL
- ☐ CONTINUING PROJECT
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FISHING INDUSTRY RESEARCH TRUST ACCOUNT

TITLE OF PROPOSAL/PROJECT: Bacterial pathogens of oyster larvae and spat

ORGANISATION: University of Tasmania

PERSON(S) RESPONSIBLE: CD GARLAND and TA McMEEKIN

FUNDS SOUGHT/GRANTED

YEAR	SOUGHT	GRANTED
1983/84		$41,233

RELATED APPLICATIONS:

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Secretary
Fishing Industry Research Committee

F139–12/84

FINAL REPORT

FIRTA Grant 82/48

Bacterial pathogens of oyster larvae and spat

by

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Period of original Grant: July 1982 - June 1984
Extended period: July 1984 - June 1985

General Aims:
To examine the role of bacteria as agents of infection in larval Pacific oysters (*Crassostrea gigas*) cultured under high density conditions. To study bacteriological aspects of post-larval and juvenile blacklip abalone (*Haliotis ruber*) laboratory-reared on natural substrata.

General Comment:
Most of the major results have been reported earlier (see FIRC Annual Report 1983-84). This report is a more detailed account. The project followed 4 distinct strands. Please note that the first 3 strands, commenced under FIRTA Grant 82/48, have been completed only very recently under FIRTA Grants 84/48 and 85/19. The delay in completion was due to a change in the original aims of the project, due to very severe losses of larval oysters from bacterial disease sustained at a commercial hatchery in Tasmania between August 1983 and April 1984. Accordingly, the major emphasis of the project shifted from the original plan for laboratory/experimental studies to hatchery-based "problem-solving" studies.

Strand 1: Bacterial disease in hatchery-reared larval Pacific oysters (*C. gigas*).

Increased production and bacteria in feed microalgae

These studies are conducted at a commercial hatchery for rearing larval Pacific oysters (*C. gigas*) operated by Shellfish Culture Pty. Ltd. at

Bicheno, Tasmania. Hatchery production can be measured as the number of eyed larvae ready-to-set. As shown in Table 1, production for 1984-85 was 106.5 x 10^6 ready-to-set larvae. Compared to the previous season, production was increased by a factor of 3.5 x, due to the increase in larval survival from 1.4% (of total eggs spawned) in 1983-84, to 3.2% in 1984-85. Larvae were produced in a significantly shorter time per batch in 1984-85 (18.5 days, first ready-to-set larvae seen) compared to the previous season (23.5 days), resulting in further cost savings. The improved larval survival was due mainly to greatly decreased losses from bacterial disease.

The major vector for bacterial disease in the hatchery has been identified as the feed microalgae. As shown in Table 1, the mean bacterial level in the feed microalgae was 2.4 x 10^6 ml^{-1} in 1983-84 but was reduced to 6.2 x 10^5 ml^{-1} in 1984-85 (significantly different, Students t-test, 0.001 level). In 1984-85, the safer feed microalgae were grown in a culture medium of much lower initial bacterial content (viz. 0.2um membrane-filtered seawater) compared to 1983-84 (see Table 1). In 1984-85 axenic (bacteria-free) starter cultures of microalgae were used routinely whereas in the previous season starter cultures were already contaminated with bacteria (Table 1).

The improved production seen in 1984-85 has been sustained in the first half of the 1985-86 season. In 6 batches of C. gigas reared, 4.6% of the eggs spawned have survived to the ready-to-set larval stage. Such larvae have been first seen at a mean age of 18.0 days per batch. The mean bacterial level in feed microalgae, grown in 0.2um membrane-filtered seawater medium, was 8.9 x 10^5 ml^{-1}, similar to that in 1984-85.

Safety level of bacteria in feed microalgae

Feed microalgae are cultured in 500-litre bags. Evidence gained in late 1985 indicates that if any one bag exceeds a bacterial level of 2 x 10^6 ml^{-1}, it is very likely that the bag will induce disease in larvae. This safety level of 2 x 10^6 is shown in Figure 1. In batch a, not one bag used for feeding larvae exceeded a level of 2 x 10^6 bacteria ml^{-1}. 7.5% of larvae survived to the ready-to-set stage. (Note that those larvae lost were culled out by selective screening; no larvae were lost due to bacterial disease.) In batch b, larvae grew poorly over the first 7 days and several million were lost due to bacterial disease; the bacterial level exceeded 2 x 10^6 ml^{-1} in 4 bags at this time. Further losses were sustained and only 2.5% of larvae

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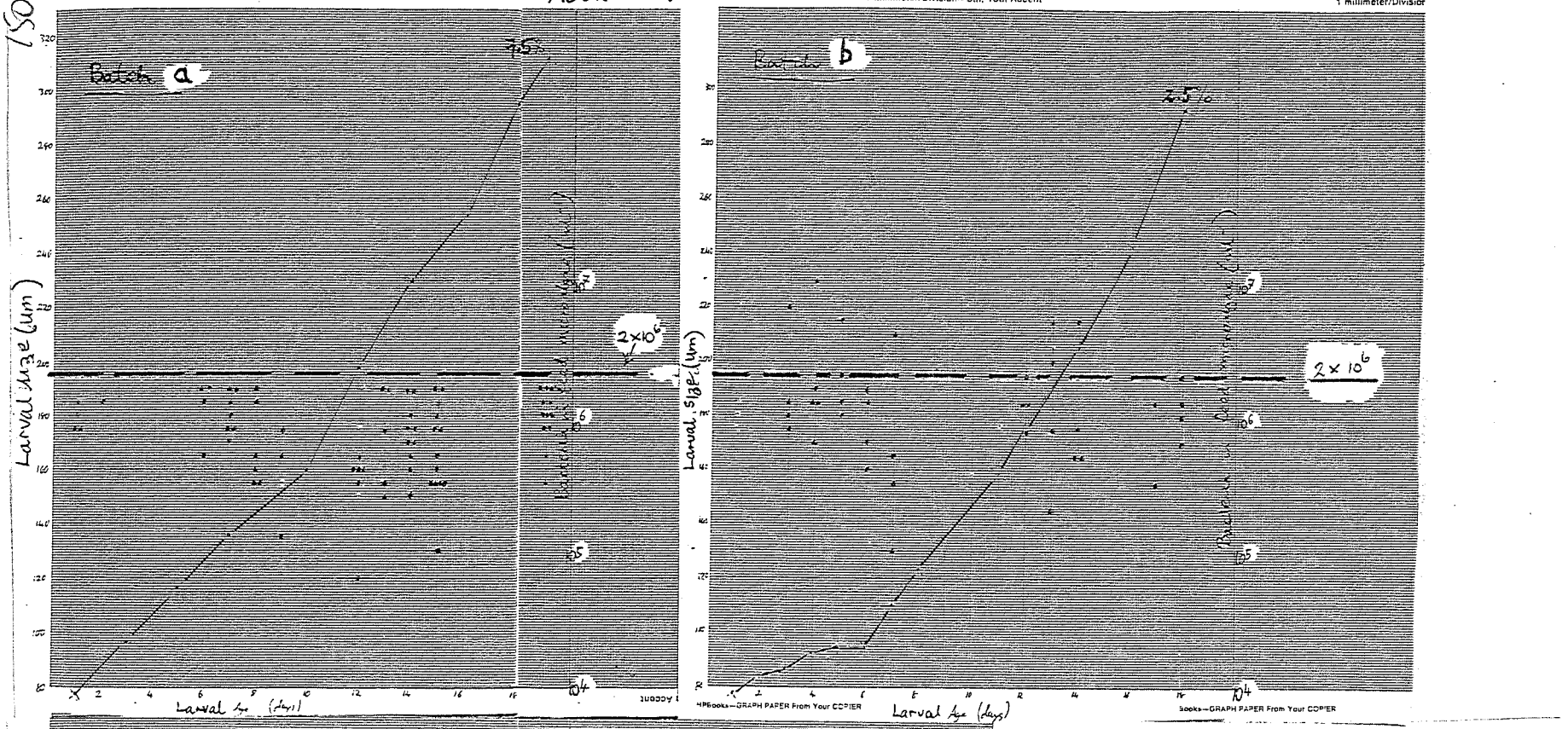
Table 1 Comparision of production of "ready-to-set" oyster larvae in 1983-84 and 1984-85 at Shellfish Culture Pty. Ltd, Bicheno (FIRTA 84/48, Garland and McMeekin).

Season	No. of eggs spawned	% Survival to ready-to-set larval stage	No. ready-to-set larvae produced	Days to produce ready-to-set larvae (per batch)	No bacteria in culture medium before microalgae inoculation	Use of axenic microalgae starter cultures	No. bacteria in "feed" microalgae
1983-84	23.3×10^8	1.4	30.5×10^6	23.5 days	2.6×10^3/ml	No	2.4×10^6/ml
1984-85	33.8×10^8	3.2	106.5×10^6	18.5 days	0.2/ml	Yes	6.2×10^5/ml

FIGURE 1.


Batch a
Batch b
Batch c
Larval size (μm)
Larval age (days)
Bacteria (cells/ml)
2×10^6
2 × 10^6
2×10^6
25°C
25°C
8°C
10^7
10^6
10^5
10^4
1 millimeter/Division—5th, 10th Accent
HPBooks—GRAPH PAPER From Your COPIER

reached the ready-to-set stage. 3 "unsafe" bags were fed to larvae on days 13 and 14. Batch c also was fed "unsafe" bags during the growth cycle (at days 1, 3, 6, 15, 16). Several million larvae were lost early. The 2 bags fed at day 15 and 16 appear to have caused the total loss of animals.

It should be noted that no Vibrio were detected in the feed microalgae given to batches a, b, or c, indicating that mortalities were due to a total dose of bacteria rather than specific types (e.g. Vibrio). Interestingly, in 1983-84 55% of bags exceeded a bacterial level of 2×10^6 ml^{-1} and production was 1.4%, in 1984-85 20% of bags exceeded 2×10^6 ml^{-1} and production was 3.2%, and in late 1985 approximately 10% of bags exceeded 2×10^6 ml^{-1} and production was 4.6%.

NOTE. These data are to be submitted as a scientific paper to Aust. J. Mar. Freshw. Res. in February, 1986.

Strand 2 Bacterial disease in laboratory-reared larval Pacific oysters (C. gigas).

This work was conducted over 3 seasons in an experimental laboratory at the University of Tasmania, Department of Agricultural Science. The work has been submitted as a scientific paper recently to the Australian Journal of Marine and Freshwater Research under the title "Undesirable effects of 0.2um membrane-filtered seawater as a culture medium on oyster (Crassostrea gigas) fertilized eggs and larvae" by C.D. Garland, S.L. Cooke, T.A. McMeekin and J.E. Valentine. The Abstract of the paper follows:

Abstract

Early stages of the Pacific oyster (Crassostrea gigas) were laboratory-reared at 24.5°C in 500 ml culture vessels devised to exclude contaminating microorganisms, and were fed axenic microalgae. Raw seawater, or 0.2 um membrane-filtered (m-f) seawater obtained from a running (fresh) or recirculating source, was used as the culture medium. The bacterial content of m-f seawater was < 1 $\log_{10}$ unit ml^{-1}. Fertilized eggs, produced in raw seawater, transformed at normal rates into trochophore and D shape larvae when suspended in m-f seawater from a running source. Fertilized eggs suspended in m-f seawater from a recirculating source formed abnormal trochophores which failed to develop into veligers. Scanning electron microscopy revealed more fragmented algae and microalgae on filters from the recirculating system than on those from the running system. M-f seawater (recirculating system) contained trace amounts of compounds normally found

within the algal cell and which were absent from m-f seawater from the running source, as determined by HPLC-spectrophotometry. Such compounds were considered responsible for the undesirable effects on embryonic oysters, and on normal veliger larvae which also developed abnormally in m-f seawater (recirculating source).

Normal D shape larvae also failed to grow normally in m-f seawater from a running source. Compared to larvae reared successfully in raw seawater, the animals grew minimally, exhibited shell and velum abnormality, and mostly were dead by 9 days of age. After 2 days use by animals of certain ages, significantly higher numbers of bacteria were found in m-f culture medium than in raw medium. During the 2-day periods, total bacterial levels ($\log_{10}$ units) increased from <1 to >6 ml^{-1} in m-f seawater, but from 3.95 to <6 in raw seawater. Fatal disease of oyster larvae appeared to be due more to mixed bacterial populations than to Vibrio strains.

Strand 3 The influence of H_2O_2 on the induction of spawning by Pacific oysters (Crassostrea gigas)

This study was conducted over three seasons and completed in December 1985. The major aim was to improve the conventional thermal method of induction of spawning which we considered to be rather slow and unpredictable. This was also the view of hatchery personnel at Shellfish Culture Pty. Ltd., Bicheno.

A simple technique using H_2O_2 was developed and found to be very successful. Animals are left overnight at 17-19°C out of seawater. Next morning, static (non-flowing) seawater is warmed to 25°C, the pH is adjusted to 9.0 (with 5M NaOH) and 6% H_2O_2 added to give a final concentration of 0.006%. The animals are then placed in the treated seawater, approximately 30 animals per 20 litres. As animals begin to spawn, they are placed in isothermal, untreated seawater and allowed to continue spawning.

The results of normal thermal-induced and H_2O_2-induced spawnings at 25°C are shown in Tables 1 and 2. As shown in Table 1 for animals with excellent (+++) gonad condition, H_2O_2 resulted in little difference in the time for induction of spawning. In the case of animals in moderate (++) condition, spawning was induced at a significantly faster rate (Student's t-test, 0.005 level). The mean spawning time for both males and females was also much more

predictable, as indicated by the smaller value of the standard deviation in respect of H_2O_2-induced ++ animals. Attempts to induce animals in poor (+) condition were not facilitated by H_2O_2.

As shown in Table 2, the number of eggs spawned by ++ animals was almost double in respect of H_2O_2-induced oysters. When examined by scanning electron microscopy, these eggs appeared morphologically indistinguishable from conventionally spawned eggs. As shown in Table 2, eggs transformed into D-shape veliger larvae at very similar rates, independent of the gonad condition of the broodstock or the use of H_2O_2 as an inducing agent.

Figure 1 shows the growth curve for the 3 different treatment groups in Table 2. The growth curves are very similar, further indicating that H_2O_2-induced eggs are normal. For the 3 treatment groups, similar values were obtained for the % survival to the read to-set larval stage (range 3.5-5.2%) and for the time at which the ready-to-set stage was first seen (range 17.3-19.7 days).

The results will be submitted as a scientific paper to the Australian Journal for Marine and Freshwater Research in January 1986, with the title "Rapid induction of spawning by Pacific oysters (Crassostrea gigas) in still seawater containing H_2O_2" by C.D. Garland, T.D. Obrien, M.I. Fraser, P.A. Tong and C. Ward.

Table 1. Influence of 0.006% H_2O_2 (final concentration) on the time for induction of spawning by Pacific oysters (Crassostrea gigas) at 25°C.

Gonad condition index of animals[a]	Use of 0.006% H_2O_2	pH	Mean ± SD time to spawning (mins) ♂[a]	♀[a]	No. of spawning trials
+++	+	9.0 ± 0.1	12.5 ± 1.4	21.0 ± 5.9	6
+++	-	7.9 ± 0.1	15.4 ± 5.3	24.95 ± 10.3	6
++	+	9.0 ± 0.1	32.3 ± 2.4	56.9 ± 5.3	6
++	-	7.7 ± 0.1	56.2 ± 18.4	90.2 ± 24.0	4
+	+	9.0 ± 0.1	NS[b]	NS	4
+	-	7.7 ± 0.1	NS	NS	2

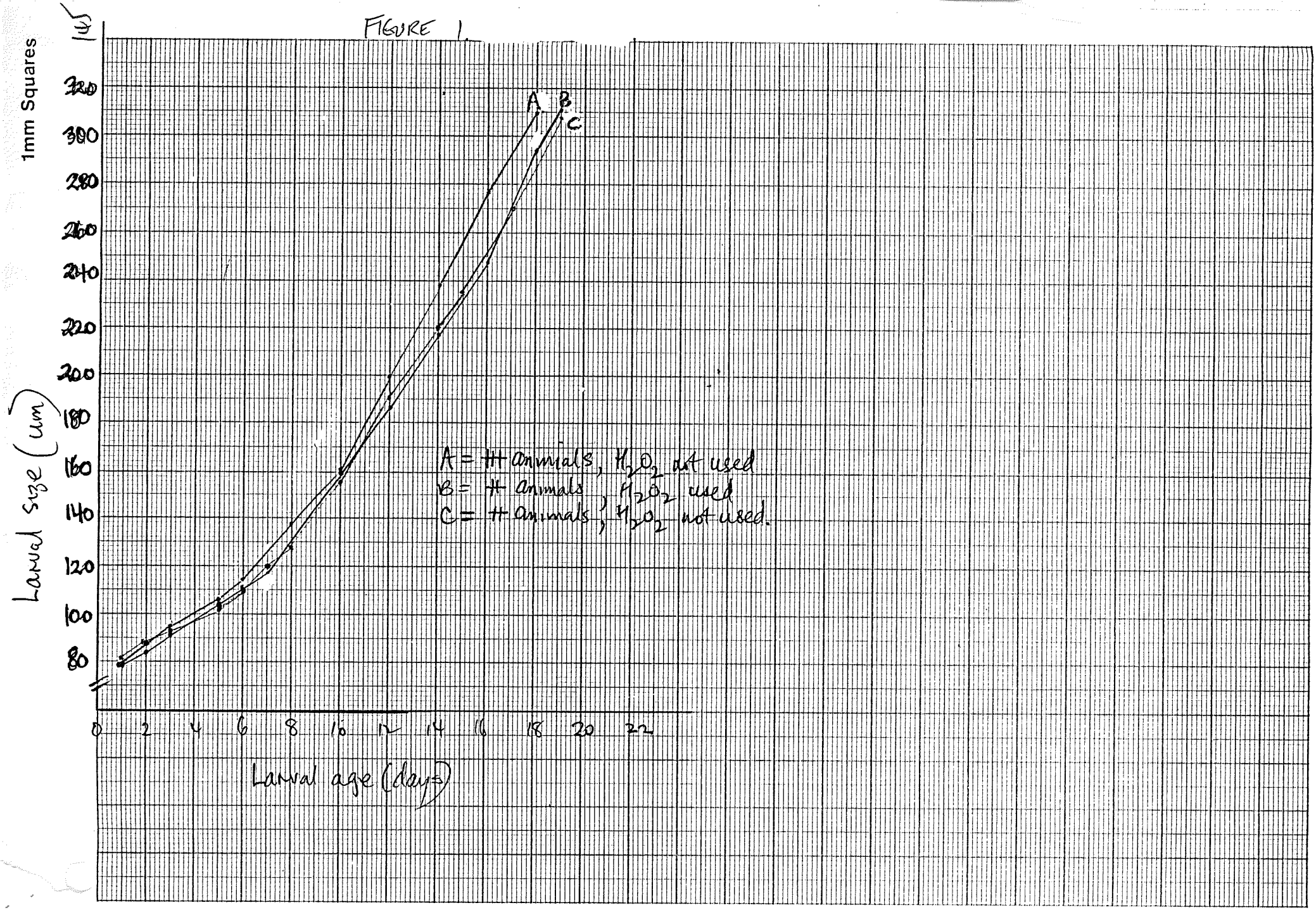
[a]Defined in Materials and Methods.

[b]Not one animal had spawned after 2 hours.

Table 2. Influence of 0.006% H_2O_2 (final concentration) on the number of eggs spawned by Pacific oysters at 25°C.

Gonad condition / index of animals	Use of 0.006% H_2O_2	pH	Mean ± SD no. of eggs produced per ♀ (10^6 x N)	Mean ± SD % transformation to D-shape veliger larvae (at 24 hours)	No. of spawning trials
+++	-	7.7 ± 0.1	15.0 ± 5.3	65.8 ± 19.8	6
++	+	9.0 ± 0.1	9.5 ± 3.2	69.5 ± 5.0	4
++	-	7.7 ± 0.1	5.0 ± 0.7	66.3 ± 13.7	4

FIGURE 1.


1mm Squares
Larval size (um)
320
300
280
260
240
220
200
180
160
140
120
100
80
A
B
C
A = # animals, H_2O_2 not used
B = # animals, H_2O_2 used
C = # animals, H_2O_2 not used.
0
2
4
6
8
10
12
14
16
18
20
22
Larval age (days)

Microb Ecol (1985) 11:221–230

MICROBIAL ECOLOGY

The Bacterial Biota on Crustose (Nonarticulated) Coralline Algae from Tasmanian Waters

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Abstract. The bacterial biota associated with the cuticle surface of healthy benthic samples of crustose nonarticulated coralline algae from the east coast of Tasmania (Australia) was examined by bacteriological cultivation and electron microscopy. In 32 samples studied, the viable count on Zobell's marine agar (supplemented with vitamins) was 3.3×10^6 bacteria g^{-1} wet wt. (range 2.9×10^4–2.7×10^7). Of 732 strains isolated from 16 out of 32 samples and identified to genus level, *Moraxella* was the predominant genus (66%). In contrast, *Moraxella* comprised only 11% of 217 strains isolated from benthic seawater samples collected at the same time as coralline algae. In 22 out of 32 algal samples examined by scanning electron microscopy, the total count was 1.6×10^7 bacteria g^{-1} wet wt. (range 5.1×10^6–3.8×10^7); the major morphotype was cocco-bacilli (80%). Several environmental factors did not significantly influence the viable count or generic distribution, or the total count or morphotypic distribution of bacteria on the cuticle. These factors included geographical site, season, storage of samples in aquarium conditions, and the presence or absence of abalone from shells that the coralline algae encrusted. The microbiota, consisting mostly of the nonmotile bacterial genus *Moraxella,* appeared to be highly adapted to its calcerous plant host.

Introduction

Crustose (nonarticulated) coralline algae (Corallinaceae, Rhodophyta) are a benthic subtidal marine alga. They grow very slowly as a red encrusting (calcerous) layer on shells, rocks, and other surfaces in areas of low light intensity. Very little is known about the coralline algae. A limited number of studies have been concerned with morphological, physiological, and reproductive aspects. The first monograph on coralline algae did not appear until very recently [10]. To our knowledge, nothing has been reported about the bacteriology of the group, apart from a single transmission electron micrograph presented in a morphological study [1]. The micrograph shows several bacteria on the thallus surface of an articulated genus (*Corallina*).

In this report we characterize, for the first time, the microbiota associated with the cuticule surface of crustose (nonarticulated) coralline algae. Algae that encrusted the external shell surface of adult abalone in Tasmanian waters were

selected for study. Genera present included *Mesophyllum* and *Lithophyllum*. Samples of algae were examined bacteriologically to determine the viable count, and representative isolates were identified to genus level. Parallel algal samples were investigated by scanning (SEM) and transmission (TEM) electron microscopy to determine the total microbial count and to describe the distribution of morphotypes on the surface. For electron microscopy (EM), specimens were fixed and prepared using methods that optimize the preservation of surface-associated microorganisms [6, 7]. We also examined the influence of various environmental factors on the size and composition of the microbiota on coralline algae. This included obtaining samples from different geographical sites and during different seasons, storing samples in aquarium conditions over several weeks, and examining algae on abalone shells that housed a living animal or were empty.

Methods

Collection of Samples of Coralline Algae

Benthic abalone (*Haliotis ruber*) shells encrusted with crustose (nonarticulated) Corallinaceae were collected by SCUBA diving from Bicheno, Safety Cove, and Dunalley. These sites are located on the eastern coast of Tasmania between latitudes 42°S and 43°S, on longitude 148°E. The shells were immersed in 20 l fresh seawater at ambient temperature and transported to the laboratory in Hobart within 4 hours. Certain shells from Dunalley were stored for up to 15 weeks in a running seawater aquarium at the Tasmanian Fisheries Development Authority's research laboratory at Taroona (Hobart).

The algal samples were collected between September 30, 1982 and January 4, 1984 at intervals of 1–2 months. In certain instances, samples of benthic seawater were also collected over this period. They were examined in the laboratory in the same manner as algae.

Bacteriological Cultivation

Approximately 0.5 g. algae was aseptically excised from the external encrusted surface of the abalone shell with a sterile scalpel blade and vigorously washed three times in sterile seawater to remove debris. The segments were weighed and hand-homogenized in 4.5 ml seawater in a grinding tube (Corningware) to produce particles no greater than 1.5 μm in diameter, as determined by phase-contrast microscopy. Serial tenfold dilutions of the homogenate were inoculated (0.1 ml spread plate) onto duplicate plates of Zobell's 2216 marine agar (Difco) supplemented with thiamine HCl (0.5 $\mu g\ l^{-1}$), biotin (2.5 $ng\ l^{-1}$) and vitamin B12 (2.5 $ng\ l^{-1}$). (Trials indicated that the addition of these vitamins to marine agar greatly improved the subculture recovery rate, from 60% on marine agar to 80–95% on marine agar with vitamins, termed MAV medium.) The plates were incubated at 22° ± 2°C for 7–10 days and total viable counts were then determined. For samples requiring identification of the bacterial biota, approximately 50 colonies were randomly selected from suitable plates and subcultured onto MAV. Pure cultures were stored on MAV slopes at 10°C.

Bacterial Identification

Isolates were identified to genus level using the schemes described in references [9] and [15]. The taxonomic tests included colonial morphology, Gram's stain, catalase and oxidase reaction, sen-

sitivity to vibriostatic agent 0/129 and growth on TCBS medium (Oxoid), glucose utilization in marine oxidation-fermentation medium and glucose-peptone-seawater medium, and fluorescein production in King's B medium. *Alteromonas* was distinguished from *Pseudomonas* by DNase production [14]. For studies of motility and flagellar arrangement, mildly turbid suspensions prepared in seawater broth [2] were examined by phase contrast microscopy and then stained with 1% phosphotungstic acid pH 7.0 for 15 *s* and examined on parlodion-coated grids in a Hitachi H-300 transmission electron microscope at 87 kV.

Preparation of Specimens for Electron Microscopy (EM)

At the same time as algal specimens were prepared for bacteriology, parallel samples were processed for EM. Washed samples were placed on bronze mesh supports above 10% aqueous acrolein, and vapor-fixed for 1 hour, then immersed overnight in 3% formaldehyde-3% glutaraldehyde fixative containing 0.1 M cacodylate buffer, 0.05% ruthenium red and marine salts (1.6% NaCl, 0.6% $MgCl_2$, 0.1% $CaCl_2$), pH 7.4 [7]. Fixed tissues were washed in 0.1 M cacodylate, pH 7.4, buffers of decreasing salt concentration: buffer 1 contained the salts at fixative concentration; buffer 2 contained 1.0% NaCl, 0.4% $MgCl_2$, 0.07% $CaCl_2$; buffer 3 contained 0.5% NaCl, 0.2% $MgCl_2$, 0.03% $CaCl_2$. Samples were stored in buffer 3 at 4°C.

For SEM, fixed specimens were dehydrated in a graded ethanol-acetone series [7], critical point dried in CO_2 and sputter-coated with gold [6], and examined in a Philips 505 scanning electron microscope at 15 kV.

For TEM, fixed specimens were decalcified by immersion for 4 hours at room temperature in the aldehyde fixative described above to which 2.5% EDTA [1] had been added and from which the marine salts had been omitted. Samples were then stained with OsO_4 and uranyl acetate, dehydrated, and embedded in Spurr's low viscosity epoxy resin [7]. Ultrathin sections (LKB Ultratome 8800) were stained with 2% uranyl acetate in 50% aqueous ethanol for 20 min in the dark and then with Reynold's lead citrate for 10 min, and examined in a Philips 201 transmission electron microscope at 60 kV.

In situ Quantitation of Microorganisms on the Algal Surface by SEM

Areas on the algal surface were randomly selected at low magnification, then examined at a screen magnification of 7800×. The cuticle surface was focused and arbitrarily aligned normal to the viewing plane. At 7800× each field measured 250 μm^2 in area. All microorganisms within the field were counted, including cocco-bacilli (<1 μm in diameter and <1 μm long), rods and spiral-shaped bacteria (≤ 1 μm in diameter and 1–5 μm long), filamentous microorganisms (1–3 μm in diameter and 1–5 μm long, per cell), and diatoms. In fields of low microbial colonization, counts were made using the SEM's TV scanning mode. In densely colonized fields, the reduced raster viewing system was used in a 3 × 3 grid pattern; the total area of the 9 reduced raster fields corresponded to the area of one full field. For each algal sample, 36 full fields were enumerated.

The counts per unit surface area were calculated after adjustment for a 29% shrinkage factor (see below).

Weight and Surface Area of Algal Segments

Four fresh, excised algal segments were weighed. Their total surface area was measured by macrophotography. Analysis of the data (not shown) produced a correlation coefficient of 2.3 cm^2 surface area per g wet weight. The same segments were then fixed and prepared for SEM. After critical point drying, the surface area of the segments was again measured; the dried segments were found to have shrunk by 29%.

Table 1. Estimates (mean ± 1 SD unit) of the viable bacterial count and generic distribution of the bacterial biota on coralline alge

Sample source/ condition	Viable count		Generic distribution (%)[a]					
	No. of samples	Log_{10} nos. g^{-1} wet wt	No. of samples	M	F/C	P	V	A
Site								
Taroona	16	6.2 ± 1.5	2	72 ± 21	22 ± 24	4 ± 6	2 ± 2	—
Bicheno	7	6.8 ± 0.6	5	61 ± 26	13 ± 4	14 ± 7	11 ± 14	3 ± 3
Safety Cove	9	6.6 ± 0.9	9	69 ± 12	16 ± 16	13 ± 10	6 ± 7	2 ± 3
Season								
Spring	10	6.6 ± 0.5	4	67 ± 12	19 ± 8	19 ± 6	2 ± 4	1 ± 2
Summer	4	7.2 ± 0.1	4	68 ± 14	18 ± 20	10 ± 11	4 ± 5	3 ± 4
Autumn	11	6.3 ± 0.8	3	54 ± 36	20 ± 17	10 ± 12	13 ± 20	3 ± 5
Winter	7	6.6 ± 0.3	5	75 ± 7	8 ± 7	7 ± 4	7 ± 8	1 ± 1
Shell[b]								
+	19	6.7 ± 0.5	15	67 ± 18	14 ± 12	13 ± 9	7 ± 10	2 ± 2
−	13	6.2 ± 0.7	1	56	40	—	4	—
Storage								
Stored (1–15 wk)	19	6.4 ± 0.8	4	56 ± 30	19 ± 15	11 ± 10	11 ± 16	1 ± 1
Fresh	13	6.8 ± 0.4	12	70 ± 11	15 ± 14	12 ± 10	6 ± 6	2 ± 3
Mean of all samples	32	6.5 ± 0.6	16	66 ± 18	16 ± 14	13 ± 10	6 ± 9	2 ± 3

[a] M *Moraxella*, F/C *Flavobacterium/Cytophaga*, P *Pseudomonas*, V *Vibrio*, A *Alteromonas*
[b] Abalone shell; + with animal, − without animal (empty)

Statistical Analysis of Data

Analyses of variance, estimates of the mean and standard deviation, and Student's *t* test were computed as described elsewhere [6]. Regression analyses were computed as described in reference [3]. The accuracy of the in situ quantitative method was assessed as the ratio, standard error (mean): mean [5].

Results

Bacteriological Studies

Over a 16-month period, 32 samples of crustose (nonarticulated) coralline algae from Tasmanian waters were cultured to determine the number of plate-viable marine bacteria by which they were colonized. The mean estimate was 3.3×10^6 bacteria g^{-1} wet weight, range 2.9×10^4–2.7×10^7. The mean estimates (log_{10} values) gained from algal samples from different sites and seasons, from samples that were fresh or had been stored in aquarium conditions for up to 15 weeks, and from shells in which the host abalone was present or absent, are presented in Table 1. It can be seen that these environmental conditions did not greatly influence the size of the bacterial population on the algal surface.

Statistical analyses (variance ratio, Student's t tests) revealed no significant differences (0.05 level) either between or within the different parameter groups.

Seven hundred thirty-two strains of bacteria isolated from 16 out of 32 samples were identified to genus level. As shown in Table 1, 66% of the isolates belonged to the nonmotile genus *Moraxella*. Interestingly, the predominance of *Moraxella* colonization of coralline algae did not appear to be strongly influenced by the environmental factors of site, season, storage, and shell. Strains of the *Flavobacterium/Cytophaga* group, *Pseudomonas* group, *Vibrio* and *Alteromonas* were also associated with the algal surface but in much lower numbers than *Moraxella* (Table 1).

In some instances benthic seawater was sampled during collection of coralline algae. The plate-viable count in 12 samples had a mean estimate 2.3×10^3 bacteria ml^{-1} seawater, range 1.9×10^2–6.5×10^3. Two hundred seventeen representative isolates from 6 out of 12 seawater samples were identified to genus level and found to belong mostly to *Vibrio* (43%) and *Pseudomonas* (22%) groups. Only 11% of isolates were *Moraxella* spp. The significant difference between bacteria on coralline algae and those in the adjacent seawater was confirmed statistically (regression analysis).

EM Studies

The surface of coralline algae and the microbial biota associated with it was examined by EM. At low magnification, the topography was typically undulating with occasional conceptacles and protuberances (Fig. 1). The surface was free of epiphytic macroalgae and a single mollusc, probably a gastropod, was observed in only one sample. At high magnification, the cuticle was seen as a dense cover over the epithallium cells (Fig. 2). These cells secreted the finely granular cuticle as a layer up to 3 μm in thickness (Fig. 3).

The microbial biota on the cuticle was quantified in situ by SEM in 22 out of 32 samples of coralline algae. The mean estimate of the total microbial count was 1.6×10^7 g^{-1} wet weight (which represents 2.3 cm^{-2} surface area), range 5.1×10^6–3.8×10^7. The mean estimates ($\log_{10}$ values) of total counts on algal samples from different sites and seasons, from samples that were fresh or stored, and from shells in which the host abalone was present or absent, are presented in Table 2. It can be seen that these environmental factors did not greatly influence the total microbial count. No statistically significant differences (0.05 level) either between or within the factor groups were found by analysis of variance or Student's t test. It should be noted that the in situ counting technique used here was regarded as statistically accurate because the ratio, standard error of the mean : mean, was always <0.10.

The microbiota on the cuticle was composed of various morphotypes. As shown in Table 2, the frequency of the morphotypes was determined in 22 out of 32 samples of coralline algae. Cocco-bacilli predominated (Fig. 4, 6), whereas rods and spiral-shaped bacteria comprised 19% of the microbiota (Fig. 2, 5), filamentous organisms represented 3% (Fig. 5), and diatoms were seen very rarely (Fig. 4). The environmental factors (site, season, shell, storage) did not appear to greatly influence the morphotypic distribution (Table 2).

The different morphotypes were seen mostly on the cuticle, infrequently in

Table 2. Estimates (mean ± 1 SD unit) of the total microbial count and morphotypic distribution of the microbial biota on coralline alge

Sample source/condition	No. of samples	Log_{10} nos. g^{-1} wet wt (or 2.3 cm^{-2})[a]	Morphotypic distribution (%)[b]			
			Cocco-bacilli	Rods and spiral-shaped bacteria	Filamentous micro-organisms	Diatoms
Site						
Taroona	7	7.0 ± 0.2	66 ± 12	30 ± 11	5 ± 2	<1
Bicheno	6	7.2 ± 0.1	81 ± 13	17 ± 11	2 ± 2	<1
Safety Cove	9	7.4 ± 0.1	88 ± 4	13 ± 4	1 ± 1	<1
Season						
Spring	4	7.2 ± 0.1	92 ± 3	8 ± 3	1 ± 1	<1
Summer	4	7.4 ± 0.2	87 ± 5	14 ± 5	1 ± 1	<1
Autumn	8	7.0 ± 0.2	65 ± 11	30 ± 16	5 ± 2	<1
Winter	6	7.3 ± 0.1	83 ± 8	16 ± 8	1 ± 1	<1
Shell[c]						
+	17	7.3 ± 0.2	84 ± 9	15 ± 8	2 ± 2	<1
−	5	7.0 ± 0.2	63 ± 15	25 ± 11	2 ± 3	<1
Storage						
Stored (1–15 wk)	10	7.3 ± 0.2	72 ± 14	34 ± 15	6 ± 3	<1
Fresh	12	7.1 ± 0.2	88 ± 5	12 ± 4	1 ± 1	<1
Mean of all samples	22	7.2 ± 0.2	80 ± 13	19 ± 11	3 ± 2	<1

[a] For each sample, the counts were obtained in 36 randomly selected fields at 7,800×; the area of the field was adjusted in terms of the 29% shrinkage factor (see Methods section)
[b] Dimensions of the different morphotypes are given in the Methods section
[c] See Table 1

the cuticle (Fig. 7), and never in the epithallium cells. All morphotypes were very firmly associated with the cuticle. The simple bacteria appeared to attach by means of polymer bridges (Fig. 8), whereas certain filamentous bacteria (probably cyanobacteria) attached by means of holdfastlike structures (Fig. 9).

It was notable that the density of cuticle cover illustrated in Fig. 2 was seen in nearly all fields in the 22 coralline algae samples examined by SEM.

Discussion

Microorganisms colonize numerous biological surfaces in marine environments, including those of soft and stony corals [4] and macroalgae and phytoplankton (reviewed in reference 11). Thus it is not surprising that this first microbiological study of crustose (nonarticulated) coralline algae demonstrates microbial colonization of the secreted surface (cuticle). The microbiota was primarily bacterial, however, three aspects of the data are striking. The first concerns the relatively constant composition of the microbiota. The ability of a particular bacterial genus to consistently populate the cuticle of alga samples

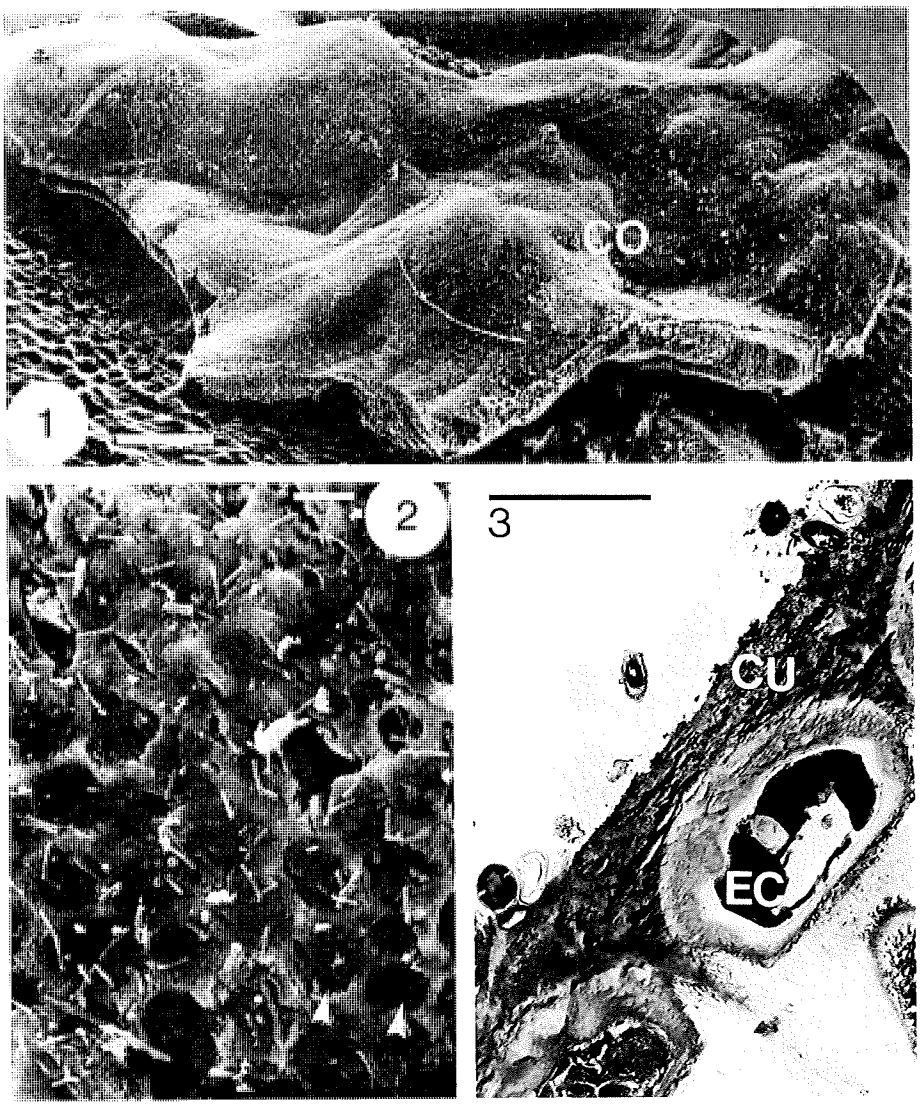


Fig. 1. The surface of crustose coralline algae is typically undulating, with occasional conceptacles (*CO*) (containing the gametes) and protuberances. The surface was free of epiphytic macroalgae. SEM × 40, bar = 0.5 mm.

Fig. 2. Typical cuticle cover on the surface of coralline algae; the epithallium cells (*arrowheads*) are outlined below. High numbers of cocco-bacilli and rods colonize the cuticle. SEM × 1,600, bar = 5 μm.

Fig. 3. Secretion of cuticle (*CU*), 0.5–3 μm thick, by epithallium cells (*EC*). Various bacteria, including dead cells (no cytoplasmic content), are attached to the cuticle. TEM × 8,500, bar = 2.5 μm.

obtained from different geographical sites and during different seasons indicates a stable association between specific microorganisms and the host. EM observations showed the microbiota to be firmly attached. In addition, the predominant bacterial genus tolerated a change in habitat, since encrusted shells taken

from the benthos and placed in an aquarium for up to 15 weeks remained colonized by the initial population. Furthermore, the association between the major bacterial genus and the coralline alga was not altered by removal of the living abalone within the encrusted shell. The capacity of the microbiota to tolerate changes in these environmental conditions indicates that it is highly adapted to its habitat.

The second striking aspect concerns the predominant bacterial genus *Moraxella*. Little is known about the general ecology of this nonmotile organism in marine environments [8, 16, 18]. Although it was the major organism on the cuticle, we found it as only a minor member of the population in the water column. This further supports the suggestion that *Moraxella* is specifically adapted to its habitat and is autochthonous to the secreted surface of crustose (nonarticulated) coralline algae.

The third interesting feature relates to the similarity between the total microbial count (1.6×10^7 g^{-1} wet weight) and the plate-viable bacterial count (3.3×10^6 g^{-1} wet weight). In several marine habitats, including seawater [12, 17], particulates in the water column and sediments [12], the total count can be 50–200× (and more) greater than the plate-viable count. Our finding of similar counts may reflect that a large proportion of the bacterial residents have nutritional needs provided for by the host, possibly in the form of photosynthate which may leak into the cuticle from the photosynthetically active epithallium cells below. The *Moraxella* population is not nutrient-deprived in situ. Accordingly, it can be recovered on Zobell's marine agar (supplemented with certain vitamins).

Laboratory cultures of *Moraxella* typically consist of cocco-bacilli [13]. It is possible then, that the major morphotype seen on the cuticle (viz., cocco-bacilli) correlates to the major genus isolated from it (viz., *Moraxella*). Clearly, further investigations are needed on the association between this poorly studied genus and its unusual calcerous host.

Acknowledgments. This project was supported by the Fishing Industry Research Trust Account, Department of Primary Industry, Canberra, Australia. We thank the Tasmanian Fisheries Development Authority (Hobart) for aquarium facilities at Taroona, Dr. D. Challis (Royal Hobart Hospital, Department of Anatomical Pathology) for the use of the Philips 201 transmission electron microscope, and Dr. W. J. Woelkerling (Botany Department, La Trobe University, Bundoora, Australia) for identifying genera of coralline algae present on abalone shells.

Fig. 4. Cocco-bacilli (*arrowheads*) on the cuticle. A rarely seen diatom (*Skeletonema*) is also present. SEM × 2,600, bar = 2.5 μm.

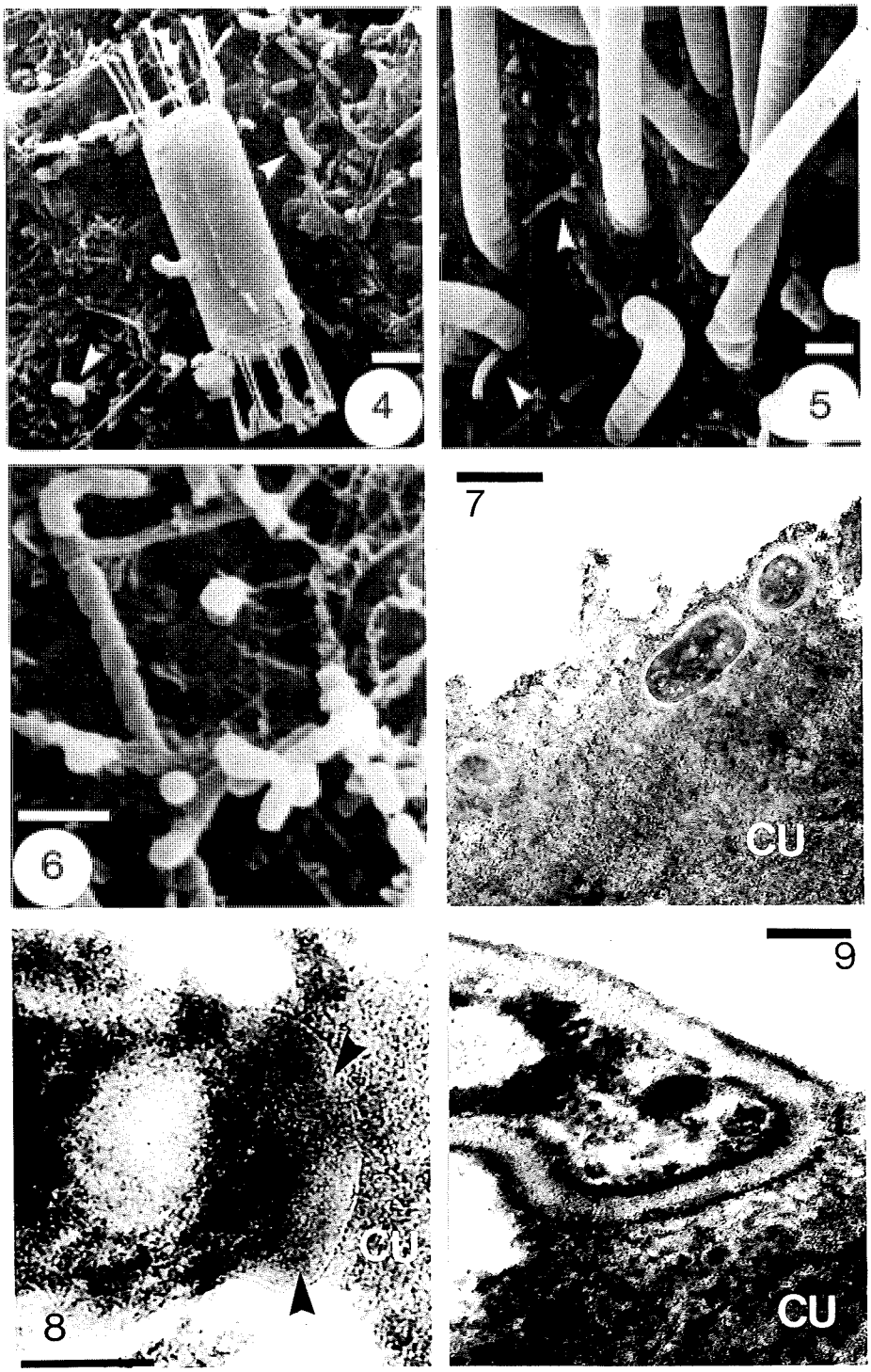
Fig. 5. Filamentous microorganisms (probably cyanobacteria) attached to the cuticle. Slender rod-shaped bacteria are also present (*arrowheads*). SEM × 2,600, bar = 2.5 μm.

Fig. 6. Typical cocco-bacilli in pairs and chains on the cuticle. SEM × 5,200, bar = 2.5 μm.

Fig. 7. Rarely seen bacteria within the cuticle (*CU*). TEM × 24,300, bar = 500 nm.

Fig. 8. Bacterium attached to the cuticle (*CU*) by means of a polymer bridge (*arrowheads*). TEM × 192,000, bar = 100 nm.

Fig. 9. A filamentous microorganism (possibly cyanobacteria) attached to the cuticle (*CU*) by means of a holdfastlike structure. TEM × 48,600, bar = 250 nm.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INGESTION OF THE BACTERIA ON AND THE CUTICLE OF CRUSTOSE (NON-ARTICULATED) CORALLINE ALGAE BY POST-LARVAL AND JUVENILE ABALONE (*HALIOTIS RUBER* Leach) FROM TASMANIAN WATERS

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Abstract: Larvae of *Haliotis ruber* Leach 1814 (gastropod mollusc) were reared in the laboratory and at 6–8 days of age, competent larvae (250 μm diameter) were induced to settle and metamorphose on crustose (nonarticulated) coralline algae. Post-larvae and juveniles were reared on this substratum in continuously flowing fresh sea water in the laboratory. The population density of animals on coralline algae was ≈ 0.9 animal $\cdot$ cm^{-2} and 0.6 animal $\cdot$ cm^{-2} at 6 and 13 wk of age respectively, by which time abalone had grown to a mean size of 692 ± 33 and 2435 ± 448 μm, respectively. Scanning electron microscopy was used to examine *H. ruber*, specimens of the radula, and grazed coralline algae. In 6-wk-old animals, the range of the distance between the axial rows of rachidian and lateral teeth of the radula was 9–11 μm, similar to the distance (8–13 μm) between the parallel tracks seen on the grazed algal surface. In lightly grazed areas, the animals rasped and ingested a layer 1–2μm thick, consisting of the algal cuticle and the bacterial biota (mostly *Moraxella*) indigenous on it. In heavily grazed areas, the cytoplasmic contents of the epithallium (underlying the cuticle) were also removed and ingested. In 6- and 13-wk-old animals, bacteria were found between the teeth on the radula tip and around the oral disc, but rarely on the external shell surface. Cultivation of rinsed homogenized abalone on bacteriological media showed they were colonized by 1.5×10^3 bacteria per animal (6-wk-old) and 8.3×10^3 bacteria (13 wk old). In the younger group, *Vibrio* and *Moraxella* predominated, whereas in older animals *Moraxella* alone predominated.

Key words: marine bacteria; *Moraxella*; *Vibrio*; *Haliotis*; abalone; Corallinaceae; crustose coralline algae

INTRODUCTION

Microorganisms can be of great importance for the normal development of very young marine invertebrates. Bacterial-organic films on substrata are essential for the successful recruitment of many larval animals, including certain bryozoans (Mihm *et al.*,

1981; Brancato & Woollacott, 1982), spirorbids (Kirchman *et al.*, 1982a,b), scyphozoans (Brewer, 1984) and bivalve molluscs (Weiner *et al.*, 1985). After settlement and metamorphosis, the diatoms and flagellate microalgae, due to their nutritional value are highly significant to post-larvae and juveniles (Bardach *et al.*, 1972; Ryther & Goldman, 1975; Kohn, 1983). These microorganisms may be planktonic or associated with substrata. Apart from their role as infectious agents, the importance of bacteria to post-larvae and juveniles is not well understood. Due to the limited biomass of bacterial populations in marine environments (ZoBell & Feltham, 1938), they may be of minor nutritional significance. However, within the gut they may perform metabolic transformations essential to the host (e.g. Fong & Mann, 1980).

We are interested in the importance of microorganisms, particularly bacteria, to very young abalone (gastropod mollusc). Members of the commercially important genus *Haliotis* begin life as planktonic larvae but after several days they settle on and graze suitable benthic substrata, where over the next few weeks they metamorphose into the creeping juvenile form (Mottet, 1978). In the case of certain *Haliotis* spp., competent larvae preferentially settle and metamorphose on the crustose coralline red algae, crustose non-coralline red algae and related algal species (Crofts, 1929; Morse & Morse, 1984). Recruited *Haliotis* larvae can use their radula to graze the surface of the substratum immediately (Crofts, 1937; Morse, 1984). *Haliotis* post-larvae and juveniles rasp such encrusted substrata, as well as delicate red seaweeds (Crofts, 1937) and artificial substrata to which very small benthic diatoms are attached (Bardach *et al.*, 1972; Mottet, 1978; Ebert & Houk, 1984). Of the microorganisms ingested after grazing, the diatoms are considered very important, due to their nutritional value (Crofts, 1937; Mottet, 1978; Ebert & Houk, 1984; Morse, 1984). However, the significance of bacteria to competent larvae, post-larvae and juveniles is poorly understood, either in terms of their influence as bacterial-organic films on settlement and metamorphosis, their nutritional value, their metabolic or other activities within the alimentary tract, or their pathogenicity (Elston & Lockwood, 1983).

Coralline (non-articulated) red algae form crustose substrata which are preferred for settlement, metamorphosis and early development by *Haliotis ruber* in Tasmanian waters (T.E. Lewis & P. Whyte, pers. comm.) and by *H. iris* in New Zealand waters (L.J. Tong, pers. comm.) In an accompanying paper, we used electron microscopy and bacteriological methods to describe the microbial biota indigenous on the cuticle surface of crustose coralline algae from Tasmanian waters (Lewis *et al.*, in press). Phytoplankton were seen very rarely on the cuticle. The biota consisted almost entirely of bacteria, mostly small cocco-bacilli of the genus *Moraxella*. *Moraxella* was the dominant microorganism of crustose coralline algae sampled in different seasons, in different geographical locations, and on segments which were fresh or maintained in aquarium conditions for up to 4 months. As *Moraxella* occurred infrequently in the water column, its colonization of crustose coralline algae suggested it was highly adapted or autochthonous to that habitat (Lewis *et al.*, in press). Krieg (1984) describes *Moraxella* as pathogenic to terrestrial animals but we did not assess this characteristic for our marine isolates (see also Gibson *et al.*, 1977).

In the present study, we examined 6- and 13-wk old *Haliotis ruber* Leach 1814 and the coralline alga encrusted substrata on which the animals were reared in the laboratory. Scanning electron microscopy (SEM) was used to investigate the cuticle surface of rasped algae, the bacterial biota on the cuticle, and the epithallium responsible for cuticle secretion (Johansen, 1981). The oral disc and radula of actively grazing post-larvae and juveniles were also studied by SEM. In addition, we homogenized 6- and 13-wk-old *H. ruber* and cultivated them on bacteriological media to determine whether the bacteria present in the animals closely resembled the biota (mostly *Moraxella*) indigenous on the coralline alga substratum.

Materials and Methods

HATCHERY REARING OF ABALONE

Gravid adult specimens of *Haliotis ruber* were collected off the Tasmanian coast by commercial hooker diving. At a hatchery at Bicheno (Tasmania) animals were placed in bins containing fresh running sea water and induced to spawn at night using a thermal/UV irradiation stimulus (Grant, 1981). After fertilization embryos were allowed to develop into normal larvae (Grant, 1981). Settlement and metamorphosis were induced by methods similar to those of Morse & Morse (1984); γ-amino butyric acid was not used. At 6–8 days of age, competent larvae (250 μm diameter) were presented with coralline algae in fresh still sea water in 1000-l culture bins, maintained at 15–17 °C, at a density of 1 animal $\cdot$ ml^{-1}. After 18–24 h, fresh running sea water was introduced into the culture bins. The outlet pipes were not screened to prevent loss of larval animals which had failed to settle. Thereafter, culture of the post-larvae and juveniles on coralline algae was continued in fresh running sea water at 15–17 °C.

SAMPLING PROCEDURES

More than 80 specimens from each of two batches of animals, spawned from adults on 14 April 1983 and 30 April 1983, were examined at a mean age of 6 $\pm$ 1 wk and later at 13 $\pm$ 1wk. The animals were transported from the hatchery within 4 h to the microbiology laboratory while still attached to coralline algae (in sea water at ambient temperature) and were processed immediately. Abalone which were exhibiting side-to-side scraping, as seen through the dissecting microscope, were judged to be actively feeding (Morse, 1984). To estimate the population density for each age group, the number of animals on 5–10 specimens of substratum of $\approx$25 cm^2 surface area, was counted.

PREPARATION OF SPECIMENS FOR SEM

For each age group, 10 specimens consisting of abalone and the surrounding substratum on which they were feeding, were removed by gentle excision of the coralline

alga with a scalpel blade. The samples were placed on bronze-mesh supports, fixed in acrolein vapour, then further fixed and prepared for SEM as described elsewhere (Lewis *et al.*, in press).

To obtain the radula, a method was devised based on suggestions by L.J. Tong (Department of Agriculture and Fisheries, New Zealand). Whole animals which had been fixed and stored in 0.1 M cacodylate buffer at 4 °C for several weeks were transferred to a solution of 5 M NaOH at room temperature and viewed through the dissecting microscope. The oral disc was located near the foot and gently dissected out. The tissue was allowed to dissolve in 5 M NaOH for 1–2 h, then pressed gently with a probe to extract the radula. In this way the radula (or fragments of it) was obtained from six animals. For SEM, the samples were washed in several changes of distilled water, placed on double-sided tape and allowed to air dry, sputter-coated with gold and examined.

BACTERIOLOGICAL CULTIVATION AND IDENTIFICATION

For these studies it was essential to obtain animals which were not only healthy but also free from contaminating coralline algae and other debris. Animals which were feeding on the coralline algal surface, were removed by placing a blunt-nosed metal probe next to them on the algal surface and allowing them to creep onto it. Excess sea water was allowed to run off the probe and attached animals. The probe was then placed in a grinding tube (Corningware) containing 4 ml of sterile sea water. The animals climbed off the probe and attached to the inner surface of the glass tube. In this way 60–70 animals were collected from each age group. The sea water was tipped out of the tube and replaced with sterile sea water, a process repeated five times to remove all debris and any abalone not sufficiently healthy as to remain attached to the tube. For each age group the animals were counted then hand homogenized in 2 ml of sterile sea water to produce particles no larger than 1.5 μm, as observed by phase-contrast microscopy. Serial dilutions of the homogenates were cultured on marine agar plates supplemented with vitamins (Lewis *et al.*, in press). Viable counts were made on plates incubated at 22 $\pm$ 2 °C for 10 days.

Representative bacteria were isolated in pure culture and identified to genus level as described elsewhere (Lewis *et al.*, in press).

RESULTS

Competent *H. ruber* larvae (250 μm) presented with crustose coralline red algae as a surface for settlement and metamorphosis, and subsequently reared on this substratum, were examined regularly by light (dissecting) microscopy. The population density of animals on the coralline algal surface was estimated. The mean number of 6-wk-old post-larvae was 23 (range 16–28) on 25 cm^2 substratum, i.e. 0.9 animal $\cdot$ cm^{-2}. The mean number of 13-wk-old *H. ruber* was 14 (range 7–28) on 25 cm^2,

i.e. 0.6 animal · cm^{-2}. The mean size ± SD of 6- and 13-wk-old animals was 692 ± 33 μm (n = 46) and 2435 ± 448 μm (n = 26) respectively. The shell of grazing animals was pink-red.

Representative specimens of abalone grazing on coralline algae were examined by SEM. Fig. 1 shows a 6-wk-old animal on a typical segment of substratum. Prior to preparation for SEM, several *H. ruber* had been seen to creep across this segment and stop intermittently, in the process of creating fresh grazing tracks. Figs. 2 and 3 show typical heavily grazed areas at higher magnification. Not only has most of the cuticle and its bacterial biota been removed (Fig. 2) but also apparently most of the contents of the underlying epithallial cells since these cells were almost devoid of cytoplasmic contents (Fig. 3). The appearance of the grazed algal surface is in dramatic contrast to the electron microscopic appearance of ungrazed algae, in which the bacterial biota is attached to an intact cuticle and the epithallium is barely discernible below the cuticle (see Figs. 1–3 in Lewis *et al.*, in press). The heavily grazed substratum appeared indented, with parallel V-shaped tracks 8–13 μm apart and 2–3 μm deep (Fig. 2). In areas lightly grazed by 6-wk-old abalone the tracks were measured to be 1–2 μm deep and the epithallium cells generally remained intact. Wider (11–16 μm) but more U-shaped tracks, 2–3 μm deep were found on coralline algae grazed by 13-wk-old abalone (results not shown).

The feeding organ of abalone, the radula, was considered responsible for rasping the cuticle and its associated bacterial biota, and in some instances portions of the epithallium. A large fragment of the rhipidoglossate radula extracted from a 6-wk-old animal is shown in Fig. 4. The specimen has been carefully cleaned to reveal the nature of the chitinous teeth. Fig. 5 shows the axial rows of rachidian and lateral teeth. The rows were measured as 9–11 μm apart, indicating that these teeth were responsible for the graze marks with an inter-grazing track distance of 8–13 μm, as shown in Fig. 2. The finely cusped marginal teeth are shown in Fig. 6. The sigmoid-shaped radula was up to 290 and 450 μm long in 6- and 13-wk-old *H. ruber* respectively.

The action of the rhipidoglossate radula of adult gastropods has been reviewed by Hickman (1980) and described specifically for adult *Haliotis* by Crofts (1929). Crofts (1937) has given a brief account of radula activity in *H. tuberculata* at 1, 2, and 3 wk of age. In the following text and figures, we use the data of Crofts (1929 and 1937) and Hickman (1980) to describe the mechanism by which *H. ruber* appears to feed on crustose coralline algae. Fig. 7 shows a 13-wk-old animal which had been grazing immediately prior to preparation for SEM; subsequently the animal was inverted to reveal the oral disc, located near the foot. To feed, the radula is protruded through the oral disc; the marginal teeth are erected sideways as the radula passes over the tip of the odontophore. The radula then bends back on itself. As the radula is moved axially over the algal substratum, the bacteria and cuticle would be rasped by the rachidian and lateral teeth (Fig. 5). On withdrawal of the radula into the radula sac, the finely-cusped marginal teeth (Fig. 6) would collapse to trap the fragments of cuticle and attached bacteria. Fig. 8 shows the tip of the withdrawn radula still protruding through the oral

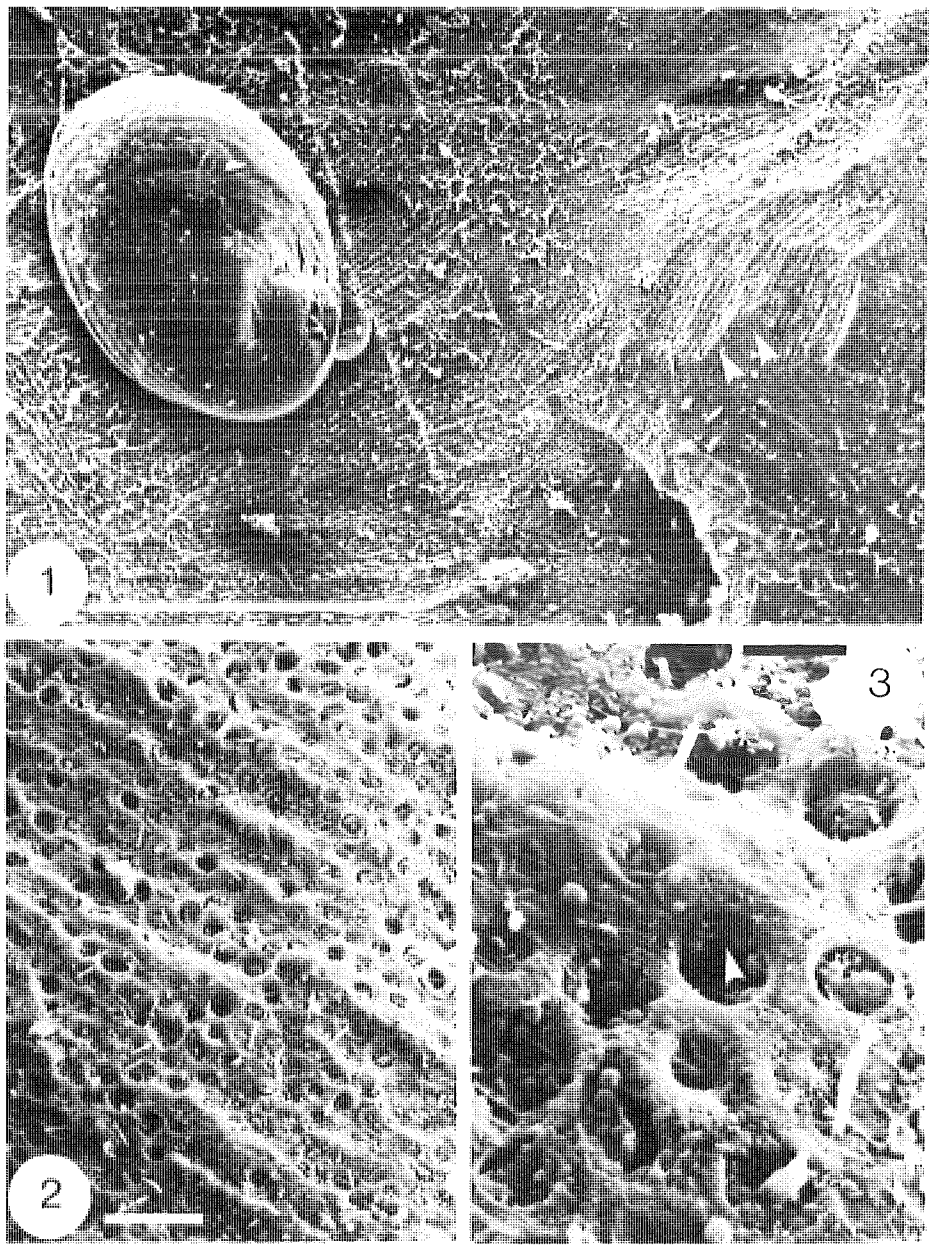


Fig. 1. Six-week-old abalone (570 μm diameter) on coralline algae: most of the visible surface in the figure has been grazed, some areas very heavily (arrowheads); SEM × 85; scale bar, 500 μm.

Fig. 2. Heavily grazed area from Fig. 1: parallel grazing tracks, ranging from 8–13 μm apart, can be seen (running from top left to bottom right of micrograph); the cuticle and its bacterial biota (see Lewis *et al.*, in press) have been completely removed; SEM × 630; scale bar, 20 μm.

Fig. 3. Heavily grazed area from Fig. 2: many of the epithallial cells (arrowheads) underlying the cuticle have lost their cytoplasmic contents; a few bacteria have colonized the newly exposed surface; SEM × 2500; scale bar, 5 μm.

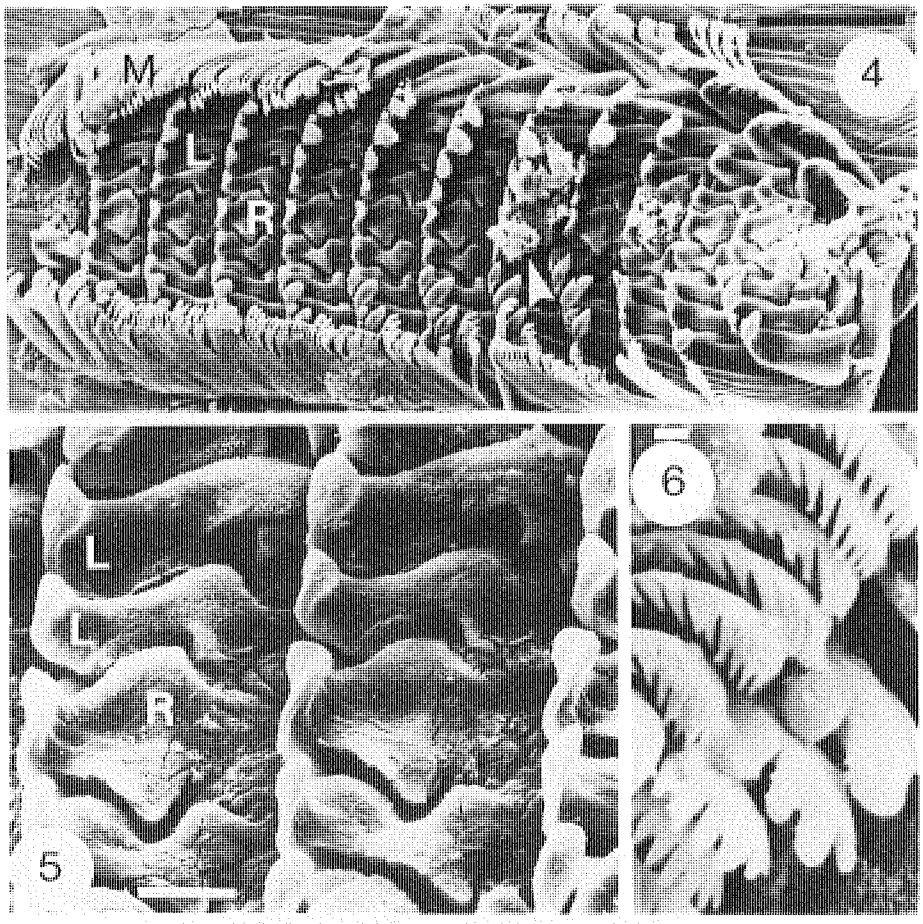
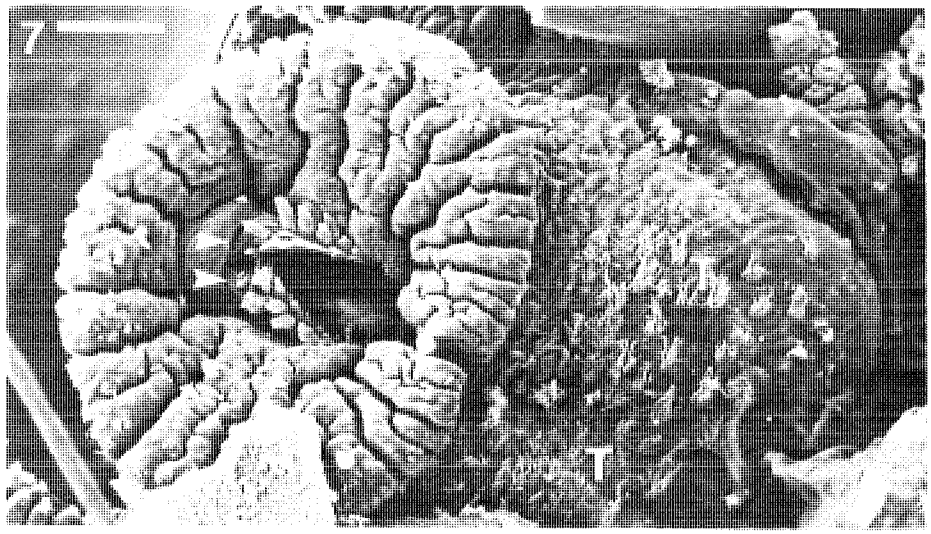


Fig. 4. Large fragment of radula extracted from a 6-wk-old abalone consisting of rachidian (R), lateral (L) and marginal (M) teeth: although the specimen was washed several times in distilled water, some debris remains caught between the R and L teeth (arrowhead); SEM × 380; scale bar, 50 μm.

Fig. 5. Enlargement of Fig. 4: rachidian (R) and lateral (L) teeth, ranging from 9–11 μm apart, from tip to tip; SEM × 1300; scale bar, 10 μm.

Fig. 6. Enlargement of Fig. 4: marginal teeth with very fine cusps; SEM × 3800; scale bar, 1 μm.

disc. Clumps of bacteria can be seen caught between the radula teeth in Fig. 8, and at higher magnification in Fig. 9. It can be readily seen that these anterior teeth, possibly marginal and outer lateral types, have lost their normal morphology, presumably due to wear during rasping (compare Figs. 8 and 9 with Fig. 4). Moderate numbers of bacteria were also found on the lips surrounding the oral disc (Fig. 8) and between the tufts of cilia at the base of the oral disc (Fig. 10). However, no microorganisms were seen on the foot nor on the tentacles radiating from it.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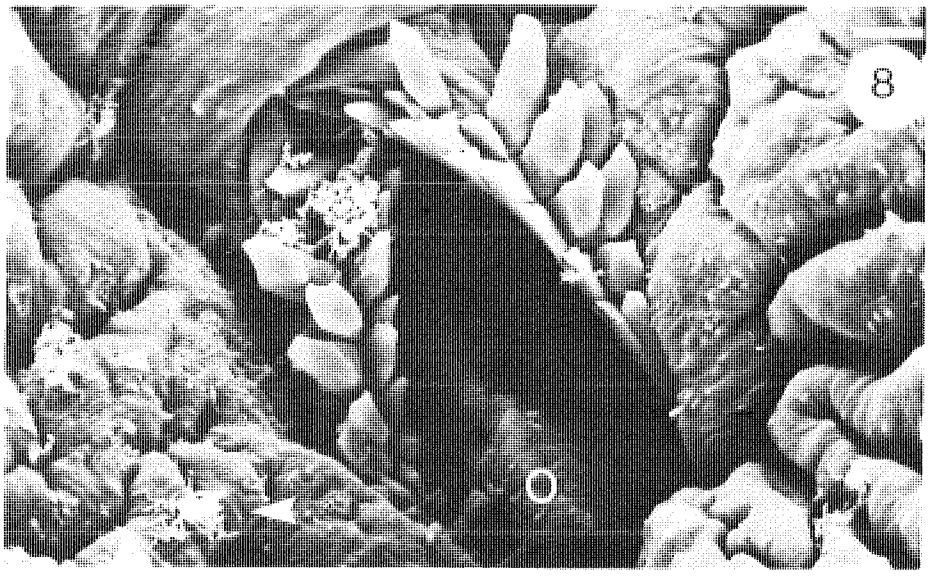
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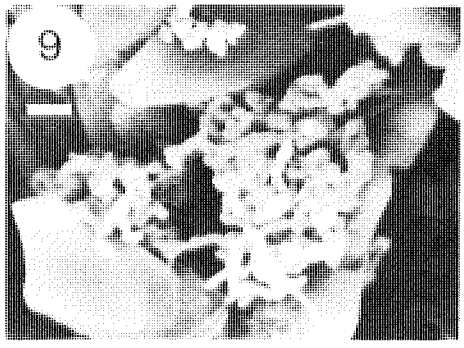
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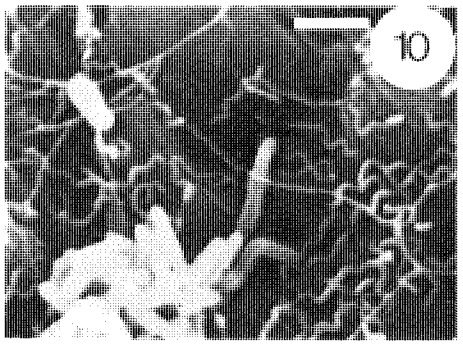
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After digestion in the gut of adult animals, waste material is rejected through the multi-purpose respiratory pores in the shell into the water column (Crofts, 1929). These pores were first seen by us in *H. ruber* at 6–7 wk of age and indicate the change from post-larval to juvenile stage (Mottet, 1978). SEM observation of the external shell surface of healthy abalone at 6 and 13 wk of age demonstrated that very few micro-organisms colonized this surface. Fig. 11 shows a 13-wk-old animal shell in which faecal material is being discharged through respiratory pores. As shown in Fig. 12, however, bacteria in fecal material have lodged transiently on the external shell surface, near a pore. It is of interest that these bacteria appear viable (i.e. not degraded).

Actively feeding animals from the two age groups were washed thoroughly, homogenised and cultivated on bacteriological media. Representative isolates were identified

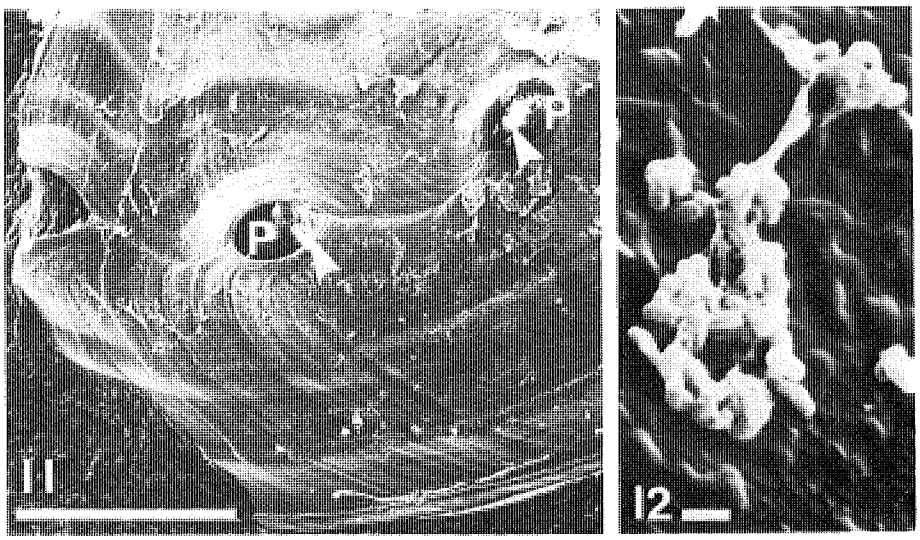

Fig. 11. Part of a 13-wk-old abalone shell: faecal material (arrowheads) is being excreted through the respiratory pores (P); SEM × 58; scale bar, 500 μm.

Fig. 12. Intact faecal bacteria lodged transiently on external shell surface near respiratory pore of same animal shown in Fig. 11: SEM × 6200; scale bar, 1 μm.

◀ Fig. 7. Oral disc of 13-wk-old abalone: the tip of the radula can be seen protruding (arrowheads); tufts (T) of cilia are located on the base of the oral disc; SEM × 280; scale bar, 50 μm.

Fig. 8. The protruding tip of the radula shown in Fig. 7: clumps of bacteria are caught between the very worn anterior teeth, and are also present on the lips of the oral disc (arrowheads); the tip of the odontophore (O) is visible; SEM × 880; scale bar, 10 μm.

Fig. 9. Bacteria caught between the radula teeth, possibly rachidian and lateral types, seen in Fig. 8: SEM × 2300; scale bar 2.5 μm.

Fig. 10. Rod and spiral-shaped bacteria located on the base of the oral disc between tufts of cilia (shown in Fig. 7): SEM × 4100; scale bar, 2.5 μm.

to genus level. As shown in Table I, each animal supported 1.5×10^3 bacteria, of which *Vibrio* (36%) and *Moraxella* (27%) were the major members. Thirteen-week-old animals were colonized by 8.3×10^3 bacteria, of which *Moraxella* (71%) was the predominant member.

TABLE I

The viable count and generic distribution of the bacterial biota of *H. ruber* reared on coralline algae; a, mean of 46 animals; b, mean of 28 animals; c, % of 67 isolates; d, % of 63 isolates; e, identified tentatively on a morphological basis (Krieg, 1984).

	Age and mean size (diameter ± SD) of abalone	
	6 ± 1 wk, 692[a] ± 33 μm	13 ± 1 wk, 2435[b] ± 448 μm
Viable count (bacteria/animal)	1.5×10^3	8.3×10^3
% Generic distribution		
Moraxella	27[c]	71[d]
Vibrio	36	5
Pseudomonas	17	15
Alteromonas	11	3
Flavobacterium–Cytophaga	4	6
Hyphomicrobium[e]	1	0
Other appendaged forms	3	0
Aeromonas formicans	1	0

DISCUSSION

The rhipidoglossate radula is a robust chitinous organ (Purchon, 1977; Hickman, 1980) which adult marine gastropods use to graze various hard biological substrata including crustose algae (e.g. Bertness *et al.*, 1983). Our data show that post-larval and juvenile *Haliotis ruber* possess a radula surprisingly long in relation to total body size: up to 290 μm in 6-wk-old animals of 692 ± 33 μm diameter, and up to 450 μm long in 13-wk-old animals of 2435 ± 448 μm diameter. By means of the radula, *H. ruber* rasps the surface of crustose coralline algae and removes a layer 1–3 μm thick, which consists of bacteria and the cuticle secreted by the epithallium. In instances of heavy grazing, *H. ruber* also appears to remove the cytoplasmic content of the epithallium. Most material is then ingested although some bacteria lodge on the lips of the oral disc (Fig. 8), probably because the radula is withdrawn with a licking action (Purchon, 1977).

It appears a general finding for *Haliotis* that post-larval and juvenile forms can graze very small items present on local benthic substrata and use them for adequate nutrition. In English waters, Crofts (1937) reported that newly-settled *H. tuberculata* larvae feed on diatoms and foraminifera present on the surface of stones on which the animals creep. Post-larval *H. tuberculata* was able to rasp off fragments of delicate red seaweeds. Morse & Morse (1984) noted that newly-settled *H. rufescens* larvae can use surface

mucus and microorganisms present on crustose coralline algae from Californian waters. Later, the post-larvae use the crustose material as well. Ebert & Houk (1984) state that post-larval and juvenile *H. rufescens* utilize mixed natural populations of benthic diatoms which have attached to artificial substrata. Similar observations have been made on *H. discus* in Japanese conditions (Bardach *et al.*, 1972).

We have found that *H. ruber* grazes, and presumably utilizes, material from the surface of crustose coralline algae from Tasmanian waters. The shell of post-larvae and juveniles was pink-red; very likely, these colourations were derived from the red pigments located in the epithallium, but not in the cuticle of coralline algae (Johansen, 1981). The ingested material represents a source of adequate nutrition for *H. ruber*, at least for the first 3–4 months of growth. In the case of *H. ruber* rasping a typical segment of coralline algae of, say, 10 μm wide × 10 μm long (i.e. 100 μm^2) × 2 μm deep, a total volume of 200 μm^3 would be removed. To determine the proportion of the ingested volume which consists of microorganisms, the data of Lewis *et al.* (in press) can be used. These authors studied ungrazed samples of crustose coralline algae very similar to those on which *H. ruber* was reared in the present study. Lewis *et al.* (in press) found very few phytoplankton on the algal surface, $< 10^3$ per 2.3 cm^2, i.e. $< 10^{-3}$ per 100 μm^2. The density of bacteria was estimated by SEM as 1.6×10^7 per 2.3 cm^2, i.e. 7 per 100 μm^2. Most of these bacteria were cocco-bacilli with average dimensions 0.9 μm long × 0.8 μm diameter, or average volume 0.27 μm^3. The total volume of bacteria on 100 μm^2 substratum surface would thus be 1.9 μm^3, and the proportion of bacteria in a rasped total volume of 200 μm^3 would be $\approx 1\%$.

Although the calculations above are approximate, they suggest that post-larvae and juvenile *H. ruber* depend for nutrition mostly on the cuticle and epithallial contents grazed from coralline algae. Phytoplankton form a negligible part of the diet. Bacteria form a minor component in terms of bulk. However, this does not exclude the possibility that bacteria perform metabolic activities in the gut that are highly significant to the host's development. Of necessity, such bacteria would be viable. It was of interest that bacteria in the faecal material (Fig. 12) appeared viable (undegraded). It was also of interest that spiral-shaped bacteria at the base of the oral disc appeared viable (Fig. 10). These organisms may normally reside in the gut, which in many molluscs is colonized by spiral-shaped bacteria (Breznak, 1973) and may have been disgorged during protrusion of the radula.

As shown in Table I, 13-wk-old *H. ruber* were colonized by bacteria mostly of the genus *Moraxella*. These juveniles thus reflected the bacterial biota (mostly *Moraxella*) indigenous on the coralline algal surface (Lewis *et al.*, in press). However, in 6-wk-old animals, which had not yet metamorphosed into the adult form (e.g. respiratory pores absent), *Vibrio* and *Moraxella* predominated (Table I). Although our evidence is limited, it may indicate that succession is occurring amongst the bacterial populations in the gut of *Haliotis ruber* as the animal grows older. In the gut of vertebrate animals, the succession of bacterial populations to form climax communities is not completed until the host has attained adult features (Savage, 1977). Bacteria which form climax

communities can benefit the host in numerous ways (Savage, 1977; Garland *et al.*, 1982). We are undertaking further studies on the importance of marine bacteria, particularly *Moraxella*, to competent larvae and post-larvae of *Haliotis ruber*.

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Strand 4 The ingestion of bacteria on the cuticle of coralline algae by grazing post-larval and juvenile abalone.

Coralline algae are the preferred substratum on which larval abalone (*Haliotis ruber*) settle, metamorphose and grow for at least 5 months. For successful hatchery rearing of *H. ruber*, this critical phase in the animal's development must be understood better. The bacterial biota on the surface of ungrazed coralline algae has been described by us recently. A copy of the recently published paper is appended. In a parallel study, we also described the ingestion of the bacteria on the cuticle surface of coralline algae by post-larval and juvenile abalone. A reprint of this recently published paper is included.