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The iron-encrusted microbial community of *Urothoe poseidonis* (Crustacea, Amphipoda)

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Abstract

A rust-coloured coating frequently covers the appendages and sternites of *Urothoe poseidonis*, an amphipod living in the burrow of the echinoid *Echinocardium cordatum*. Up to 80% of the collected amphipods were coated. In winter, coated amphipods were always more abundant than uncoated ones. In summer, uncoated specimens predominated. The aspect, location and development of the coating are similar in juveniles and adults. EDAX analyses and Prussian blue testing indicate that the rust-coloured coating contains iron oxyhydroxide minerals with trace metals and phosphorus. Scanning electron microscopy shows that the iron coating harbours bacterial filaments related to Beggiatoaceae (3 morphotypes were observed). Protozoans, possibly Peritrichia of the families Rovinjellidae or Vaginicolidae (one morphotype), were also observed on pereopods VI and VII. The formation of the iron coating and its potential role in the biology of the amphipod are discussed.

Keywords: *Urothoe*; Amphipod; Epibiosis; Biofilm; Bacteria; Iron oxide

1. Introduction

In marine environments most substrates are colonised by microorganisms (Cooksey and Wigglesworth-Cooksey, 1995; Dang and Lovell, 2000). It is therefore not surprising to observe epibiotic microorganisms on the body surface of various invertebrates. The list of fouled species is long and the threat of fouling is omnipresent (Wahl, 1989; Prieur, 1991; Pukall et al., 2001). Microepibionts include bacteria, diatoms, and protozoans (Getchell, 1989; Brock and Lightner, 1990; Meyers, 1990; McClatchie et al., 1990; Carman and Dobbs, 1997; Fernandez-Leborans et al., 1997; Gillan and Cadée, 2000). Microepibionts may influence the ecology of their hosts in various ways, especially when the hosts are small (Sar and Rosenberg, 1987; Wahl, 1989; Gil-Turnes and Fenical, 1992; Polz et al., 1994).

Epibiotic microbial communities are sometimes associated with ferric iron deposits. This is the case of the communities living on the bivalve *Montacuta ferruginosa* (Gillan and De Ridder, 1997; Gillan et al., 1998, 2000), on the mudsnail *Hydrobia ulvae* (Gillan and Cadée, 2000), and on some deep-sea mussels, limpets, and polychaetes (Jannasch and Wirsen, 1981; Baross and Deming, 1985). Because they immobilise the toxic sulphide, ferric deposits may be advantageous for organisms thriving in sediments or in the deep-sea (Vismann, 1991). The study of microbial

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communities living at sites where active iron deposition occurs also provides important information for geochemists and sedimentologists to understand the role of microorganisms in ancient iron microbial ecosystems such as the red matrices of different European Paleozoic and Mesozoic series (Mamet et al., 1997; Préat et al., 1999a,b).

In this paper we examine the iron-encrusted microbial community that develops on the ventral appendages of the amphipod *Urothoe poseidonis*. *U. poseidonis* is a sediment dweller that can live freely in the sediment or as a commensal in the burrow of various endofaunal invertebrates. It is regularly reported in the burrow of the echinoids *Echinocardium cordatum* and *Spatangus purpureus*, of the ophiuroid *Acrocnida brachiata*, of the holothurians *Synapta* spp., of the hemichordates *Balanoglossus* spp., and of the polychaete *Arenicola marina* (Giard, 1876; Goulliart, 1952; Vader, 1965, 1978; Lackschewitz and Reise, 1998). We describe the iron-encrusted microbial community of *U. poseidonis* living in the burrow of *E. cordatum*. The microbial community was characterised using microscopy and chemical analyses. Its development on the amphipods was followed during an annual cycle.

2. Materials and methods

2.1. Collection of specimens

Specimens of *Urothoe poseidonis* (Reibich 1905) were collected intertidally in the burrows of *Echinocardium cordatum* (Pennant 1777) (Echinoidea, Spatangoida) at Wimereux (Pas-de-Calais, France). The burrows were located at ca. 15 cm depth in the sediment. They feature a respiratory funnel and a backward blind prolongation called the sanitary drain (Nichols, 1959). All *E. cordatum* were adult individuals measuring ca. 5 cm in length. Samplings were done monthly from November 1997 to April 1999. Specimens were also collected in February 2003. Each month 30 *E. cordatum* burrows were examined (except in July 1998 when only 17 burrows were examined). The burrows were carefully opened with a spade and the amphipods living in the burrow, the sanitary drain, and the respiratory funnel, were collected with tweezers. Additional amphipods were obtained by mixing the burrow sediments with seawater. A total amount of 1263 individuals were collected, fixed in 70% ethanol, and viewed under a stereo microscope in order to determine their size (measured dorsally between the cephalothorax and the telson), their sex, the presence and the precise body location of the rust-coloured coating. A 'coating stage' was determined as follows: (−) rust-coloured coating absent; (+) less than 50% of the amphipod body covered with a rust-coloured coating, and (*) more than 50% of the amphipod body covered. Juveniles corresponded to individuals less than 2 mm in length that never presented secondary sexual characters (e.g., big eyes or long antennae for males, oostegites for females). Some specimens were further prepared for epifluorescence microscopy, scanning and transmission electron microscopy, and for energy-dispersive X-ray (EDAX) analyses.

2.2. Microscopy

For interference contrast microscopy (ICM, Nomarski), appendages of fixed specimens (70% ethanol) were observed under a Leitz Diaplan microscope.

For epifluorescence microscopy, fixed (70% ethanol) or unfixed amphipods were suspended for 5 min in a 0.01% acridine orange solution in order to visualise bacteria (Austin, 1988). Appendages were then removed, placed on a slide and observed under a Leitz Diaplan microscope equipped with an I2/3 filter block.

For scanning electron microscopy (SEM), the amphipods were fixed for 24 h in 3% glutaraldehyde in cacodylate buffer (0.1M; pH 7.4), rinsed in buffer, postfixed in 1% osmium tetroxide in buffer for 1 h and briefly rinsed in buffer. The specimens were then

Table 1
Total number of *Urothoe poseidonis* in 30 *Echinocardium cordatum* burrows according to month

Month	Number of specimens	Month	Number of specimens
Nov 97	107	July 98	12
Jan 98	55	Oct 98	6
Feb 98	95	Nov 98	202
Mar 98	135	Dec 98	229
Apr 98	56	Feb 99	224
May 98	40	Mar 99	10
Jun 98	39	Apr 99	30

dehydrated in graded ethanol (70, 90, 100%) and dried by the critical-point method using CO_2 as transition fluid. Then, they were mounted, sputter coated with gold and observed under an ISI DS 130 SEM microscope operating at 20 kV.

For transmission electron microscopy (TEM), the amphipods were fixed, rinsed, postfixed and dehydrated as for SEM. The specimens were then immersed in propylene oxide for 5 min, then embedded in Spuur's resin and thin sectioned (LEICA 'ultracut' UCT ultramicrotome). Thin sections contrasted with uranyl acetate and Reynold's lead citrate were observed under a Philips EM 300 microscope operating at 80 kV.

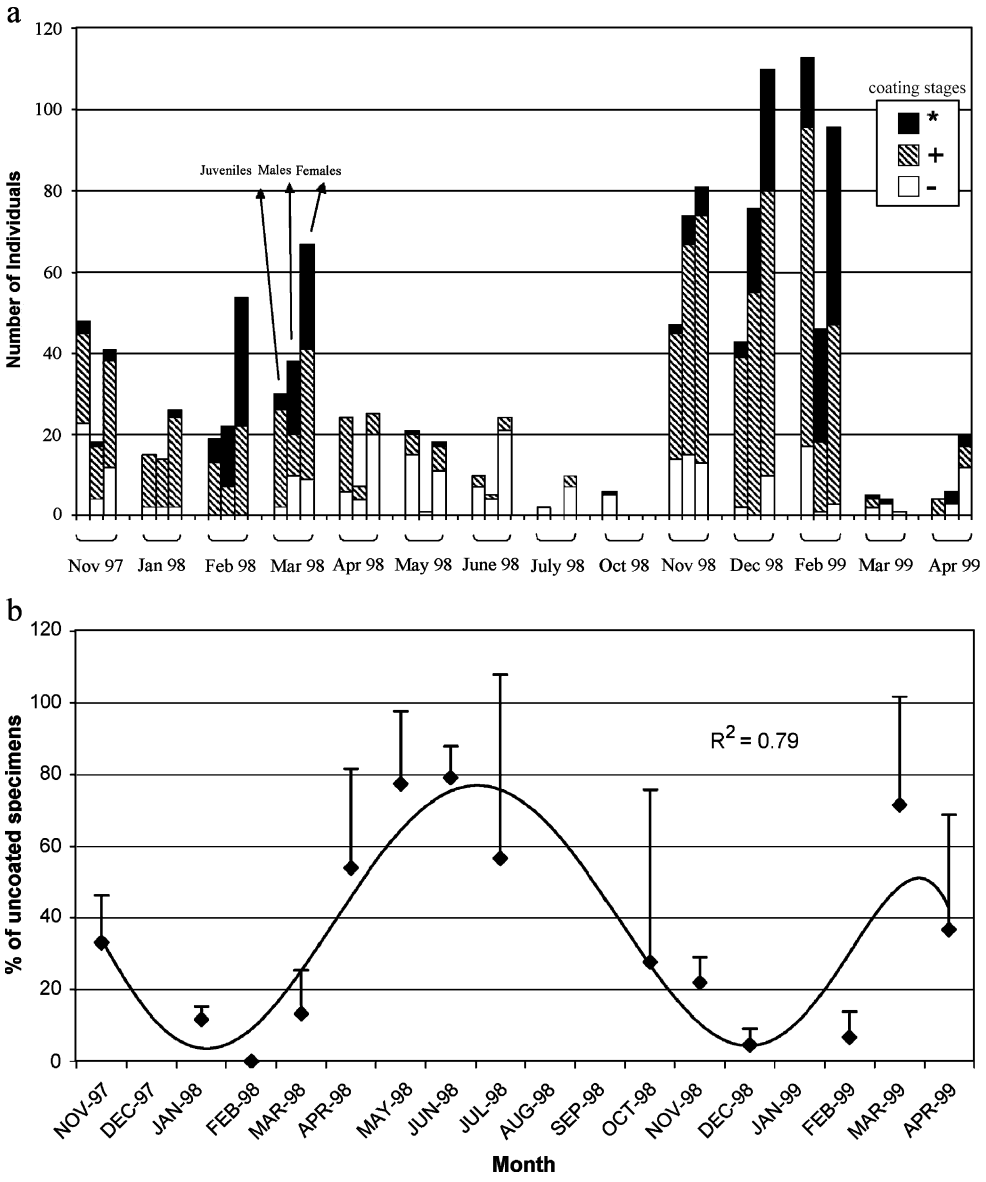

Fig. 1. (a) Distribution of the coating stages of juveniles, male and female individuals according to the month of collection. The number of burrows examined was always 30 except for July 1998, when it was 20. (b) Percentage of uncoated specimens according to the month of collection (mean of males, females and juveniles).

2.3. Chemical analyses

Energy-dispersive X-ray (EDAX) analysis was used to characterise the iron precipitate of the coating. Specimens fixed in 70% ethanol were air-dried at ambient temperature, mounted, and observed under a JEOL superprobe 733 SEM coupled to an EDS detector. The electron microprobe was pointed on the amphipod coating (carpopodite V). The analysis was done at 25 kV with a sample current of 2.5 nA. To detect the presence of Fe(III) in the mineral, we used the Prussian blue method employing 2% ferrocyanide in 1% HCl, and to detect the presence of Fe(II) in the mineral, we used the Turnbull blue method emploting 2% ferricyanide in 1% HCl (Pearse, 1972).

3. Results

3.1. Structure of the population and coating stages

The *Urothoe poseidonis* population of Wimereux was regularly sampled over an 18-month period, from November 1997 to April 1999 (4 months were not sampled: December 1997, August 1998, September 1998 and January 1999). Table 1 shows the total number of amphipods isolated each month from 30 echinoid burrows. The specimens of *U. poseidonis* were found in the sanitary drain, the respiratory funnel and on the body-surface of the echinoid. The number of amphipods per sea urchin was between 0 and 16. Minimum and maximum sizes were 1.5 and 6 mm, respectively. The biggest specimens (>4 mm) were always females. Juveniles, male and female individuals were observed each month. In most cases females were more numerous than males. Ovigerous females were observed from March 1998 to June 1998 and were only observed in the largest size group (>4 mm).

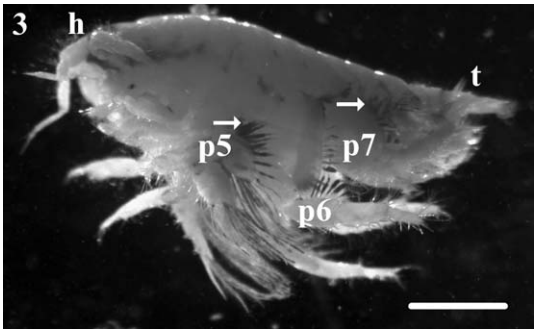

Fig. 3. Lateral view of *Urothoe poseidonis* under the binocular (female specimen). Arrows point to iron deposits. h, head; p5-7, pereopods V-VII; t, telson. Scale bar = 1 mm.

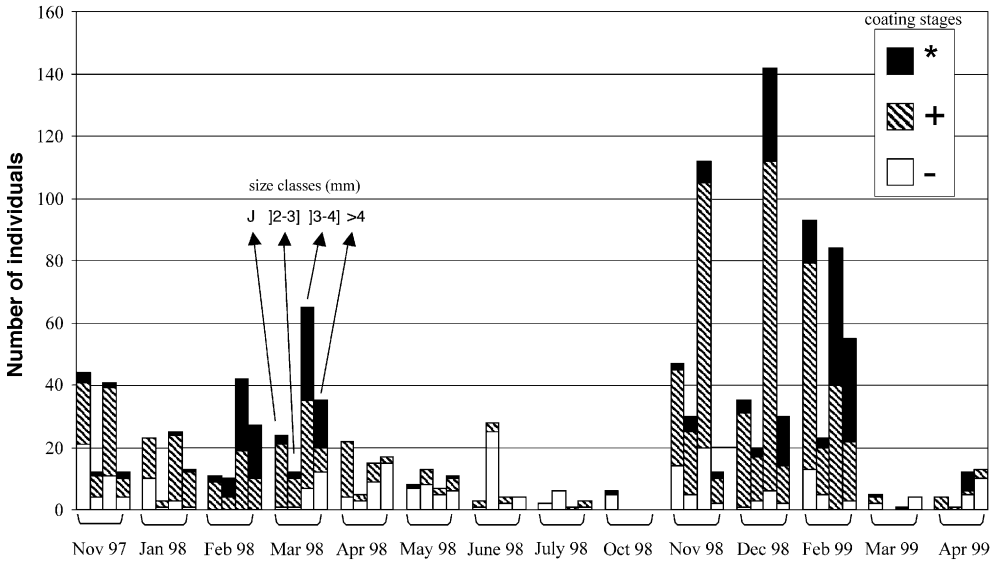

Fig. 2. Distribution of the coating stages according to the size of the amphipods.

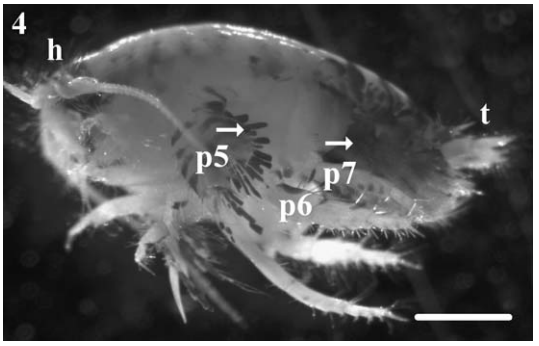

Fig. 4. Lateral view of *Urothoe poseidonis* under the binocular (male specimen). Arrows point to iron deposits. h, head; p5-7, pereopods V-VII; t, telson. Scale bar = 1 mm.

Females incubated 8.5 ± 1.5 eggs (the maximum was 10 eggs) that reached 500 μm.

Fig. 1a illustrates the distribution of the coating stages among juvenile, male and female individuals throughout a 14-month sampling period. All coating stages were observed every month and coated individuals (+ and *) were always more numerous than uncoated ones (−). During the summer months (May to July 1998) uncoated specimens predominate and well-coated individuals (+) were nearly absent. This seasonal effect is demonstrated in Fig. 1b where the percentage of uncoated specimens is calculated for each month (mean between males, females and juveniles). In Fig. 1b, a maximum of uncoated individuals is observed in June and a minimum in December and January.

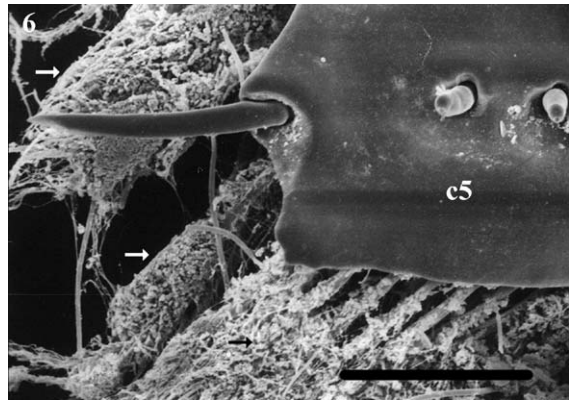

Fig. 6. Enlarged view of the carpopodite V. Arrows point to iron deposits. c5, carpopodite V. SEM, scale bar = 100 μm.

Fig. 2 shows the number of individuals of the four size classes (≤ 2 mm or juveniles, 2- mm, 3–4 mm, >4 mm) and their respective coating stages according to the month of collection. The juveniles formed the highest percentage of the population during November 1997 (44.8% of the population), April 1998 (42.8%), May (52.5%), October 1998 (100%) and February 1999 (50.4%). They were also well represented in November 1998. The three coating stages occurred in all the size classes.

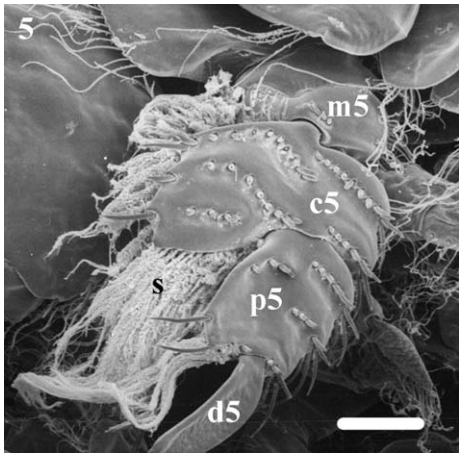

Fig. 5. Pereopod V of *Urothoe poseidonis*. c5, carpopodite V; d5, dactylopodite V; m5, meropodite V; p5, propodite V; s, setae. SEM, scale bar = 250 μm.

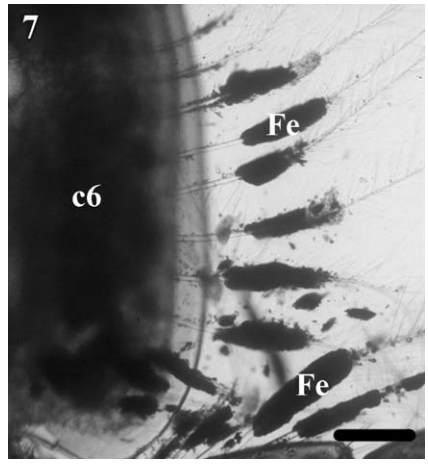

Fig. 7. Iron-encrusted setae on pereopod V. c5, carpopodite V; Fe, iron deposit. ICM, scale bar = 100 μm.

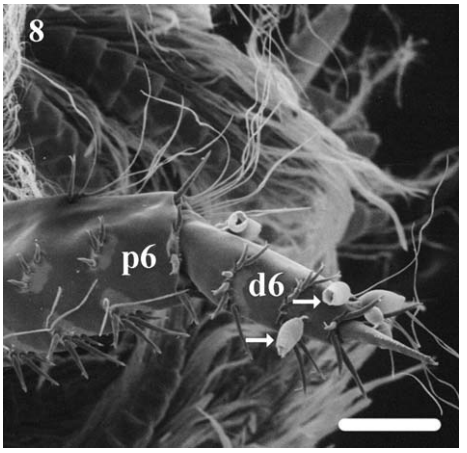

Fig. 8. Protozoans on dactylopodite VI (arrows). p6, propodite VI; d6, dactylopodite VI. SEM, scale bar = 100 μm.

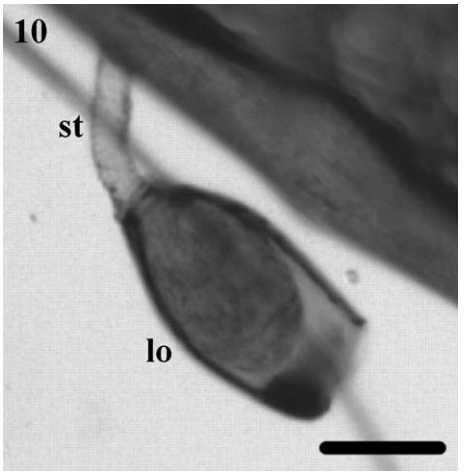

Fig. 10. Protozoa on dactylopodite VI under the ICM. st, stalk; lo, lorica. Scale bar = 25 μm.

3.2. Study of the rust-coloured coating

The majority (78.8%) of the examined individuals of *U. poseidonis* presented a rust-coloured coating. The coating was localised on pereopods V,VI, and VII, being most developed around the setae of these pereopods (Figs. 3–7). The mouth parts, the pleopods and the telson were also frequently iron-encrusted. The long setae of pereopods V were always the most heavily coated (Figs. 5–7). No difference of coating aspect, localisation and development was observed between juvenile and adult (male or female) individuals. The coating systematically included rust-coloured granular materials and microbial filaments.

The most conspicuous microorganisms associated with the amphipods were sessile protozoans (attached directly to the pereopods) and filamentous bacteria (associated with the iron coating). The protozoans are shown in Figs. 8–10. The zoids were ovoid and located inside an elongated rust-coloured lorica, with only one zoid per lorica. No operculum was detected. An uncoloured stalk was present. The stalk appeared contractile as its surface was rippled and internal

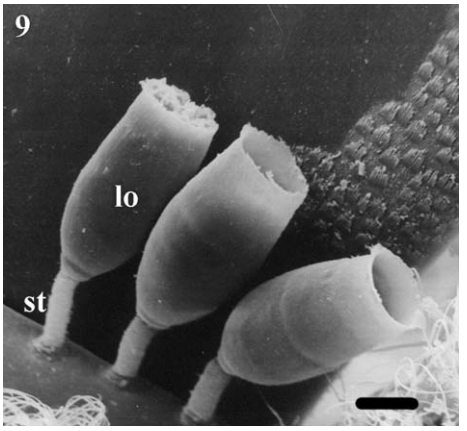

Fig. 9. Enlarged view of protozoans on dactylopodite VI. st, stalk; lo, lorica. SEM, scale bar = 10 μm.

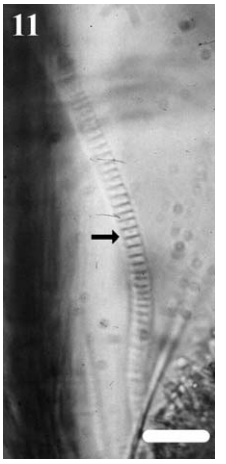

Fig. 11. Type-1 bacterial filament (arrow) from carpopodite V. ICM, scale bar = 15 μm.

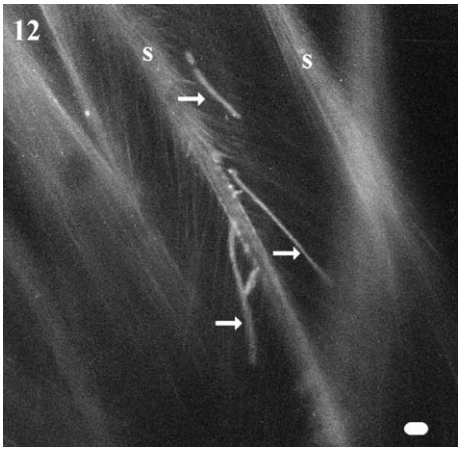

Fig. 12. Type-1 bacterial filaments (arrows) fixed on the setae of carpopodite V. Epifluorescence microscopy, scale bar = 5 μm.

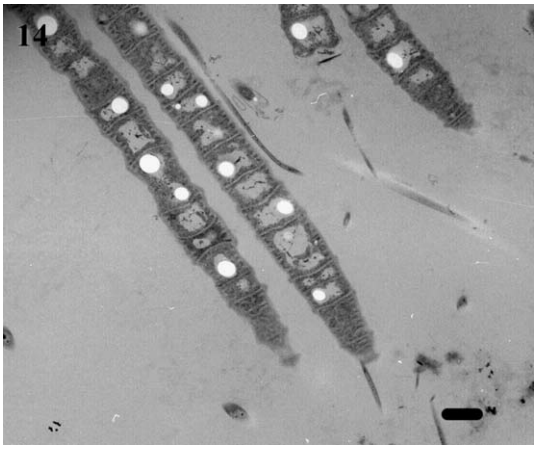

Fig. 14. Type-2 bacterial filaments (carpopodite V). SEM, scale bars = 2 μm.

longitudinal fibrills were present. Under the SEM, the total body length (lorica + stalk) was 100 to 125 μm, with a diameter of about 25 μm. The diameter of the stalk was 10 μm and its length about 25 μm. Observations in February 2003 showed that 36% of the amphipod population had these sessile protozoa (44 individuals observed in total). Both males and females were colonised: of the 33 females observed, 13 were colonised, and of the 11 males observed, 3 were colonised. The number of protozoa per amphipod may reach 11. The protozoa were located on pereopods VI and/or VII, generally on the carpopodite, the propodite or the dactylopodite. One amphipod had protozoa on the last pair of pleopods and the telson.

Three morphotypes of filamentous bacteria were observed. Type-1 filaments (Figs. 11–13) were composed of disk-like cells of about 3 μm in diameter and about 1 μm in length. Type-2 filaments (Figs. 14 and 15) were 3.5 to 4 μm in diameter with 1 to 2 μm long cells. These filaments contained large cells in division (Fig. 15). Type-3 filaments (Figs. 16–18) were composed of cylindrical cells, 0.8 to 1 μm wide, and 3 to 5 μm long. All filaments reached at least 380 μm and were fixed to the thick setae, to the spines of the

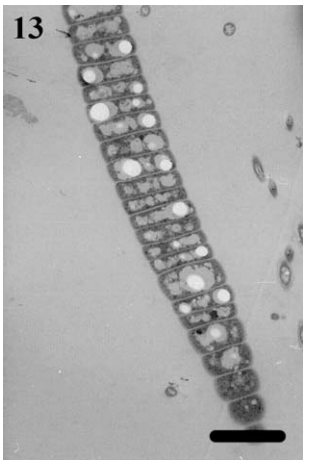

Fig. 13. Type-1 bacterial filament (carpopodite V). SEM, scale bar = 2 μm.

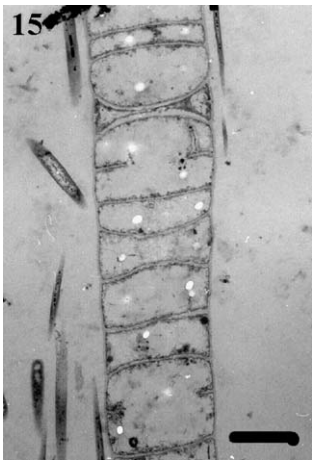

Fig. 15. Type-2 bacterial filaments (carpopodite V). SEM, scale bars = 2 μm.

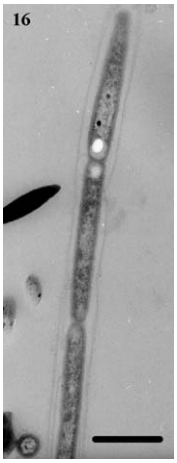

Fig. 16. Type-3 bacterial filaments (carpopodite V). Arrow points to iron precipitates. sh, sheath. SEM, scale bars = 2.5, 1.5, and 1.5 μm, respectively.

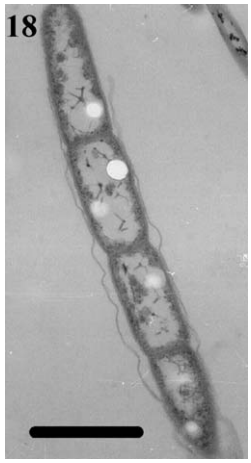

Fig. 18. Type-3 bacterial filaments (carpopodite V). Arrow points to iron precipitates. sh, sheath. SEM, scale bars = 2.5, 1.5, and 1.5 μm, respectively.

pereopods, or to the appendages themselves. A sheath was observed in type-3 filaments, with a single filament per sheath; this sheath was frequently encrusted with iron precipitates forming small granules (arrow in Fig. 17). All filaments appeared Gram-negative under the TEM. Inclusions were present in the protoplasm of all the bacterial types. Under the TEM, inclusions appeared either electron-dense (diameter, 50 nm) or electron-transparent (diameter, 0.3 to 1 μm) (Figs. 13–18).

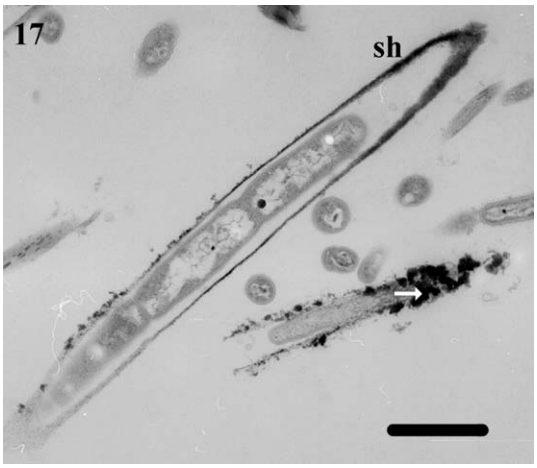

Fig. 17. Type-3 bacterial filaments (carpopodite V). Arrow points to iron precipitates. sh, sheath. SEM, scale bars = 2.5, 1.5, and 1.5 μm, respectively.

EDAX analyses indicated that the characteristic elements of the coating were iron, phosphorus, oxygen, and calcium with traces of silicon, magnesium, aluminium and potassium (Fig. 19). Some coatings were enriched in silicon and impoverished in iron and phosphorus. Treatment of specimens with acid sodium ferrocyanide led to a strong blue coloration (Prussian blue). This indicated that Fe(III) is abundant in the mineral. Treatment with ferricyanide gave no colour reaction. This indicated that Fe (II) is absent.

4. Discussion

The iron coating appears as a permanent structure of *Urothoe poseidonis* when associated with *E. cordatum* (about 80% of the individuals were coated in this study). The iron coating is not dependent on the age of the amphipods and is restricted to particular parts of the body. The uncoated individuals are possibly amphipods that have recently moulted. The fact that during the summer months (May to July 1998) uncoated specimens predominated over coated specimens may be explained by the disappearance of the well-coated specimens from the population. This may be due to the death of these specimens or to an increased predation related to the fact that swimming, burrowing or escape from predators are affected by iron-encrustation. This conclusion is supported by the

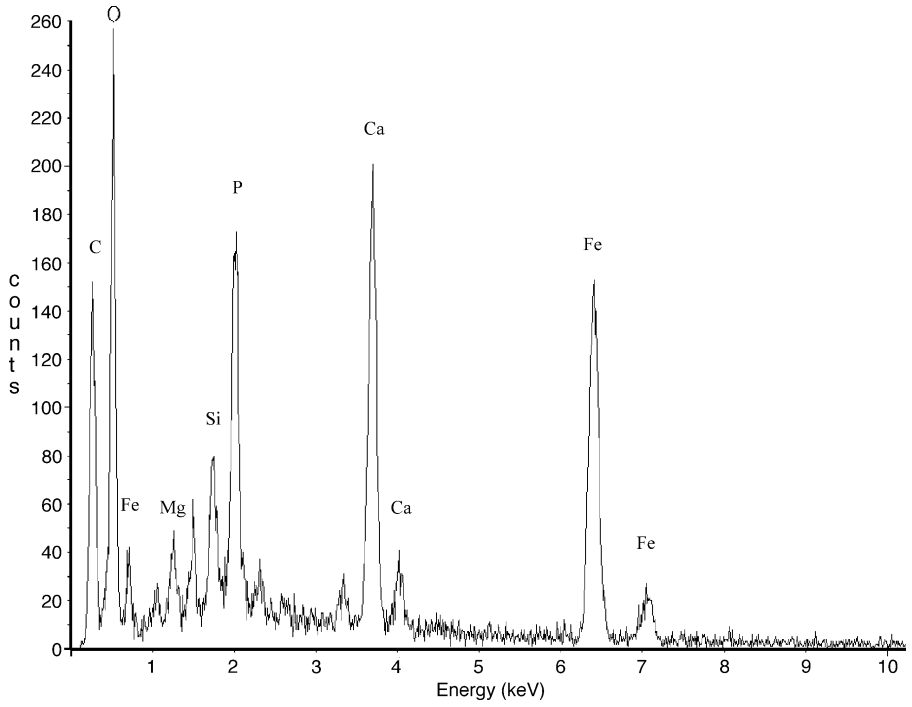

Fig. 19. EDAX spectrum of the iron-coating of *Urothoe poseidonis*.

low numbers of amphipods collected during summer (always below 40 specimens). Another possibility would be that the moulting behaviour occurs predominantly during the summer months. It is also possible that the iron coating is partly removed during summer due to increased iron solubility (Roekens and Van Grieken, 1983). But in the last two cases, the total number of amphipods collected in summer would have been higher than it was. It may be argued that the low number of amphipods obtained during the summer months is a sampling artefact. Indeed, the tide amplitude is lower in summer. As a result, sampling in summer might have been done principally at the edge of the sea-urchin population, where physico-chemical conditions are possibly not optimal for the amphipods; in contrast, in winter, the proportion of burrows located near the infralittoral would have been higher. This is theoretically true, and a sampling artefact could be possible. However, burrows located near the infralittoral were rarely accessible in winter: the wind is then stronger, and the lower part of the tidal flat constantly covered with a thin layer of seawater so that sea-urchin burrows could not be sampled. Whatever the month of sampling, amphipods were always collected in the same area ($\pm$ 100 m wide, as checked with a GPS). We conclude that a real seasonal effect was detected in the population of *U. poseidonis* associated with *E. cordatum*. On the other hand, Lackschewitz and Reise (1998) found no apparent seasonality of the *U. poseidonis* associated with *Arenicola marina*. These authors did not mention any iron-coating.

The setae of pereopods V were always the most heavily coated. These setae are entangled with bacterial filaments forming an iron encrusted net-like structure that increases the surface of the pereopod. Goulliart (1952) mentioned that *U. grimaldii* living in the burrow of *A. marina* is also encrusted with a rust-coloured mineral rich in ferric iron. The author suggested that the presence of ferric iron on amphipods could be linked to the presence of mucus in their environment. This mucus would be produced by *A. marina* and also by the glutiniferous glands situated inside the pereopods V, VI, and VII of the amphipod

(Goulliart, 1952). To support his proposal, Goulliart pointed first to *M. ferruginosa*, the iron-encrusted commensal bivalve of *E. cordatum* (in this case the mucus was produced by the sea urchin *E. cordatum*) and secondly to the sternal shields and setae of some aphroditid polychaetes (covered with mucus and ferric iron). Goulliart did not mention the presence of filamentous bacteria on *U. grimaldii*. As discussed below, we think that bacteria have an important role in the formation of these iron minerals.

The iron-deposits are granular and coat the setae, the cuticle, and the attached bacterial filaments. EDAX analyses and Prussian blue testing indicate that the deposits are possibly iron oxyhydroxide minerals with trace metals and phosphorus adsorbed on their surface. Iron oxyhydroxides are known to scavenge many trace metals (Spark et al., 1995) as well as phosphorus (Slomp et al., 1996), so the presence of Al, Mg and P in the mineral is not surprising. By its composition, the iron mineral is similar to the one present on the shell of the marine gastropod *Hydrobia ulvae* (Gillan and Cadée, 2000). The bivalve *M. ferruginosa*, sharing the same burrow as *U. poseidonis*, is covered by a mineral phase that is purer, without Al and Mg (Gillan and De Ridder, 2001).

Three morphotypes of filamentous bacteria are epibiotic on *U. poseidonis*. Although identification of bacteria based on morphology alone is not possible, type-1 and type-2 filamentous bacteria resemble *Leucothrix* and the Beggiatoaceae (such as *Thiothrix*) because they form multicellular filaments of similar morphology (Strohl, 1989). Type-3 filamentous bacteria resemble *Herpetosiphon* (Holt, 1989) or some *Flexibacter* species. *Leucothrix*-like filaments and Beggiatoaceae have previously been observed in association with ferric iron deposits (Jannasch and Wirsen, 1981; Baross and Deming, 1985; Gillan and De Ridder, 1997). Among crustaceans, the deep-sea shrimp *Rimicaris exoculata* also features ferric iron-encrusted ectosymbiotic bacteria, among which some resemble the Beggiatoaceae *Thiothrix* spp; these bacteria are located within the gill chamber and on the mouth parts (Polz and Cavanaugh, 1995; Gebruk et al., 1993). *Leucothrix mucor,* which is essentially an epiphyte of macroscopic algae (Bland and Brock, 1973; Brock, 1989), is a common epibiont of crustaceans (Johnson et al., 1971) as are filamentous bacteria of the genera *Thriothrix*, *Flexibacter*, *Cytophaga*, and *Flavobacterium* (Brock and Lightner, 1990).

One morphotype of Protozoa has been observed on the pereopods of *U. poseidonis*. It is possibly a ciliate belonging to the familiy Rovinjellidae (i.e. *Rovinjella* or *Opisthonecta*) or to the family Vaginicolidae (i.e. *Cothurnia*, *Vaginicola* and *Thuricola*), both families are in the order Peritichia. These ciliates, very similar to the protozoans of *U. poseidonis*, are stalked and loricated. However, the precise genus is hard to determine because the zoids were retracted into the lorica during the fixation. The amphipod *U. poseidonis* is not mentioned in the review of the species of protozoan epibionts commonly observed on crustaceans (Fernandez-Leborans and Tato-Porto, 2000a,b) so the present work is probably the first report on epibiotic ciliates of *U. poseidonis*. Protozoan epibionts are frequently reported on crustaceans and are predominantly members of the Ciliophora (especially the Hypostomata, Suctoria, Hymenostomata, Peritricha, and the Spirotricha - Carman and Dobbs, 1997). Among the genera of peritrich ciliates which resemble the epibiotic protozoa of *U. poseidonis*, and which have already been detected on amphipods, we find the genera *Rovinjella* and *Cothurnia* (Fernandez-Leborans and Tato-Porto, 2000a).

Although the microorganisms were not identified further in this work, it is possible that they somehow influence the deposition of ferric iron minerals. Bacteria in particular are known to complex various metals in their exopolymeric substances (EPS), which are rich in anionic groups such as carboxyls (Geesey and Jang, 1989; Ferris, 1989). This complexation may be followed by the subsequent nucleation of iron minerals (Dalas, 1990). Other possibilities include the presence of iron-oxidising bacteria, and/or the presence of heterotrophic microbes degrading iron organic complexes (Ehrlich, 1990). Despite the short life time of Fe(II) in oxic seawater, the presence of iron-oxidising bacteria on *U. poseidonis* is possible because the amphipod is living at an interface where the life time of Fe(II) is higher (Roekens and Van Grieken, 1983). Organic complexes of iron are abundant in seawater (Gledhill and Van den Berg, 1994), and their degradation by bacteria may lead to iron deposition (Ehrlich, 1990; Harding and Royt, 1990); such a process is known to occur in *M. ferruginosa* (Gillan et al., 2000). The mucus produced by the sea

urchin, and also by the glutiniferous glands of the amphipod, probably act in the same way as the bacterial EPS, i.e. as metal scavengers (mucus is rich in carboxylated sugars, able to complex iron). Degradation of this mucus by epibiotic bacteria can also lead to iron re-precipitation. The presence of glutiniferous glands on pereopods V, VI, and VII may explain the abundance of bacteria and iron precipitates on these appendages.

Whatever its origin, the iron coating may have various effects on the ecology of the amphipod. As suggested above, the swimming and burrowing behaviours might be affected, as well as recognition by predators. According to Vismann (1991), the presence of ferric iron deposits at the surface of an organism could immobilise the toxic S^{2-} ions present in the environment and prevent their diffusion into the body. This process could be of particular interest for *U. poseidonis* because it lives in sediments where S^{2-} is omnipresent, especially during summer. Interestingly, ferric deposits occupy a ventral position on *U. poseidonis*. The coating is thus well positioned to protect branchia and eggs. If the protective iron coating is partly removed during summer, the low numbers of amphipods then observed may also be explained by the toxicity of S^{2-}. Epibiotic bacteria may also damage the exoskeleton of Crustacea (Brock and Lightner, 1990). However, such damages were not observed in *U. poseidonis*.

We are currently studying the microbial community of *U. poseidonis* using molecular methods (cloning and synthesis of specific fluorescent oligonucleotide probes. We hope to be able to identify and quantify the important epibiotic micro-organisms of *U. poseidonis*. Future work will also focus on the possible seasonal effect detected in this work.

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