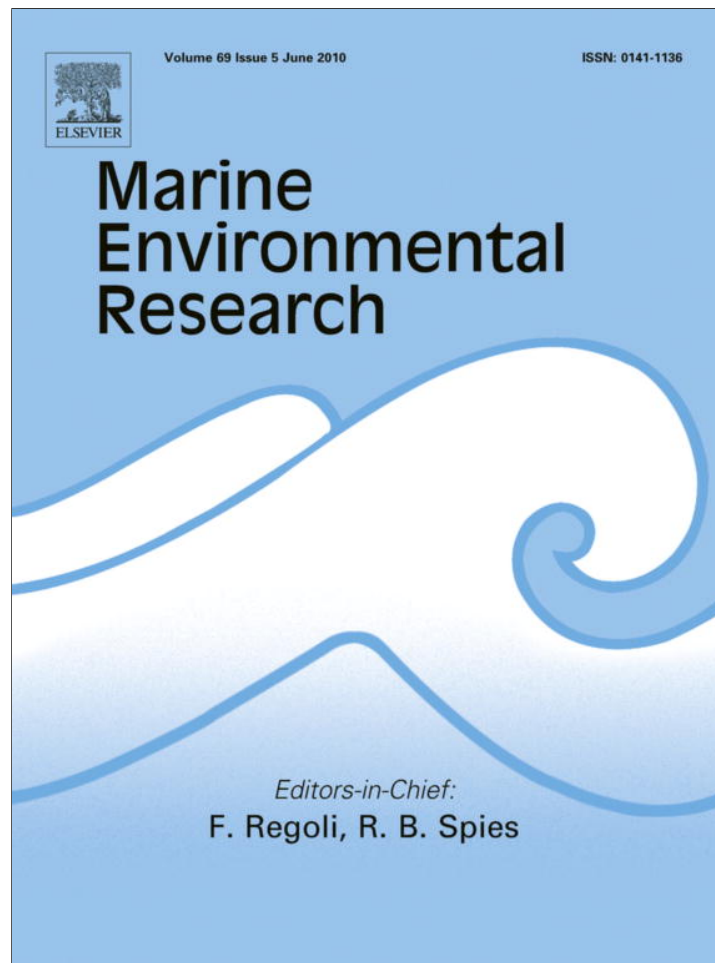


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Raman microspectrometry as a powerful tool for a quick screening of thiotrophy: An application on mangrove swamp meiofauna of Guadeloupe (F.W.I.)

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ABSTRACT

The mangrove swamp environment constitutes a sulphide rich habitat harbouring some thioautotrophic organisms. The ciliate *Zoothamnium niveum* and the nematode *Eubostrichus diana*, both known to live associated with bacterial sulphide-oxidizing ectosymbionts, were analysed as positive controls by Raman microspectrometry. The detection of the 3 Raman bands characteristic of elemental sulphur (S_8) allows us to define a positive model of sulphide-oxidizing symbiotic invertebrates and by extrapolation, of thioautotrophic organisms. A fast screening using this tool was carried out on eukaryotic organisms such as hydrozoan, nematodes, annelids, copepods, and ciliate (*Pseudovorticella* sp.) and on free-living filamentous bacteria found on decomposing leaves in order to detect thioautotrophic organisms. The Raman microspectrometry permits us: (i) to reveal thioautotrophic metabolism of free-living bacteria (*Beggiatoa* sp.) and even for Archaea and (ii) to detect sulphide-oxidizing endosymbiotic and ectosymbiotic bacteria associated with the Bivalve *Lucina pectinata* and *Pseudovorticella* sp., respectively.

Raman microspectrometry represents a fast, easy and non destructive technique which can be used on living organisms without constraints of sample size. The Raman analysis can also be completed by ultrastructural analysis (SEM, TEM) on the same sample.

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1. Introduction

The Caribbean mangrove fringe forests are characterized by one dominant tree species, *Rhizophora mangle*. It represents an original ecosystem occurring between land and marine habitats. The decomposition of submerged fallen leaves and branches by bacterial and fungal communities (Ashton et al., 1999) in the mangrove swamp supports a diverse invertebrate collection, numerically dominated by meiofauna (Alongi, 1990; Gee and Somerfield, 1997). These sunken woods have been recently described to constitute an environment suitable for sulphide-oxidizing symbioses due to the production of sulphides by the decomposition of organic matter (Laurent et al., 2009).

Previous investigations conducted in mangrove swamps have shown the presence of a specific meiofauna (sessiles or vagiles) including copepods, nematodes, polychaetes, protozoans, etc. colonizing immersed vegetal substrates from *Rhizophora mangle* (Alongi, 1990; Zhou, 2001; Torres-Pratts and Schizas, 2007). More particularly, the ciliate *Zoothamnium niveum* (Hemprich and Ehrenberg, 1831) and the nematode *Eubostrichus diana* (Hopper and Cefalu,

1973) were found on this organic decomposing matter (Bauer-Nebelsick et al., 1996a; Ott et al., 2004). These two organisms are remarkable for their obligatory association with sulphide-oxidizing ectosymbiotic bacteria covering their entire body (Bauer-Nebelsick et al., 1996a,b; Polz et al., 1999; Rinke et al., 2006). In the same way, some symbiotic bivalves such as *Lucina pectinata* (Gmelin, 1791) and *Anodontia alba* (Link, 1807) live in the sediment of mangrove swamps (Frenkiel et al., 1997; Gros et al., 2003a).

Recently, Raman microspectrometry was used by Himmel et al. (2009) to characterize the speciation and the location of sulphur S_8 in the nematode *E. diana* and the bivalve *Codakia orbicularis* (Linné, 1758). This technique, particularly efficient for sulphur S_8 detection, appears to be attractive since it can be applied without damage to living organisms. It does not need any preliminary chemical preparation of samples that may be involved in the dissolution of sulphur compounds (Truchet et al., 1998; Pasteris et al., 2001).

In our study, we used Raman microspectrometry to conduct a quick screening of mangrove swamp organisms in order to detect elemental sulphur S_8 and, by extrapolation, thioautotrophic organisms possibly involved in symbiotic relationships. In the case of a positive signal, this technique was coupled with electron microscopy analyses (SEM and TEM) in order to point out the presence of spherical periplasmic inclusions in bacteria that correspond to

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sulphur globules found in the chemoautotrophic symbionts (Lawry et al., 1981; Vetter, 1985; Giere et al., 1988; Lechaire et al., 2008): (i) inside of symbiotic bacteria (ecto- or endosymbiotic), (ii) in free-living bacteria or (iii) in sulphur-containing eukaryotic cells.

2. Materials and methods

2.1. Sample collections

Sampling was done in the “Manche-à-Eau” lagoon localized in the mangrove swamp of Guadeloupe (F.W.I). Animals were collected from branches and leaves of red mangrove (*Rhizophora mangle*) fallen on the muddy sediment in approximately 1 m depth. The animals were collected from their wood substrates using a dissect-

ing microscope. Bivalves were manually collected in the black mud of mangrove swamps. The collected living organisms on vegetal substrate were re-suspended in filtered seawater (0.22 μm) before analysis and the gill of bivalves were dissected in seawater.

2.2. Raman microspectrometry

Living animals or dissected part of the bivalve's gill were either directly analysed in seawater or preliminary fixed in 2% glutaraldehyde (or 2% paraformaldehyde) in filtered seawater (0.22 μm) and washed 3 times in sterile seawater before analysis. Seawater constitutes both an isotonic medium for marine invertebrates and a good cooling medium to avoid irradiation damages of the samples

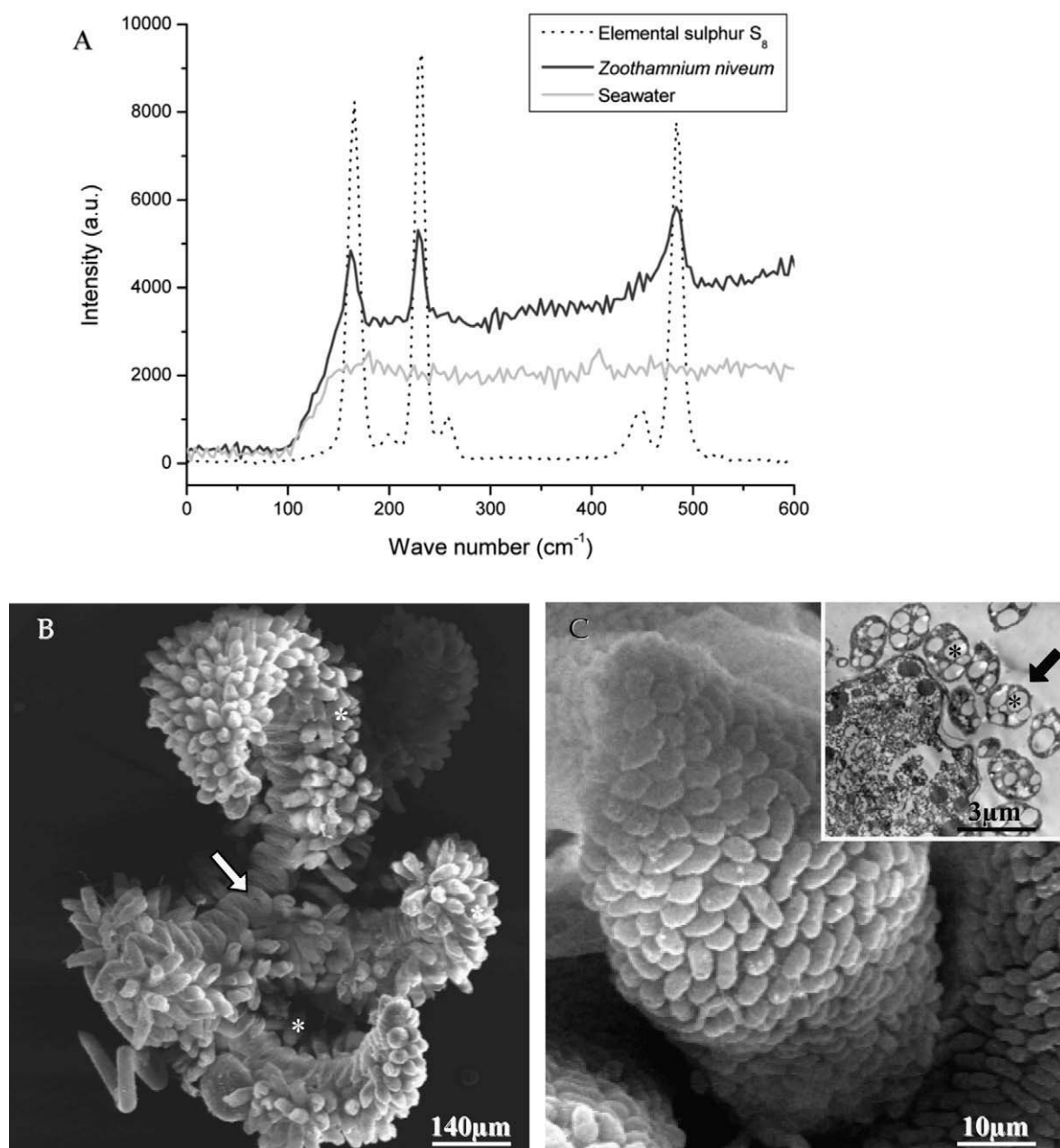


Fig. 1. Analyses of the ciliate *Zoothamnium niveum*. (A) Raman spectrum of elemental sulphur S_8 with the three characteristic Raman bands at 160 cm^{-1} , 225 cm^{-1} and 480 cm^{-1} and Raman spectrum recorded on zooids of slightly fixed *Z. niveum*. (B) SEM observation of *Z. niveum* which is composed of several zooids (white stars) attached to a central stalk that appears sinusoidal when retracted (white arrow). (C) SEM high magnification of one zooid covered by a single layer of bacteria. Inset: TEM observation of a transversal section of a zooid with the ectosymbiotic bacteria (black arrow) containing empty inclusions (black stars).

during Raman analysis. Damage can result from the high energy density focussed on the samples (10^8 W m^{-2}).

Raman spectra were recorded as described by Himmel et al. (2009). The LASER power at the sample surface was 30 mW and acquisition time 5 s. Spectra of the seawater surrounding the samples are acquired as controls.

2.3. Scanning electron microscopy (SEM)

Animals were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 to 3 h at 4 °C. Samples were washed twice in sodium cacodylate buffer at room temperature and dehydrated in a graded acetone series for 10 min each (30%, 50%, 70%,

95% and two times 100%) before critical point drying (Biorad, Polaron critical Point drier). Then, they were coated with a thin gold layer (Sputter Coater SC500, Biorad) and investigated with Hitachi S-2500 SEM running at 20 kV.

2.4. Transmission electron microscopy (TEM)

Animals were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 to 3 h at 4 °C. Samples were washed twice in sodium cacodylate buffer at room temperature and dehydrated through an ascending ethanol series before embedding in epoxy resin (Glauert, 1975; Gros et al., 2000). Blocks of each sample were sectioned with an ultra-microtome (Ultracut E Leica) and

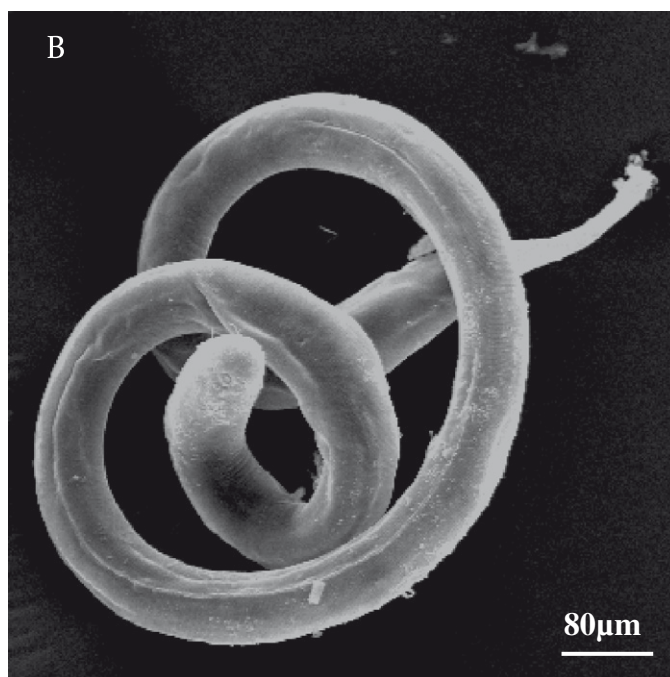
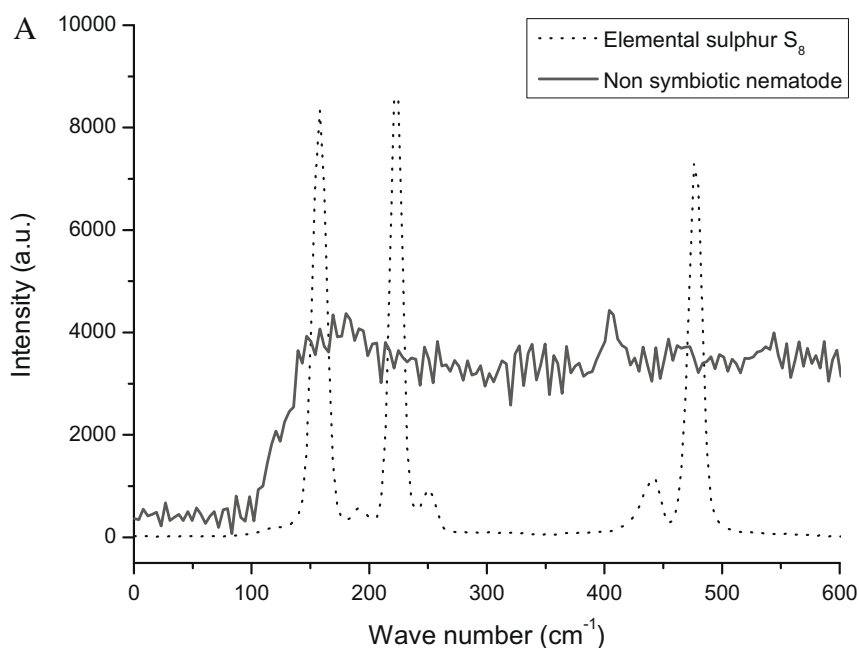


Fig. 2. Analyses of a non-symbiotic nematode. (A) Raman spectra of elemental sulphur S₈ and of the non-symbiotic nematode which is tested on the middle part of nematode. (B) SEM view of a non-symbiotic free-living nematode colonizing the mangrove swamp sediment.

thin sections (60 nm) were contrasted 30 min in 2% aqueous uranyl acetate and 10 min in 0.1% lead citrate before examination in a Philips 201 microscope running at 75 kV.

3. Results

3.1. Confirmation of sulphur detection by Raman microspectrometry in live specimens

The Raman microspectrometry analyses were carried out on the nematode *Eubostrichus diana*. Sulphur is clearly detected in the organism with the detection of the three S_8 characteristic Raman bands at 160 cm^{-1} , 225 cm^{-1} and 480 cm^{-1} (Poborchii, 1996),

whereas no sulphur is detected in the surrounding seawater. Recently, Himmel et al. (2009) demonstrated that this elemental sulphur S_8 is located in the bacterial coat covering the entire body of the nematode. *E. diana* is used as the positive control during Raman microspectrometry in this study.

Raman microspectrometry was used on *Zoothamnium niveum* which is composed of several zooids attached to a central stalk (Fig. 1b and c). The Raman spectra obtained on different parts of the body's ciliate (Fig. 1a) show the presence of elemental sulphur S_8 since the three characteristic Raman bands of this compound were observed. After this positive Raman detection, electron microscopy observations (SEM and TEM) were performed. As already described by Bauer-Nebelsick et al. (1996a,b), our SEM and TEM investigations confirm the presence of ectosymbiotic bacteria

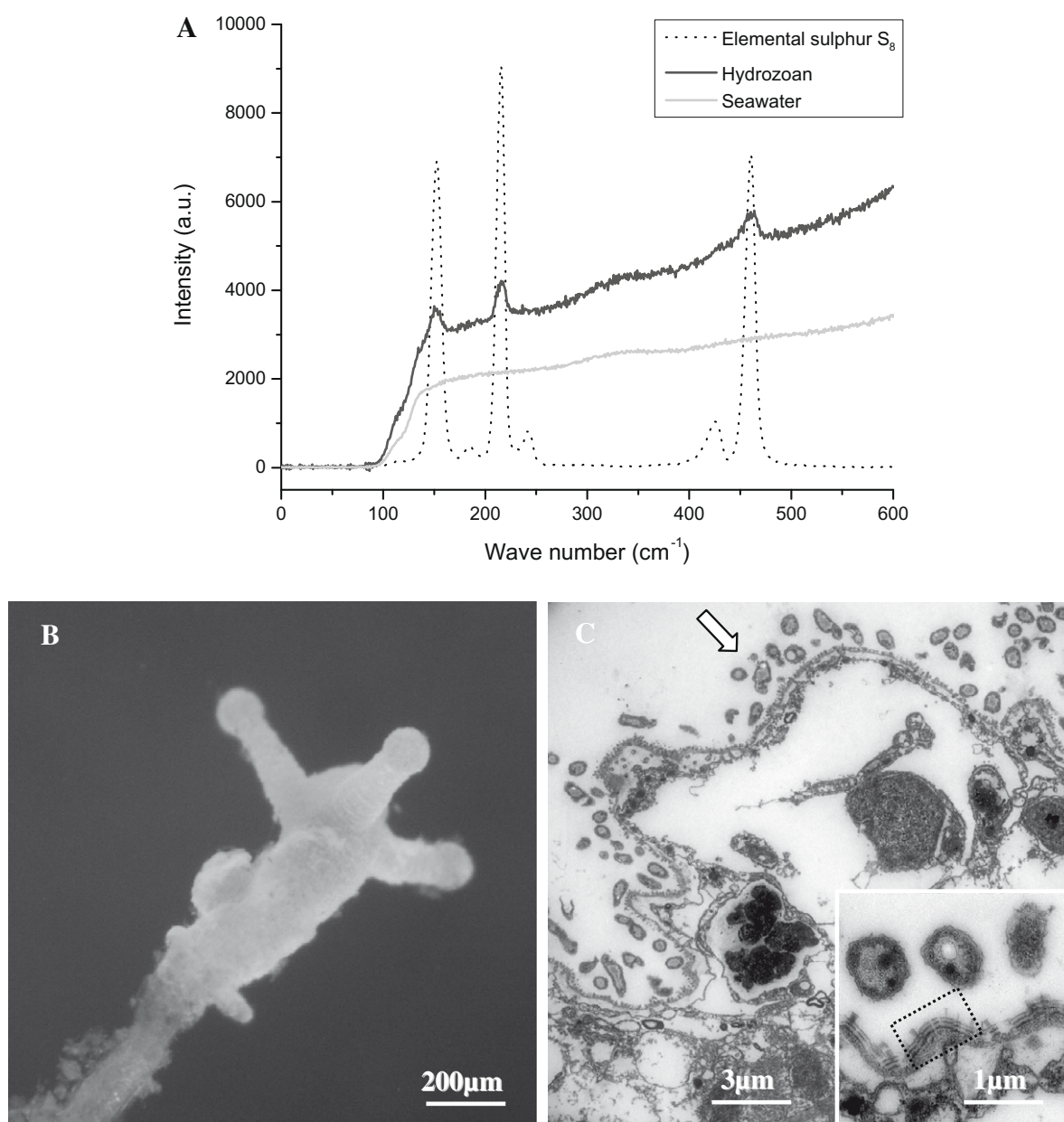


Fig. 3. Analyses of an unidentified hydrozoan. (A) Raman spectra acquired on the hydrozoan. The Raman analysis points out the presence of elemental sulphur S_8 in the animal whereas no sulphur is detected in the surrounding water. (B) Stereomicroscope view of a eukaryotic organism not identified to date. The hydrozoan appears white in incident light. (C) TEM observation of a transversal section of the animal's body. Bacteria surrounded the hydrozoan body (white arrow). Insert: these bacteria contain empty sulphur granules in the periplasmic space. An external structure (black rectangle) can be observed on the organism suggesting a tie between ectosymbionts and the hydrozoan host.

on the ciliate's body (Fig. 1b and c) and electron lucent inclusions in bacterial ectosymbionts (Fig. 1c). The Raman detection of S_8 in bacteria confirms the thiotrophic metabolism of the ectosymbionts as previously suggested by Rinke et al. (2006) based on phylogenetic analyses. *Z. niveum* represents a second positive model confirming the usefulness of Raman microspectrometry for the detection of sulphide-oxidizing ectosymbiotic bacteria.

Raman analyses carried out on non-symbiotic nematodes collected in mangrove swamp environment (Fig. 2) did not show any S_8 in the specimen. This non-symbiotic nematode represented a negative control in the analyses.

To prove the efficiency of Raman microspectrometry to detect elemental sulphur in live specimens, the screening by Raman microspectrometry was used on lucinid bivalves colonizing the sediment around mangroves. This has been described by Himmel et al. (2009) in the bivalve *C. orbicularis* colonizing seagrass bed sediments. *L. pectinata*, a bivalve also known to harbour sulphide-oxidizing bacteria (Frenkiel et al., 1996), was also analysed and elemental sulphur S_8 was detected in its gills (results not shown). These results confirm the detection of elemental sulphur by Raman microspectrometry in eukaryotic organisms containing ecto- or endosymbiotic bacteria.

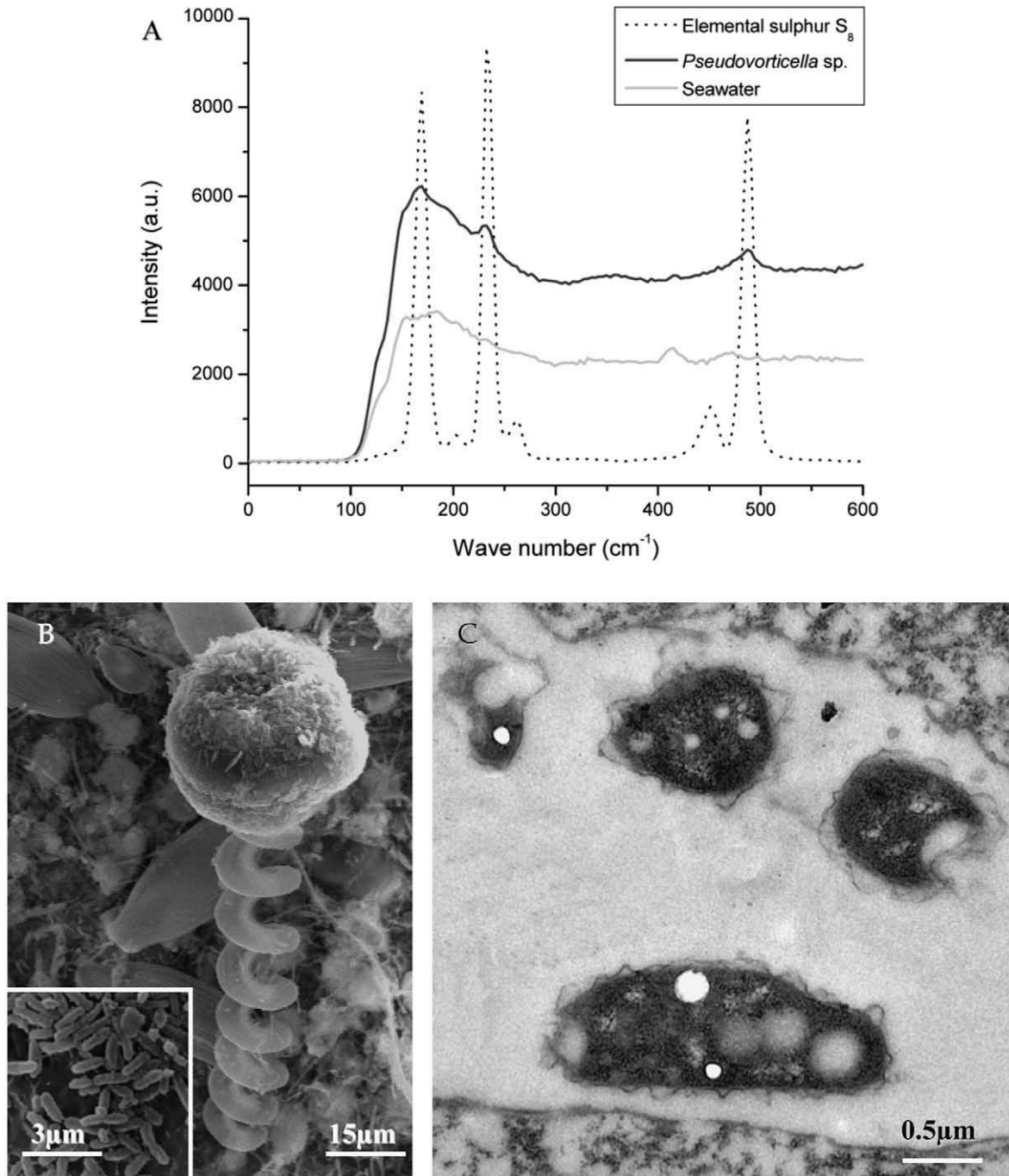


Fig. 4. Analyses on the ciliate *Pseudovorticella* sp. (A) Raman spectrum of elemental sulphur S_8 and Raman spectrum recorded on *Pseudovorticella* sp. zooid. A Raman spectrum realized on *Pseudovorticella* sp. stalk revealed sulphur presence (result not shown). (B) SEM observation of the single inverted bell-shaped zooid carried by a long spring-shaped stalk. Insert: SEM high magnification of the zooid covered by a single layer of bacteria. (C) TEM observation of a part of zooid surrounded by ectosymbionts containing empty granules.

3.2. Detection of sulphur in additional live specimens of various species

3.2.1. Screening for thioautotrophic bacteria-bearing organisms

Several annelids and copepods were analysed by Raman microspectrometry and gave the same result as the non-symbiotic nematode. No sulphur Raman bands were observed and allowed us to deduce unambiguously that these organisms had no thiotrophic metabolism.

An undescribed single hydrozoan collected on a leaf presented a positive sulphur Raman signal (Fig. 3a and b). The TEM investigation carried out on the same individual revealed the presence of bacteria surrounding the animal (Fig. 3c). Due to the non-damaging Raman analysis, the same specific individual could be prepared

for TEM investigations. The TEM micrograph presented in Fig. 3c confirms the presence of ectosymbiotic bacteria containing electron lucent granules classically associated with S_8 storage and a particular structure between the hydrozoan's body and ectosymbionts.

The Raman spectrum obtained on a vorticellid (*Pseudovorticella* sp.) shows the present of the three peaks corresponding to the Raman bands of elemental sulphur (Fig. 4a). SEM and TEM observations show that this ciliate is covered by a monolayer of bacteria on both its stalk and zooid (Fig. 4b and c). The Raman spectra appear less intense than the others recorded on *Z. niveum* and *E. dianae*. This may be due to the small size of this organism (comparatively to the other organisms studied above) and its thin

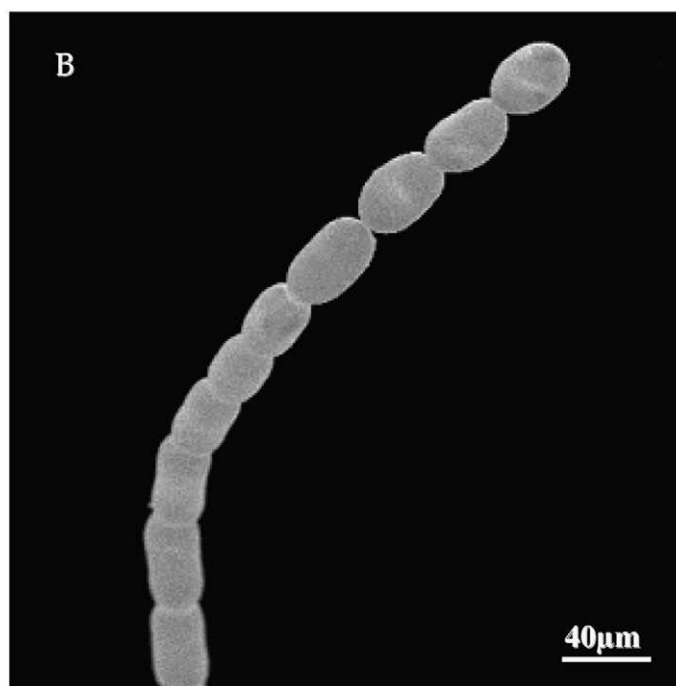
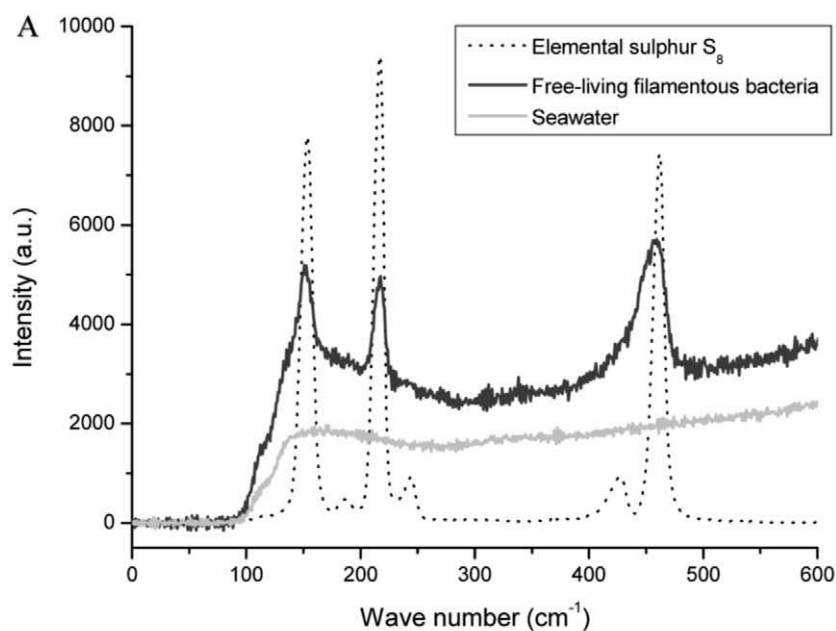


Fig. 5. Analyses on the Filamentous free-living bacteria. (A) Raman spectra of elemental sulphur recorded on a filamentous unidentified archaeon belonging to the Thaumarchaea group. (B) SEM observation of the same species.

ectosymbiotic bacterial coat. The positive sulphur Raman signal and the evidence of electron lucent granules in these ectosymbiotic bacteria indicate that this invertebrate lives in association with ectosymbiotic sulphide-oxidizing bacteria.

These results obtained on an unidentified hydrozoan and on vorticellids represent preliminary assumptions concerning the nature of the association between invertebrates and ectosymbiotic bacteria. They have to be confirmed by further phylogenetical analyses based on alternative techniques such as 16S rDNA sequences of the ectosymbionts.

3.2.2. Screening of thioautotrophic free living micro-organisms

Decomposing leaves and branches are covered by bacterial mats which appear white in light microscopy. This bacterial mat is usually constituted of several kinds of filamentous bacteria. Two shapes of these bacteria are investigated by Raman microspectrometry and give a positive sulphur Raman answer (Fig. 5). The phylogenetic analyses (Muller, pers. comm.) of the two kind of free-living bacteria allowed us to determine the presence of S_8 in free-living bacteria *Beggiatoa* sp. and Archaea belonging to the Thaumarchaea group (Fig. 5). In these two cases Raman analysis confirms the thiotrophic metabolic pathway previously suggested by phylogenetic analysis (Muller, pers. comm.).

4. Discussion

Mangrove swamp environments and sunken woods (leaves and branches) in particular are considered a suitable habitat for thiotrophic symbioses (Laurent et al., 2009). The main problem for the detection of sulphur is that solvents, such as ethanol, used during dehydration of samples before epoxy-resin embedding dissolve sulphur compounds (Truchet et al., 1998; Pasteris et al., 2001). In thioautotrophic symbioses and especially in sulphide-oxidizing bacteria, it was proven by X-ray emission spectroscopy (EDXS) that the major element extracted from the gills of symbiotic clams is the orthorhombic S_8 elemental sulphur (Vetter, 1985). Moreover, Pasteris et al. (2001) used LASER Raman microprobe spectroscopy and LASER scanning confocal microscopy in order to determine the presence and speciation of sulphur in sulphide-oxidizing marine bacteria. Recently, Himmel et al. (2009) used Raman microspectrometry on a complete symbiotic nematode *E. diana* to identify and locate sulphur S_8 in the sample. Before this recent analysis on *E. diana*, the unique method used to locate and preserve sulphur at the cellular level was the cryo-Energy Filtered Transmission Electron Microscopy (cryo-EFTEM) technique (Krieger et al., 2000; Lechaire et al., 2000, 2006, 2008). It requires a high level of technical skill and a long involved sample preparation method. It is therefore time consuming and expensive.

Raman microspectrometry appears to be an attractive tool to investigate living organisms of the meiofauna or dissected parts of macro-invertebrates collected from mangrove swamp environments or others ecosystems. The detection of elemental sulphur S_8 will point out probable sulphide-oxidizing bacterial symbioses or thioautotrophic metabolic pathways.

The non destructive character of Raman microspectrometry will allow us to recover the sample after Raman experiments in order to conduct complementary analytical (EELS, EDXS, Electron diffraction, etc.), enzymological, and ultrastructural (i.e. TEM, SEM, immunochemistry) studies. This procedure represents a significant advantage when dealing with samples that are rare due to collecting constraints or low abundance.

Raman microspectrometry analyses conducted on the two positive models, *E. diana* and *Z. niveum*, permits quick detection of elemental sulphur present in this thiotrophic ectosymbiosis. This method is reliable to quickly detect the sulphur compound on an

entire animal without damaging the biological specimen. Likewise, Raman microspectrometry detects with the same accuracy the elemental sulphur in endosymbiotic organisms. Himmel et al. (2009) applied this technique to pieces of gill tissue from the bivalve *Codakia orbicularis* colonizing a seagrass bed environment and detected sulphur S_8 . Other invertebrates like the lucinid bivalve *L. pectinata* living in mangrove mud were also tested. This bivalve is known to harbour sulphide-oxidizing gill-endosymbionts (Durand et al., 1996; Gros et al., 2003b). The Raman microspectrometry analyses conducted on these bivalves demonstrated the presence of elemental sulphur S_8 . This confirms that Raman microspectrometry is also efficient for the fast detection of sulphur in endosymbiotic organisms. The eukaryotic host *L. pectinata* contains elemental sulphur in both bacterial endosymbiont and extracellular eukaryotic organites (Frenkiel et al., 1996). In this case, Raman microspectrometry allowed us to detect elemental sulphur, but due to the spatial resolution of the analyses (10 μ m), the cells containing sulphur cannot be identified. Specifically, it did not allow us to determine if elemental sulphur is contained in internal sulphur granules that have been developed by eukaryotic organisms themselves, e.g. the nematode *Oncholaimus campylocercoides* (Thiermann et al., 2000), or if it is contained in storage granules in ectosymbiotic bacteria, e.g. in *E. diana* (Himmel et al., 2009) or endosymbiotic ones, e.g. the mouthless nematode *Astomonema jenneri* (Ott et al., 1982).

Carefully applied, Raman microspectrometry will not induce any damage to the studied specimens. This quick sulphur detection technique can be used to reduce experimentation time on symbiotic animals and in particular will allow us to acquire quantitative results on sulphur contents of specimens as a function of the sulphide time exposure (*in vitro* or natural exposure).

The Raman microspectrometer can also be used in extreme conditions such as the deep ocean for *in situ* analyses. White et al. (2005) have shown the capabilities of Raman spectrometry to investigate composition of many rocks, minerals, and seafloor gas hydrates but also to reveal the presence of sulphur S_8 in biological materials in deep-sea systems, e.g. bacterial mats (White et al., 2006). In this case, from the compound analyses (detection of sulphur, ferrous, methane, etc.) it was possible to analyse the environment of the samples of interest *in situ* and collect live animals that needed further analyses in laboratory.

5. Conclusion

The results obtained by Raman microspectrometry on two ectosymbiotic thioautotrophic animals and on the negative control demonstrate the capabilities of Raman microspectrometry as a fast selection of autotrophic organisms based on elemental sulphur S_8 detection.

Raman microspectrometry can be considered as an important tool to conduct quick screening on living organisms, easily identifying thioautotrophic animals. However other complementary techniques such as SEM and TEM must be used to precisely locate the sulphur compounds in the specimens. Since Raman microspectrometry is a non-damaging technique, these complementary techniques can be applied on the same specimen.

In conclusion, Raman microspectrometry is easy to apply for the detection of the metabolic nature of symbiotic organisms or free-living bacteria and can constitute an advantageous first step before phylogenetic analyses.

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