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A microbiological and biogeochemical investigation of the cold seep tubeworm *Escarpia southwardae* (Annelida: Siboglinidae): Symbiosis and trace element composition of the tube

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Abstract:

Tubeworms within the annelid family Siboglinidae rely on sulfur-oxidizing autotrophic bacterial symbionts for their nutrition, and are among the dominant metazoans occurring at deep-sea hydrocarbon seeps. Contrary to their relatives from hydrothermal vents, sulfide uptake for symbionts occurs within the anoxic subsurface sediment, in the posterior „root" region of the animal. This study reports on an integrated microbiological and geochemical investigation of the cold seep tubeworm *Escarpia southwardae* collected at the Regab pockmark (Gulf of Guinea). Our aim was to further constrain the links between the animal and its symbiotic bacteria, and their environment. We show that *E. southwardae* harbors abundant sulfur-oxidizing bacterial symbionts in its trophosome. Symbionts are able to fix inorganic carbon using the Calvin-Benson cycle, as reported in most other Siboglinidae, but can also use the reverse Tricarboxilic Acid Cycle. Surprisingly, the observed bacteria appear to be more closely related to symbionts of *Escarpia* and *Lamellibrachia* species from very distant sites located in the Gulf of Mexico and eastern Pacific, than to symbionts of a siboglinid occurring at a nearby methane seep site, only a few hundred km away from Regab. Then, by combining scanning electron microscopy and trace element (Mn, Fe, Sr, Zr) analyses of *E. southwardae* tube, we also show that two distinct oxidation fronts occur along the tube. The first one, near the posterior end of the tube, corresponds to the interface between oxic bottom waters and the underlying anoxic sediment. In contrast, the second redox front is located in the most anterior part of the tube, and could result from active oxygen uptake by the plume of the tubeworm. We speculate that intense oxygen consumption in this region could create favorable conditions for sulfate reduction by specialized bacteria associated with the plume, possibly leading to an additional source of dissolved sulfide that would further enhance the productivity of bacterial symbionts.

Highlights

► We characterize symbionts associated with the tubeworm *Escarpia southwardae*. ► We investigate profiles of trace elements (Mn, Fe, Sr, Zr) along the tube. ► Symbionts have genes for sulfur metabolism and autotrophic carbon fixation. ► Closest relatives are symbionts of tubeworms from the Gulf of Mexico and Pacific. ► Two distinct redox fronts occur in the anterior and posterior regions of the tube.

Keywords : Sulfur-oxidizing bacteria ; rTCA ; Trace elements ; RubisCO ; Symbiosis

1. Introduction

Siboglinidae annelids are part of the dominant fauna at submarine methane seeps around the world (Boetius, 2005 and Hilario et al., 2011). Tubeworms have adapted to a variety of oxygen-depleted marine habitats at all water depths in the ocean. All the species investigated so far are known to harbor symbiotic bacteria in their trunk region, within a specialized organ called the trophosome. Bacteria fix or convert carbon into molecules that can be used by their metazoan hosts. Apart from “bone-eating” *Osedax* species (Goffredi et al., 2005, Goffredi et al., 2007 and Katz et

al. 2011), whose metabolism involves collagen and maybe also lipid digestion, and a reported case of methanotrophy (Schmaljohann et al. 1990), bacterial metabolism associated with tubeworms most often relies on chemoautotrophy, the energy resulting from oxidation of reduced sulfur species (Fisher 1990; Boetius 2005; Hilario et al. 2011).

In comparison with, the potential role of the tube hosting the siboglinid worm in the functioning of its symbiotic system remains poorly known. The tube consists in giant beta-chitin crystallites embedded in a protein matrix (Gaill et al. 1992; Shillito et al. 1995). A series of works have investigated the role of the 'root' part of the tube in seep tubeworms (*Lamellibrachia luymesi*) which corresponds to the portion of the tube located within the substrate in subsurface sediment (Julian et al. 1999; Freytag et al. 2001; Cordes et al. 2005; Dattagupta et al. 2006). They showed that this section of the tube was permeable to dissolved sulfide, and that the posterior part of the trunk could be also uptaking reduced sulfur species. In fact, the tube itself probably acts as a direct conduit for seawater sulfate down to the subsurface environment (Bayon et al. 2009a). This mechanism is thought to enhance sulfate reduction and, as a consequence, self-sustained production of dissolved sulfide. All the above processes promote increasing sulfide availability to the tubeworms, which, in turn, help to sustain large bushes of individuals for long periods of time (Fisher et al. 1997; Cordes et al. 2005). In this regard, seep tubeworms differ from those encountered at hydrothermal vents. At cold seeps, dissolved sulfide concentrations are generally very low in ambient seawater surrounding the plume of tubeworms (Olu-LeRoy et al. 2007). In comparison, dissolved sulfide levels are typically much higher in bottom waters at hydrothermal sites, so that vent species can usually take up sulfide directly via their plume (Stewart & Cavanaugh 2005). Therefore, in contrast with their seep analogs at ocean margins, hydrothermal vent species have not developed any 'root' system. A few studies provided preliminary description of tube-associated bacterial communities in *Riftia pachyptila* and *Lamellibrachia anaximandri*, respectively, emphasizing the occurrence of both aerobic and anaerobic lineages (Lopez-Garcia et al. 2002; Duperron et al. 2009). Diverse bacterial morphotypes and lineages occur on both the interior and exterior surfaces of the tube, and also between chitin layers (Duperron et al. 2009). The tube thus appears as a complex system, which probably plays an important, but poorly known, role in the metabolism of the metazoan host and its associated symbionts.

During the last decades, several seeps have been discovered and explored in the Gulf of Guinea (Ondreas et al. 2005; Olu-LeRoy et al. 2007; Bayon et al. 2007; Sahling et al. 2008). One of the most studied seepage site in this area is Regab, a gas hydrate-bearing pockmark

located at ~3160 m deep off the Congo margin (Charlou et al. 2004; Gay et al. 2006; Pierre & Fouquet 2007; Marcon et al. 2014). At Regab, tubeworms described as *Escarpia southwardae* (Andersen et al. 2004) form dense bushes in close association with dense beds of the dual-symbiotic mussel *Bathymodiolus* aff. *boomerang.* Based on standard barcoding markers such as COI, *E. southwardae* is genetically indistinguishable from *E. spicata* occurring at sedimented vents in the Gulf of California and *E. laminata* from seeps in the Gulf of Mexico. All three were suggested to represent a single polymorphic species with a broad distribution and strong connectivity (Andersen et al. 2004; Olu et al. 2010). However, recent evidence based on microsatellites has questioned this hypothesis, suggesting instead a rather limited gene flow (Cowart et al. 2013). The symbiosis in *Escarpia southwardae* has not received much attention despite the ubiquitous occurrence of this species at Regab. The composition and biogeochemical characteristics of the tube have also not been explored previously, apart from a study which investigated tube mineralization processes at a neighboring site (Haas et al. 2009).

In the present study, we report on a combined microbiological and geochemical investigation of the seep tubeworm *Escarpia southwardae* collected at the well-studied Regab pockmark. Our aim was to document the identity of the bacterial symbionts associated with this species and to provide a detailed biogeochemical investigation of its tube in order to better understand how symbionts and tube can both contribute to the functioning of the animal symbiotic system. Bacterial symbionts were characterized using 16S rRNA-encoding gene analysis and fluorescence *in situ* hybridization (FISH). Their metabolic potential was investigated by sequencing fragments of bacterial metabolic genes. Adenosine 5'-phosphosulfate (APS) reductase, involved in sulfur metabolism, was sequenced, as well as ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) and ATP citrate lyase (ACL), both involved in carbon fixation pathways (Thiel et al., 2012). The nutritional role of symbionts was addressed by measuring stable isotopic ratios of carbon, nitrogen and sulfur. In addition to this microbiological characterization, the geochemical composition of the tube was also investigated by combining scanning electron microscopy and trace element analyses along its entire length. In particular, the abundances of manganese (Mn) and iron (Fe), two geochemical tracers of redox processes, were measured for providing further constraints on the environmental conditions related to the tube growth. Altogether, this multidisciplinary study provides new insights into the functioning of the symbiotic system consisting in *Escarpia southwardae*, its bacterial symbionts and its tube.

2. Material and methods

2.1.- Samples and fixation

Tubeworms tentatively assigned to *Escarpia southwardae* (Andersen 2004) were sampled at Regab during the 2011 WACS cruise (RV Pourquoi Pas?, chief scientist K. Olu). Bushes up to 1.5m high can include tens to hundreds of specimens (densities of 250-557 ind. m^{-2}), and cover large areas paved with authigenic carbonate crusts (up to 1,400 m^2) within the pockmark (Olu-LeRoy et al. 2007; Marcon et al. 2014). Specimens were part of a large bush sampled using a BushMaster device (Penn State University, C.R. Fisher) manipulated by the arm of the ROV *Victor6000* (dive 426; 5.798°S, 9.711°E, 3152m depth) in the central zone of the pockmark (Duperron et al. 2005; Olu-LeRoy et al. 2007; Cowart et al. 2013). Hydrogen sulfide is undetectable in bottom waters at Regab, but present in anoxic methane-rich sediment below the seawater/sediment interface (Olu-LeRoy et al. 2007; Cambon-Bonavita et al. 2009; Ristova et al. 2012). Specimens were removed from their tube. Trunk tissue was dissected from 8 specimens and frozen for DNA and stable isotope analyses, or fixed using 4% formaldehyde for 2 hours, rinsed and dehydrated in increasing ethanol series for FISH. Tubes were dried at room temperature for elemental analysis and electron microscopy.

2.2 -Host and symbiont marker gene sequencing and phylogenetic analysis

DNA was extracted from animal tissue using a DNAeasy Kit (Qiagen, CA). A fragment of host 28S rRNA-encoding gene was amplified and sequenced; fragments of genes encoding bacterial 16S rRNA, APS reductase and RubisCO form II were amplified by PCR, cloned using a TOPO TA Cloning Kit™ (Invitrogen, CA), and inserts from 5 clones per gene and specimen were sequenced in both directions by GATC-Biotech (Germany). A fragment of the gene encoding putative type II ATP citrate lyase (ACL) was amplified, cloned, and sequenced using primers acl2F1 and acl2R1 (Thiel et al. 2012). Attempts to amplify Type I RubisCO and particulate methane monooxygenase following published protocols failed to yield products (Blazejak et al. 2006; Duperron et al. 2007). Primer sets and PCR conditions are summarized in Table 1. For each PCR, three reactions per specimen were run in parallel and pooled prior to direct sequencing or cloning.

For each bacterial gene, a dataset including obtained sequences, best BLAST hits, and reference sequences was generated. Sequences were aligned using CLUSTALX, alignments

were checked by eye, and phylogenetic relationships were estimated under a Mximum Likelihood approach using the PHYLIP package (Felsenstein 2002). For each tree, bootstrap values were based on 1000 replicates.

2.3- Fluorescence in situ hybridization

Embedding of animal trunk tissue, sectioning and fluorescence *in situ* hybridization were performed as described previously (Duperron et al. 2008). Transverse sections (8µm-thick) were hybridized with four probes: the Eubacteria-specific EUB-338, the Gammaproteobacteria-specific GAM-42, and a dual probe set consisting of LaSp-60 and LaSp-640, which co-hybridize only in Siboglinidae symbionts (Amann et al. 1990; Manz et al. 1992; Duperron et al. 2009). Hybridizations were performed according to the protocol described previously, in a buffer containing 20 to 40% formamide, and observed under an Olympus BX-61 epifluorescence microscope (Olympus, Japan).

2.4 - Stable isotope analyses

Frozen tissue from the trunk region was dried (4 days, 60°C) and ground to powder. Values of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ were determined and are expressed as relative per-mil (‰) differences between samples and Pee Dee Belemnite (PDB) for carbon, air N_2 for nitrogen and Canyon Diablo Troilite for sulfur according to the following equation:

$$\delta(X) = \left[\left(\frac{R_{sample}}{R_{standard}} \right) - 1 \right] \times 1000$$

where X (‰) is ^{13}C, ^{15}N or ^{34}S abundance and *R* is the ^{13}C:^{12}C, ^{15}N:^{14}N ratios or ^{34}S:^{32}S. Analyses were performed on a GV Isoprime stable isotope mass spectrometer at Iso-Analytical (Crewe, UK).

2.5 - Determination of trace element concentrations in the tube

Trace element concentrations for manganese (Mn), iron (Fe), zirconium (Zr), and strontium (Sr) were determined along a ~ 1-meter long tube recovered from the Bushmaster sampling. As expected, the posterior root part of the tube was coiled, indicating that we recovered relatively intact tubes. However, it may be that a few centimeters of the most posterior part were lacking. The chitin-rich tube was cut on board in 55 sections perpendicular to the length of the tube. The end sections S1 and S55 correspond to the most anterior and posterior parts of the tube, respectively. The length of each cut section ranged between 1 to 3 cm. Prior to

acid digestion using twice sub-boiled nitric acid solution, samples were ultrasonicated in deionized water to remove any attached particles. Trace element abundances were measured by sector field ICPMS (Element2) at the Pôle Spectrométrie Océan (Brest, France). Concentrations were determined using the thulium (Tm) addition method (Bayon et al. 2009b; Freslon et al. 2011). The accuracy and reproducibility of the measurements were assessed by replicate analyses of the MA-A-1 Copepod Homogenate reference material (Atomic Energy Agency).

2.6- Scanning electron microscopy observations and microanalyses

Five sections of the same tube (S2, S15, S28, S48 and S55) were mounted for scanning electron microscopy (SEM) observations and energy dispersive spectrometer (EDS) micro-analyses (Oxford Instruments). Prior to analyses, samples were coated with gold. The internal (oriented towards the lumen of the tube and the animal tissue) and the external sides of each section (exposed to seawater or sediment) were investigated separately. The chemical composition of the tube (e.g. C, O, Na, Mg, Si, S, K, Ca, Fe) was determined as weight percentages, normalized to 100%.

3. Results

Specimens were identified as *Escarpia southwardae* based on their general morphology as well as geographic localization at Regab (Ann Andersen, pers. comm., Cowart et al, 2013). The 28S rRNA -encoding gene sequence displayed above 99% identical positions with *Lamellibrachia* sp. from the Guiness site (Gabon Margin, Gulf of Guinea), while no *Escarpia* sequence was available.

3.1 - Symbiont characterization

For each bacterial gene, all inserts sequenced from all three specimens yielded the same nucleotide sequence, suggesting low heterogeneity. The 16S rRNA-encoding gene sequence recovered displayed above 99% identical nucleotide positions compared to various symbiont sequences from *Escarpia* and *Lamellibrachia* species from various locations including the Mediterranean, the Gulf of Mexico and the eastern Pacific. It displayed only 2-3 distinct nucleotides compared with its close relatives (sequences DQ232902, DQ232903, HE983331), and 5 differences with a previous sequence obtained from an undescribed tubeworm

(AM888202, possibly a conspecific *Escarpia*) sampled at Regab in 2001 (Cambon-Bonavita et al. 2009). These sequences formed a well-supported clade in reconstructed phylogenies (Fig. 1). It differed by 1.7% (26 out of 1503 positions) from the sequence recovered from *Lamellibrachia* sp. from the Guiness site, its nearest neighbour geographically speaking, occurring 490 km away.

A fragment of the APS reductase-encoding gene *aprA* was amplified from all specimens, yielding a single sequence that was 97% (nucleotide) and 100% (inferred amino acids) similar to that of symbionts from *Lamellibrachia anaximandri* from the Mediterranean (Palinuro Seamount and Nile Deep-sea fan), and *Lamellibrachia* sp. from the Guiness site. All these clustered together along with *Sclerolinum contortum* in the reconstructed phylogeny, in a group distinct from that comprising *Riftia pachyptila* and *Tevnia jerichonana* symbionts (Fig. 2). The sequence displayed a single amino acid difference with that of *Escarpia* from Chapopote (GoM) over 77 overlapping amino acids (this sequence, AFB76523, was not included in the tree because of this limited overlap).

The nucleotide sequence obtained for the RubisCO form II gene *cbbM* was strictly identical to that obtained from *Escarpia laminata* from Alaminos Canyon (GoM), and 99.5% to a tubeworm from the Guaymas Basin. These formed a tight cluster in the reconstructed phylogeny based on amino acid sequences, distinct from the clade containing *Lamellibrachia* sp. from Guiness (Fig. 3).The nucleotide sequence obtained for ACL was 99% identical with that recovered from *Lamellibrachia* sp. 2 and *Escarpia laminata* from Alaminos Canyon in the Gulf of Mexico (5 out of 1114 nucleic acid positions, 1 amino acid in 350), with which it clustered in the reconstructed phylogeny (Fig. 4).

3.2 - Fluorescence microscopy

Bacteria occupied most of the volume available in the trunk region, and hybridized with all probes tested, but not with antisense or non-targeted probes. Probes LaSp-60 and LaSp-640 co-hybridized in all bacteria at 40% formamide, a pattern characteristic of Siboglinidae symbionts (Fig. 5). Bacteria were variable in size, and seemed to be grouped in larger structures likely corresponding to the lobules of the trophosome.

3.3 - Stable isotopes composition of Escarpia southwardae *tissue*

Nitrogen $\delta^{15}N$ values of 3.1 and 3.4 ‰ and carbon $\delta^{13}C$ values of -27.3 and -26.7 ‰ were measured from two specimens. Symbionts can store sulfur granules, which resulted in sulfur

content of 8.4% (3.1 to 16.2%) dry weight. Sulfur $\delta^{34}S$ averaged -21.6 ‰±0.6‰ (n=3 specimen).

3.4 - Density and trace element concentration profiles along the tube

The density and trace element profiles are displayed in Fig. 6. The tube density was low (28 mg/cm) in the most anterior part of the tube, then peaked at a maximum of 174mg/cm around 12 cm, and then decreased almost linearly down to 18mg/cm in the root part. Measured element concentrations displayed large variations along the studied tube, ranging for example from 0.2 to 7 ppm for Mn, and from 30 to 1500 ppm for Fe (Fig. 6). The posterior (the lower 2 cm) and, to a lesser extent, the anterior (i.e. the upper 3 cm) ends of the tube were enriched in Zr, Sr, and Fe, relative to the rest of the tube. The tube was also characterized by two peaks of enhanced Mn contents, in the upper (i.e. 3-31 cm; anterior part) and bottom parts (i.e. 80-85 cm), respectively. In between, the middle section of the tube exhibited much lower concentrations (Mn ~ 0.4 ppm). Fe also displayed a pronounced enrichment at the 80-85 cm interval.

3.5 - SEM-EDS observations and chemical investigation of the tube

SEM investigations of the tube matrix revealed that the internal side of each studied section was generally much smoother than the corresponding external sides (Fig. 7). C and O clearly dominated the chemical composition of the tube (mean 94.3 ± 3.0 wt%), being slightly higher on the internal (96.5 ± 1.7 wt%) than on the external side (92.1 ± 2.2 wt%), respectively (Table 2). The measured C/O ratios ranged between 1.1 and 1.9 (mean value: 1.5 ± 0.2). The relatively high levels of Br and S measured in the tube were most likely associated mainly with the chitin matrix. The presence of Na and Cl were clearly related to the occurrence of salt crystals, as shown by SEM observations (microphotograph not shown here). The outer part of the tube was also characterized by significant enrichments of Ca, Mg, Fe, K, and Si, which most likely reflected the presence of carbonate and silicate particles.

4. Discussion

4.1 – Identification and phylogenetic relationships of the symbiont of Escarpia southwardae

Most data regarding *Escarpia* symbioses are available from the Gulf of Mexico and east Pacific, and consist of 16S rRNA or ITS sequences save for a study which also employed

fluorescence *in situ* hybridization (FISH) (Di Meo et al. 2000; Raggi et al. 2012). To date, there is only little information available about the symbiosis of *Escarpia southwardae*. A possible symbiont sequence was presented in a paper investigating the bacterial diversity in various habitats at the Regab site (Cambon-Bonavita et al. 2009). We here provide a more in-depth investigation of its symbiosis. The four sequenced bacterial gene markers support a close evolutionary relationship between the *E. southwardae* symbiont and those of other *Escarpia* species (*E. laminata* from the Gulf of Mexico and *E. spicata* from the Pacific), as well as those of several *Lamellibrachia* species. This confirms that symbionts in all three reported *Escarpia* and some *Lamellibrachia* species are highly similar. Tubeworms from the Gulf of Guinea are no exception to the rule, and as already documented on hosts side, there is a remarkable homogeneity of symbionts over great geographical distances (Di Meo et al. 2000; Thiel et al. 2012). Given that symbionts are most likely acquired locally after larval settlement, it indicates that they have a wide distribution at seeps in the Pacific, Gulf of Mexico and southeast Atlantic (Nussbaumer et al. 2006; Harmer et al. 2008). *Escarpia southwardae* can be distinguished from other *Escarpia* based on certain host marker genes, and microsatellite loci suggest recent divergence of the three morphospecies and limited gene flow between the Gulf of Mexico and Gulf of Guinea populations today (Cowart et al. 2013). Because the bacterial genes used here display low levels of variation which prevent exploring bacterial biogeography, it is not yet possible to tell whether a parallel diversification occurred in their symbionts.

Interestingly, symbionts of *Lamellibrachia* sp. from the Guiness site are not among the closest relatives for 16S rRNA, Rubisco II and ACL genes. The APS reductase-encoding gene is the only sequence from the *E. southwardae* symbiont that displays high similarity with that of the Guiness species. But in this case, the only APS sequence available from an *Escarpia*, which has only limited sequence overlap, is also highly similar in the overlapping region. This is surprising given that the Guiness site is located only 490 km north from Regab. A hypothesis could be that, as for host species in the Gulf of Mexico, symbiont phylotypes cluster according to depth rather than distance. Thiel and coworkers indeed recently suggested the existence of distinct clusters of RubisCO II sequences corresponding to shallow and deep sites (Thiel et al. 2012). The *cbbM* from *E. southwardae* and *Lamellibrachia* sp. Guiness cluster accordingly, respectively in the deep and shallow clades, but this hypothesis warrants further testing using additional genes.

4.2 - Insights into symbiont metabolism

FISH micrographs on trunk sections display high densities of bacteria within the trophosome (Fig. 5). These densities are apparently much higher than reported in *Escarpia* sp. from the Chapopote site, the only other *Escarpia* for which FISH results are available, and rather comparable to the high densities observed in *Lamellibrachia* species from the Guiness site and the eastern Mediterranean (Duperron et al. 2009; Raggi et al. 2012; Duperron et al. 2012). It would be interesting to analyze more specimens using FISH, to estimate the variability of symbiont densities. The occurrence of *aprA* and *cbbM* genes strongly suggests that symbionts have the ability to oxidize reduced sulfur and to fix inorganic carbon using the Calvin Benson Bassham (CBB) cycle, as reported in other Siboglinidae. *Escarpia southwardae*-associated symbionts also seem to use the reductive tricarboxylic acid (rTCA) cycle as an alternative to the CBB for carbon fixation, as indicated by the presence of a gene encoding putative type II ATP citrate lyase. This enzyme, indicative of rTCA, was recently identified in several tubeworm symbionts (Markert et al. 2007; Robidart et al. 2008; Thiel et al. 2012).

Carbon stable isotope signatures measured here are within the range of values expected for a sulfur-oxidizing chemoautotrophic symbiosis, and comparable to values recently reported in the Gulf of Mexico, supporting that this is the main metabolism responsible for nutrition of the tubeworm (Fisher 1995; Becker et al. 2011). These values are similar to those reported from “juvenile” specimens collected at Regab in 2001 (-24.2 to -29.1‰ (Olu-LeRoy et al. 2007). According to their size (tube lengths: 62, 59 and 35 cm), the three specimens investigated here would fall within the “juvenile” group described by Olu-LeRoy et al. (2007). The negative stable isotope sulfur signature also falls into the range of previously published values for seep tubeworms and are compatible with the hypothesis of a sulfide supply resulting from seawater sulfate reduction by bacteria from the sediment (Dattagupta et al. 2006; Carlier et al. 2010). Despite the reported abundance of methane at Regab, there is no evidence for the presence of methane-oxidizing symbionts (Charlou et al. 2004).

4.3 - Biogeochemical evidence of tube growth within the sediment and in seawater

The observed density profile corresponds to a tube that would grow both at its posterior and anterior ends, as previously observed for *Lamellibrachia luymesi* (Freytag et al. 2001). Towards the posterior end, the thinning of the tube corresponds to the sulfide-permeable

'root' within the sediment, through which seep Siboglinidae acquire sulfide for their symbionts (Julian et al. 1999). Julian et al. previously reported tube wall thickness below 0.1 mm in the root part of *L. luymesi* and Haas et al. reported a similar thickness for tubes of putative *E. southwardae* collected at the Kouilou site, not far from Regab (Haas et al. 2009). SEM microchemical analyses indicate that the tube is mostly composed of carbon and oxygen (Table 2). These two elements are slightly more enriched in the internal side of the tubes relative to the outer part, which suggests that the inner side is less directly influenced by the surrounding environment. This is in agreement with evidence from SEM observations and semi-quantitative SEM-EDS analyses (data not shown here) for the occurrence of calcium carbonate minerals only on the outer part of the studied tube (Fig. 7).

The trace element concentration profiles provide interesting constraints on the environmental conditions in which the tube grows. Zr and Fe in marine sediments are typically associated with clays and other detrital silicate minerals. Sr, on the other hand, is mainly hosted by aragonite at cold seeps (Bayon et al. 2007), *i.e.* an authigenic carbonate mineral that precipitates under suboxic/anoxic conditions during the anaerobic oxidation of methane (Aloisi et al. 2002). The enrichments in these elements observed at both ends of the tube therefore reflect the presence of detrital (indicated by Fe, Zr) and carbonate particles (indicated by Sr). This is in agreement with previous studies, which reported particle accumulation around the root of *L. luymesi* (Julian et al. 1999; Haas et al. 2009). Taken together, the density profile, SEM observations of authigenic carbonate minerals (Fig. 7) and our trace element data (Fig. 6) strongly suggest that the posterior end of the tube (i.e. from about 90 cm depth onward) was formed within anoxic sub-surface sediments. As a consequence, this also implies that the overlying part of the tube must have developed in bottom waters, above the sediment/seawater interface.

4.4 - Evidence for distinct redox fronts along the Escarpia southwardae *tube and functional consequences*

A few centimeters from the aforementioned trace element enrichments, the studied Siboglinidae tube is characterized by peaks of high Fe and Mn concentrations (up to 1500 ppm and 8 ppm, respectively). Over the past decades, a large amount of work has been done on the aquatic geochemistry of Mn and Fe, hence providing useful background information for interpreting these trace element profiles along the tube (Bender et al. 1977; Burdige 1993;

VanCappellen & Wang 1996). In the marine environment, the distribution of Mn and Fe is largely controlled by redox conditions. Both Mn and Fe tend to form insoluble oxides in seawater. On continental margins, Fe-Mn oxyhydroxides are generally reduced in oxygen-depleted sediments (Burdige 1993; VanCappellen & Wang 1996). These early diagenetic processes release dissolved Mn^{2+} and Fe^{2+} into surrounding pore waters, which then diffuses towards oxic conditions near the seafloor, where they reprecipitate as solid oxide phases. Investigation of plumes collected above seeps in the Gulf of Guinea also confirmed that circulation of methane-rich fluids in sub-surface sediments was associated with precipitation of Fe-Mn oxhydroxide phases (Bayon et al. 2011; Lemaitre et al., in revision). By analogy, the observed Fe and Mn enrichments along the tube at a few centimeters above the sediment/seawater interface are probably best explained by the presence of a redox front (see redox front 2 on Fig. 6). The sub-seafloor environment of tubeworm bushes corresponds to a horizon between sulfide-rich anoxic sediments and oxygenized bottom waters. As a consequence, the portion of the tube located at the sediment/seawater interface is likely to record this redox front. Our SEM-EDS microanalyses indicate that the corresponding Fe-enrichment is located on the outer part of the tube, and not its inner part. This suggests that this redox front does not occur within the tube, where the animal lives, but rather in the surrounding external environment.

Most likely, the marked increase in Mn concentrations at the anterior part of the tube also probably reflects the occurrence of another oxidation front (redox front 1, Fig. 6). This part of the tube stands in ambient O_2-rich waters, at 1-2 m above the seafloor. This is where the branchial plume is located, which uptakes O_2 for the tubeworm and its symbionts. At least two hypotheses could explain the presence of this front. One possibility would be that the inside of the tube, from its posterior part up to the redox front 1, is characterized by anoxic conditions. The observed front would hence be the consequence of the mixing between oxygen-rich seawater in the anterior part of the tube and reduced pore-water fluids from the sub-surface environment. Movements of the animal's anterior part in a piston-like motion by action of the muscular vestimentum would create an oxic-anoxic interface extending over several centimeters as observed here. In such a case, sulfide uptake through the skin of the animal could thus occur over a much longer fraction of the trunk than previously suggested, and well above the root (Julian et al. 1999; Freytag et al. 2001).

An alternative hypothesis would be that most of the tube (at least the portion overlying the redox front 2 horizon) is oxic. The active oxidation front in the anterior part would then be the

consequence of oxygen uptake by the branchial plume of *Escarpia*. This would result in the establishment of a locally suboxic micro-environment, leading in turn to precipitation of dissolved Mn as solid Mn(IV) phases.

In contrast with the redox front 2, the anterior redox front inferred from Mn enrichments is not accompanied by enhanced Fe concentrations. This difference in behaviour between Fe and Mn can be explained when considering the response of trace element redox coupled to oxygen depletion in seawater (Rue et al. 1997). While the precipitation of Mn^{2+} to MnO_2 solid phases occurs in mild suboxic aquatic environments, Fe immobilization generally takes place under more strongly O_2-depleted conditions. This suggests that the anterior redox front (front 1) is linked to a less suboxic environment than front 2. Such moderately anoxic conditions are more likely to result from the uptake of oxygen by the plume, rather than from the mixing between seawater and fully anoxic fluids, hence supporting the second hypothesis. This would imply that the oxygen consumption related to the biological activity of the tubeworm creates moderately suboxic conditions suitable for Mn precipitation, but not for Fe, and that only the plume is responsible for oxygen uptake. Oxygen uptake has been estimated in lab-based experiments between 0.18 to 1.33 $\mu mol.g^{-1}.h^{-1}$ in *Lamellibrachia anaximandri* exposed to sulfide concentrations ranging between 0 to 80µM, and up to 10.4 $\mu mol.g^{-1}.h^{-1}$ in *L. luymesi* (Freytag et al. 2001; Duperron et al. 2009). It is possible that this could generate suboxic conditions around the plume when the plume is retracted within the anterior part of the tube. In a recent study, a colony of *Ridgeia piscesae* vent siboglinids was filmed over a 23 days period, and at any any time, between 8.5 to 78.6% of the tubes constituting the bush displayed visible plumes extending to the oxygenated seawater above the tube, while the rest were retracted. Patterns of plume extension and retraction inside the tube followed 12h and 24h periods, and in most cases retraction was not triggered by an external stimulus (such as contact with another animal) (Cuvelier et al. in press). This indicates that the plume is not always extended, explaining why it may generate a suboxic zone inside the anterior part of the tube when retracted. Under this hypothesis, the inner side of the tube would be oxic over most of its length, with the exception of the root system and the region around the plume. The above proposed scenario would have important implications for the functioning of the symbiotic system. First, the presence of oxic seawater within the tube would be in agreement with the hypothesis of seaweater sulfate injection, via the tube, below the seawater/sediment interface, as postulated in some previous studies (Cordes et al. 2005; Dattagupta et al. 2006). Second, locally oxygen-depleted conditions around the plume could possibly create

appropriate conditions for the growth of sulfate-reducing bacteria (SRBs). These could lead to active seawater sulfate reduction in the immediate vicinity of the plume, and resulting dissolved sulfide could then be uptaken by the animal's plume, as in hydrothermal vent tubeworms. This would provide an additional source of sulfide besides the sulfide already taken up in the root region, further enhancing sulfide availability to *Escarpia southwardae* and its sulfur-oxidizing symbionts, and thus the metabolic efficiency of the association. Although this latter hypothesis remains speculative at this stage, it must be noted that SRB-related Deltaproteobacteria were reported in the tube of *Lamellibrachia anaximandri* (Duperron et al. 2009), which would therefore agree with the above consideration.

5 - Conclusion

Escarpia southwardae collected at the Regab pockmark (Gulf of Guinea) harbors high densities of sulfur-oxidizing, autotrophic symbionts in its trophosome. Phylogenetically, symbionts are highly similar to those found in other *Escarpia* species from the Gulf of Mexico and Gulf of California, but differ from those encountered in *Lamellibrachia* at the nearby Guiness site (Gabon margin), only a few hundred km away. The tube appears to be an important component of the Siboglinidae symbiotic system. The use of redox-sensitive trace elements (Fe and Mn) indicates that the posterior part of the tube is anoxic, further suggesting that the root and the posterior region of trunk are the location of sulfide uptake. The observation of another redox front in the anterior portion of the tube, inferred from Mn enrichments, is best explained by intense oxygen uptake, probably due to the activity of the plume. It is hypothesized that this region might also host SRBs, which could possibly serve as an additional source of dissolved sulfide to the animal. Overall, both manganese and iron appear to be useful tracers of oxidation fronts in the *Escarpia* tube. Future work should include parallel in-depth characterization of bacterial communities associated with the internal and external sides along the tubes to test for the actual presence of sulfur-metabolizing bacteria. Also, additional specimens are needed to investigate inter-individual variability and relationship with age and environmental parameters. In particular, it remains to be shown how the anterior redox front evolves over the course of a tubeworm's life, whether it corresponds to a time-integrated redox front, or whether Mn is lost from the tube during chitin degradation as the tube ages. The tube may also play a role in the processing of some elements which are important for the physiology of hosts and symbionts, such as cofactors. An example is Zn, which is involved in the functioning of siboglinids sulfide-binding hemoglobin. Investigating the distribution of these elements might improve our understanding of their metabolism.

Finally, because seep tubeworms are long-lived, their tubes may also record variations in fluid regimes over time, and their study might also be of general interest to geochemists aiming at reconstructing the evolution of past fluid seepage at any given site.

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Table 1 : PCR primers and conditions used in this study

Gene	Annealing (cycles)	Primer name	Primer Sequence (5' -> 3')	References
16S	45 °C (27)	27F	AGAGTTTGATCATGGCTCAG	(Lane 1991)
		1492R	GTTACCTTGTTACGACTT	
APS reductase	58 °C (25)	APS1-FW	TGGCAGATCATGATYMAYGG	(Meyer & Kuever 2007)
		APS4-RV	GCGCCAACYGGRCCRTA	
RuBisCO II	62 °C (25)	cbbm1_Els	ATCATCAARCCSAARCTSGGCCTGCGTCC	(Blazejak et al. 2006)
		cbbm2_Els	MGAGGTGACSGCRCCGTCRCCRGCMCGRTG	
ACL	52 to 46°C (32)	acl2F1	CGTCGCCAAGGAAGAGTGGTTC	(Thiel et al. 2012)
		acl2R1	GGCGATGGCCTCAAAGCCGTT	
28S	52°C (35)	28S-C1	ACCCGCTGAATTTAAGCAT	(Dayrat et al. 2001)
		28S-C2	TGAACTCTCTCTTCAAAGTTCTTTTC	

Table 2: SEM-EDS microanalyses (wt%) of the selected sections of the tube (see Fig. 6 for the exact localization of sections).

Tube section	C	O	Na	Cl	Br	S	I	Ca	Mg	Si	K	Fe	C/O
Internal side													
S2-int	57.72	38.55	0.45	0.73	1.16	1.39	-	-	-	-	-	-	1.49
S15-int	55.26	43.07	0.17	0.5	-	1.01	-	-	-	-	-	-	1.28
S28-int	58.26	39.83	0.51	0.64	-	0.76	-	-	-	-	-	-	1.46
S48-int	61.81	32.69	1.24	2.8	-	1.46	-	-	-	-	-	-	1.89
S55-int	55.38	40.08	0.83	1.43	0.36	1.05	-	-	-	0.16	-	-	1.38
External side													
S2-ext	52.7	42.28	0.78	0.43	0.84	1.53	-	0.45	0.24	0.6	0.15	-	1.25
S15-ext	57.63	34.6	0.48	0.86	3.27	1.92	0.76	0.26	0.21	-	-	-	1.67
S28-ext	57.17	31.87	0.71	1.45	5.04	2.77	-	-	-	0.21	0.79	-	1.79
S48-ext	47.79	43.42	1.76	1.51	1.63	0.79	0.74	0.2	0.26	1.17	0.15	0.57	1.10
S55-ext	56.49	36.66	0.73	0.94	0.82	3.32	-	0.22	-	0.38	-	0.45	1.54

Fig. 1: Phylogenetic tree based on 16S rRNA-encoding bacterial genes. Topology is based on 1353 nucleotide positions. Bootstrap values at nodes are given as percentages (>60 shown). Scale bar corresponds to 10% estimated sequence divergence.

Fig. 2: Phylogenetic tree based on APS reductase -encoding genes. Topology is based on the analysis of 127 amino acid positions using a JTT model. Bootstrap values at nodes are given as percentages (>60 shown). Scale bar corresponds to 10% estimated sequence divergence.

Fig. 3: Phylogenetic tree based on *cbbM* genes (RubisCO). Topology is based on the analysis of 118 amino acid positions using a JTT model. Bootstrap values at nodes are given as percentages (>60 shown). Scale bar corresponds to 10% estimated sequence divergence.

Fig. 4: Phylogenetic tree based on ATP citrate lyase (ACL)-encoding genes. Topology is based on the analysis of 350 amino acid positions using a JTT model. Bootstrap values at nodes are given as percentages (>60 shown). Scale bar corresponds to 10% estimated sequence divergence.

Fig. 5: FISH image of a cross section through the trunk of *Escarpia southwardae*. Host cell nuclei are in blue, bacteria of variable size, co-hybridized with probes LaSp-60 and LaSp-640, appear yellow. Bacteria occupy most of the surface of the hybridized section.

Fig. 6: Profiles of tube density (mg/cm tube length), Zr, Sr, Fe and Mn concentrations (in ppm) in the tube of *Escarpia southwardae*. Length is measured from the anterior (0 cm) to the posterior end (100 cm). The anterior part (0-85 cm) was exposed to seawater, the posterior part (85-100 cm) was the 'root' buried in the sediment.

Fig 7: SEM micrographs of the surface of the tube. See Fig. 6 for localization of the segments (S). Ext corresponds to the external, outer side of the tube, facing the seawater or the sediment, int corresponds to the internal, inner side of the tube, facing the animal body. Note the precipitates appearing white in D and crystalline in E and F observed on the outer side of the tube, in the posterior part.

Highlights

- We characterize symbionts associated with the tubeworm *Escarpia southwardae*
- We investigate profiles of trace elements (Mn, Fe, Sr, Zr) along the tube
- Symbionts have genes for sulfur metabolism and autotrophic carbon fixation
- Closest relatives are symbionts of tubeworms from the Gulf of Mexico and Pacific
- Two distinct redox fronts occur in the anterior and posterior regions of the tube

Figure 1

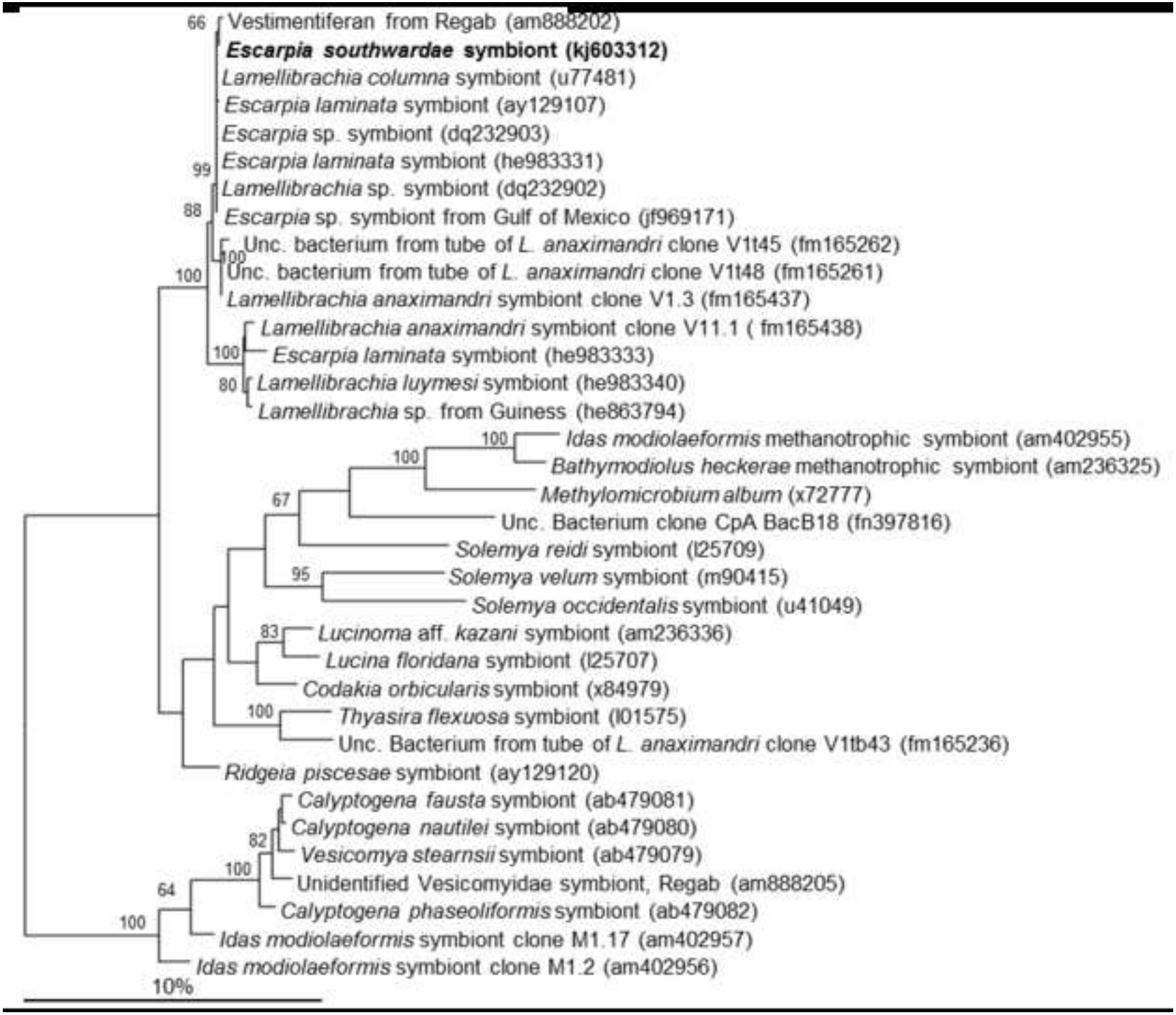


Figure 2

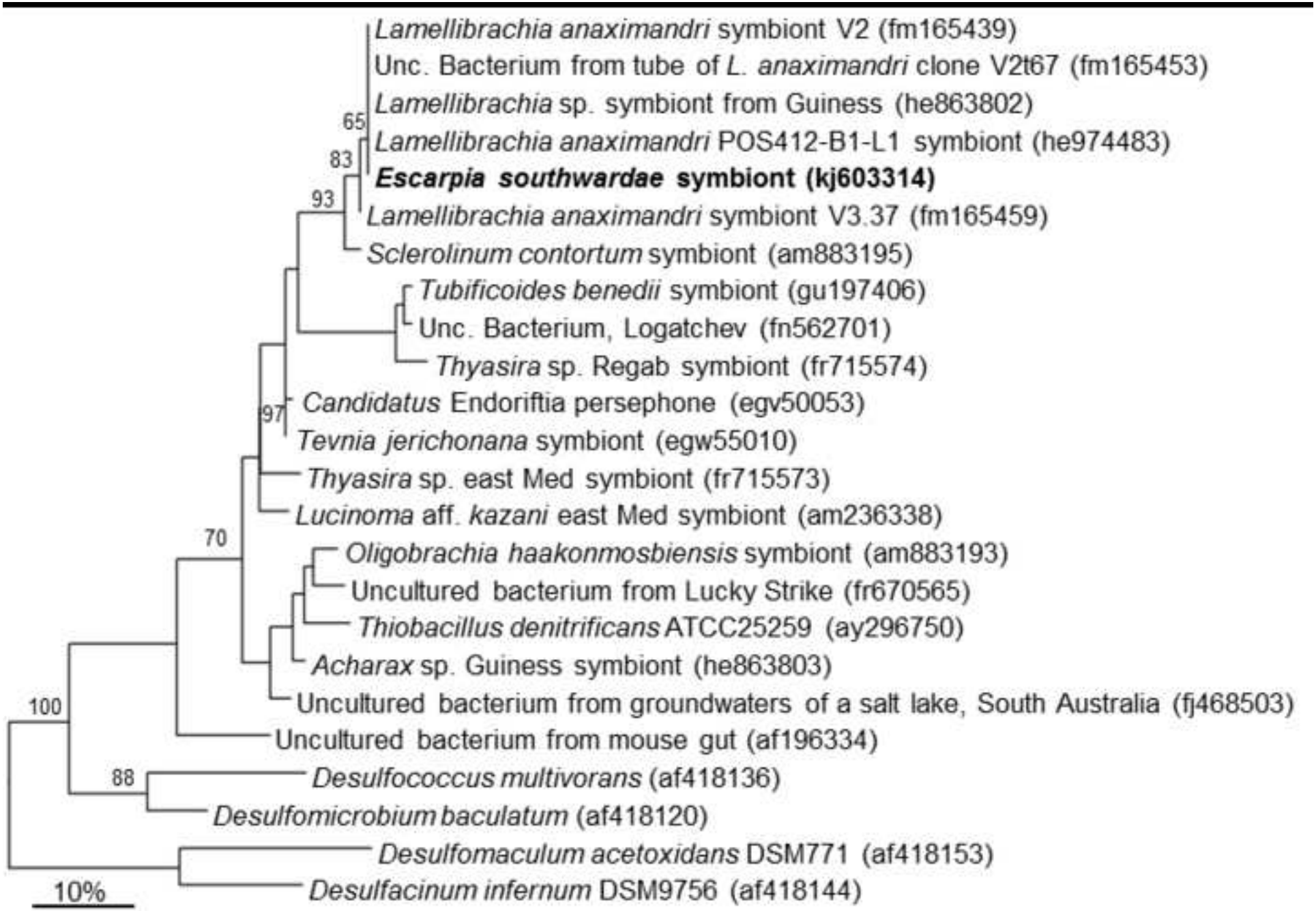


Figure 3

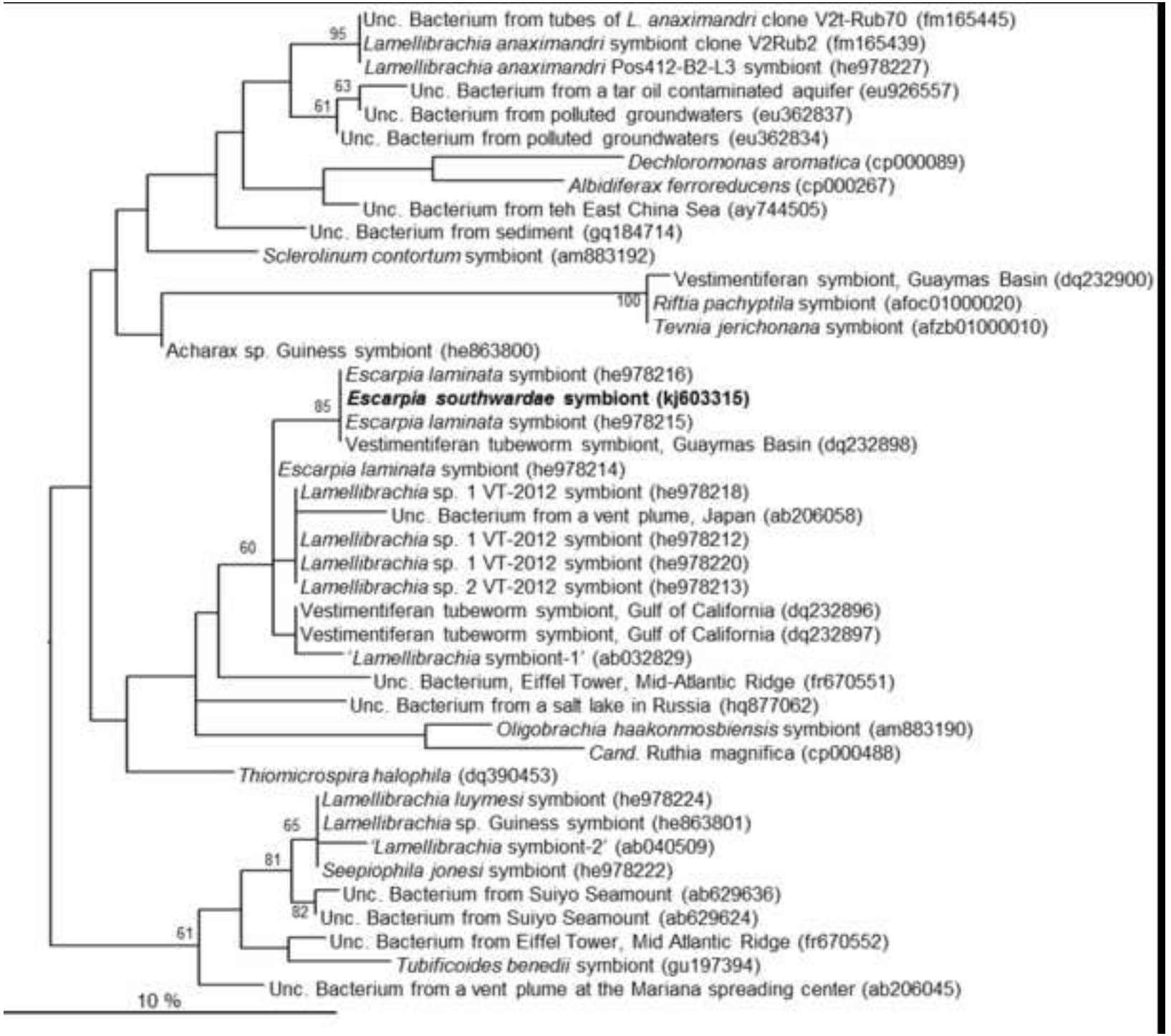


Figure 4

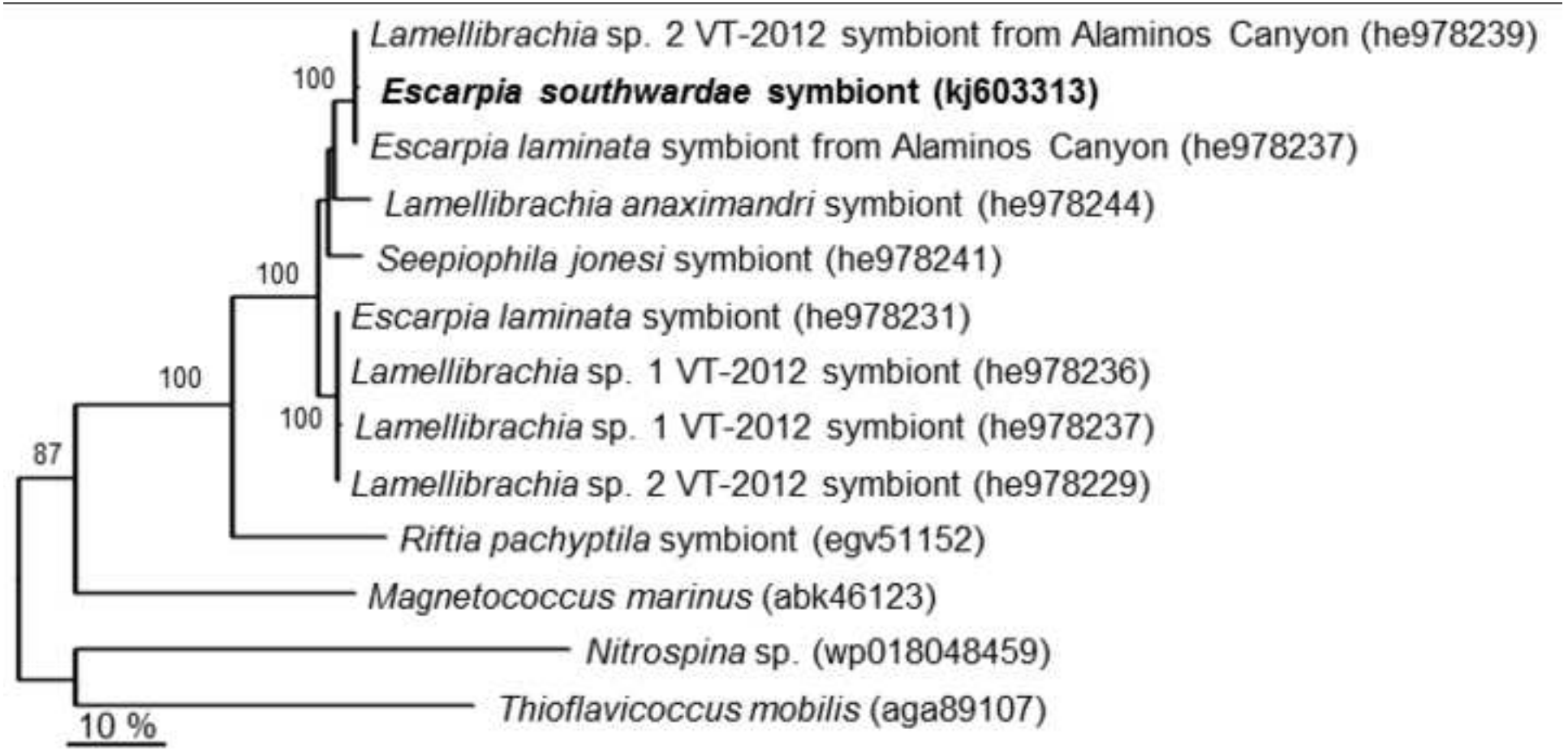
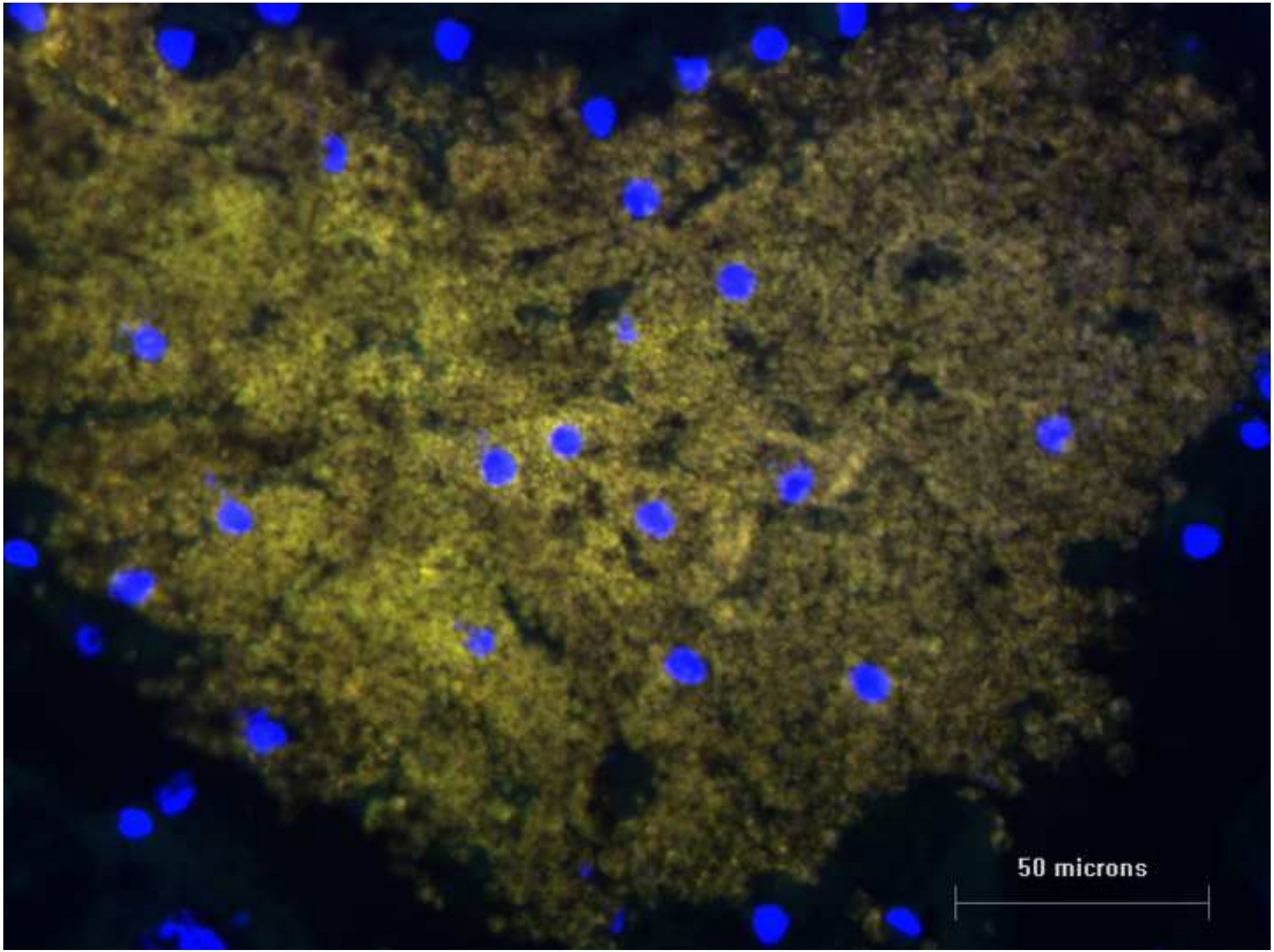


Figure 5



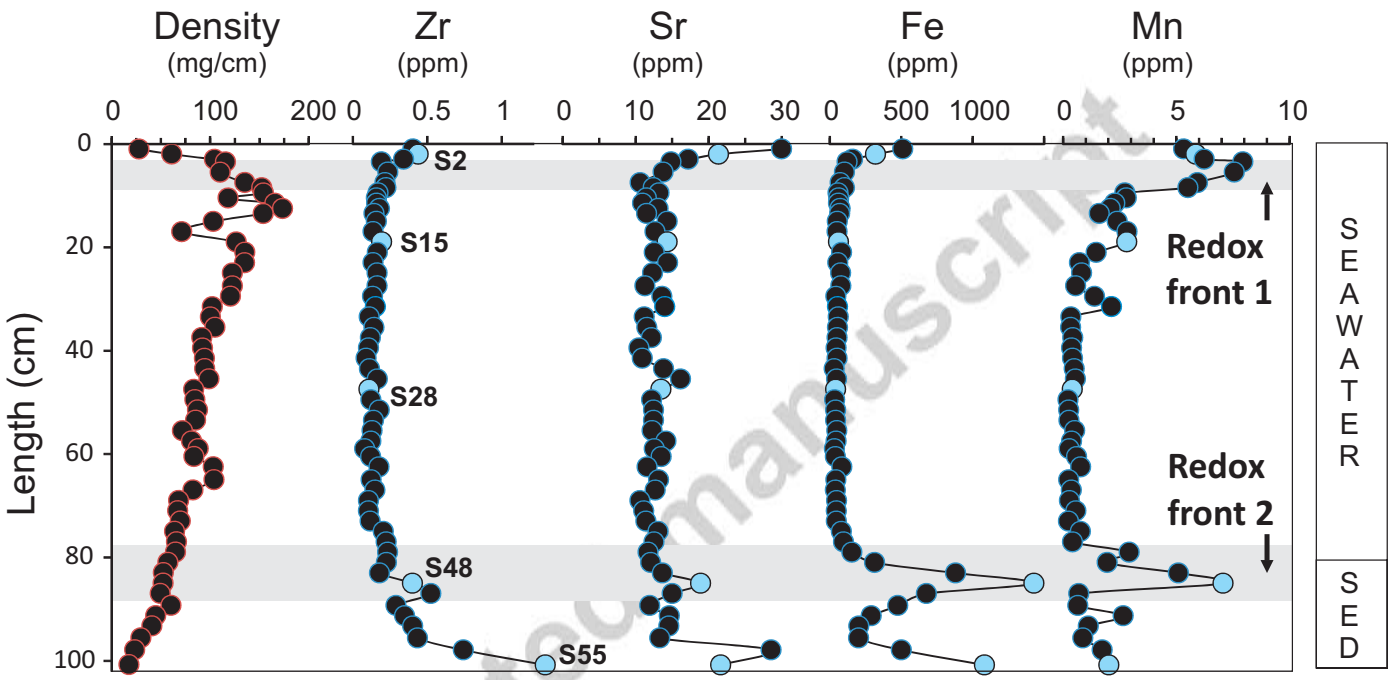


Figure 7