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Published in: PLoS ONE

DOI: 10.1371/journal.pone.0035975

Publication date: 2012

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
The Potent Respiratory System of *Osedax mucofloris* (Siboglinidae, Annelida) - A Prerequisite for the Origin of Bone-Eating *Osedax*?

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Abstract

Members of the conspicuous bone-eating genus, *Osedax*, are widely distributed on whale falls in the Pacific and Atlantic Oceans. These gutless annelids contain endosymbiotic heterotrophic bacteria in a branching root system embedded in the bones of vertebrates, whereas a trunk and anterior palps extend into the surrounding water. The unique life style within a bone environment is challenged by the high bacterial activity on, and within, the bone matrix possibly causing O₂ depletion, and build-up of potentially toxic sulphide. We measured the O₂ distribution around embedded *Osedax* and showed that the bone microenvironment is anoxic. Morphological studies showed that ventilation mechanisms in *Osedax* are restricted to the anterior palps, which are optimized for high O₂ uptake by possessing a large surface area, large surface to volume ratio, and short diffusion distances. The blood vascular system comprises large vessels in the trunk, which facilitate an ample supply of oxygenated blood from the anterior crown to a highly vascularised root structure. Respirometry studies of *O. mucofloris* showed a high O₂ consumption that exceeded the average O₂ consumption of a broad line of resting annelids without endosymbionts. We regard this combination of features of the respiratory system of *O. mucofloris* as an adaptation to their unique nutrition strategy with roots embedded in anoxic bones and elevated O₂ demand due to aerobic heterotrophic endosymbionts.


Introduction

The unique bone-eating organism, *Osedax* (Siboglinidae, Annelida) was first described in 2004 from a whale fall located at 2891 m depth in Monterey Bay, Pacific Ocean [1]. Since its first discovery it has been found on multiple whale falls in the Pacific and Atlantic Oceans, artificially deployed cow bones [2] as well as on other vertebrate bones such as those of teleost [3]. There are five formally described species, with at least a further 12 species known from genetic evidence [4,5]. In addition, convincing fossil traces of *Osedax* have been found in Oligocene and Pliocene mammal bones [6,7].

Evidence suggests that *Osedax* females utilize the complex organic compounds of the bone through endosymbiotic aerobic heterotrophic bacteria (Oceanospirillales) located in bacteriocytes in a root system that is embedded in the bone matrix [9,9]. The O₂ microenvironment around the embedded root system of *Osedax* has not been studied, yet this knowledge is crucial for understanding the function of *Osedax* in its natural habitat. The O₂ supply within the bone matrix is presumably strongly diffusion limited, and with whale bone lipid content reaching 45% in Sperm whales (closely related to Minke whales) [10] the bone interior may become O₂ depleted due to high heterotrophic microbial activity, including sulphur-reducing bacterial processes that generate sulphide, further reducing O₂ below the bone surface. External O₂ supply to the roots of *Osedax* via the bone matrix is thus highly unlikely. Yet, symbiosis with detoxifying sulphide-oxidizing bacteria has so far not been proven, though *Osedax* has been observed along with sulphophilic species and mats of white Beggiatoa-resembling bacteria (Figure 1) or bones exhibiting ferrous sulphide precipitiation [11–14]. The highly folded epidermis of the root structure of *Osedax* has been observed along with sulphophilic species and mats of white Beggiatoa-resembling bacteria (Figure 1) or bones exhibiting ferrous sulphide precipitiation [11–14]. The highly folded epidermis of the root structure of *Osedax* has been observed along with sulphophilic species and mats of white Beggiatoa-resembling bacteria (Figure 1) or bones exhibiting ferrous sulphide precipitiation [11–14]. The highly folded epidermis of the root structure of *Osedax* has been observed along with sulphophilic species and mats of white Beggiatoa-resembling bacteria (Figure 1) or bones exhibiting ferrous sulphide precipitiation [11–14]. The highly folded epidermis of the root structure of *Osedax* has been observed along with sulphophilic species and mats of white Beggiatoa-resembling bacteria (Figure 1) or bones exhibiting ferrous sulphide precipitiation [11–14].
muscular ventilation of the chitinous tube and expanded branchial structures with extensive blood vascular systems and large respiratory surface areas [16,18–20]. Whereas the tube of Osedax is gelatinous and only covers the trunk, the vascularised and pinulated palps of Osedax are believed to have a similar function as the anterior branchial plumes of Vestimentifera [21]. However, neither these structures nor the blood vascular system have been examined in detail, nor has the O₂ consumption been measured previously in any Osedax species.

Osedax and Vestimentifera are tunicobulous species and apart from the ability to retract into their tubes when disturbed, they show very little motile activity. However, Riftia pachyptila has been shown to metabolize much faster than other resting annelids (e.g. double aerobic endosymbionts and furthermore is hypothesised to be the chemoautotrophic endosymbionts. Since Osedax are believed to have a similar function resembling Beggiatoa, living in the restricted interface between hydrogen sulphide presence and oxygenated water [51].

In the present study we investigated whether Osedax mucofloris is exposed to an inhospitable hypoxic or anoxic microenvironment by measuring the O₂ distribution surrounding the embedded root system. We assessed possible morphological and/or physiological adaptations to the environmental conditions through detailed morphological studies of the respiratory surfaces and the blood vascular system as well as respiratory measurements of O₂ consumption. The results are discussed in relation to the unique environment, endosymbionts, and embedded root structure of Osedax as well as compared with studies of related annelids.

Results

Ventilating mechanisms

Ciliary bands and sensory structures (anti acetylated α-tubulin staining and histology). In the description below, we use a dorsal-ventral definition opposite to the one used by Rouse et al. [1,21], based on a new interpretation (Rouse & Worsaae, unpublished).

We found two previously unreported broad longitudinal ciliary bands dorso-laterally on each side of the ovicid on the anterior part of the trunk (Figure 2A, B, E). The ciliary bundles of the bands consist of multi ciliated cells, appearing pillow-like with numerous, conspicuously short cilia (<10 μm long) projecting outwards from the centre of the elliptical bundles (Figure 2C). The bundles are organized in an anterior-dorsal diagonal pattern within the longitudinal bands (Figure 2B). The ciliary bands narrow toward the posterior part of the trunk and are replaced by single tufts of cilia scattered basally across the trunk surface (Figure 2A). The short cilia appeared immotile, and with several longitudinal nerves running beneath, (Worsaae & Rouse, unpublished) their function may be sensory rather than ventilatory.

Two dense ciliary bands are found on each palp along their epidermal longitudinal lobes on each lateral side, as previously reported [12] (Figures 3A, B, E, 4G, 5A, F, G). The bands consist of multi ciliated cells with 70–90 μm long cilia (Figure 3E). The ciliary bands extend from near the basal part of the palps to the distal tip, beating in metachronal waves.

Two nerves, originating at the anterior part of the brain, innervate each palp. One nerve runs abfrontally, between the two lateral ciliary bands and the other nerve runs laterally underneath one of the ciliary bands (Figure 5A). Further details on the female Osedax nervous system will be described elsewhere (Worsaae & Rouse, unpublished).

Pinnules (up to 100 μm wide) project perpendicularly from the frontal palp surface between the lateral ciliary bands, with a density of approximately eight pinnules across the palp per 50 μm palp length (Figures 3A, 5F, G). At the proximal end of the palps, pinnules are less developed than at the distal end, seemingly growing in length (up to 170 μm) synchronously with the growth of the palp (Figure 3A). A sensory cell extends through the centre of the pinnule with a distal perikaryon and a few external, presumably sensory, cilia (Figure 4G, D). Its axon seems to connect with one of the two major longitudinal palp nerves, possibly the

Figure 1. Osedax mucofloris penetrating a bacterial mat resembling Beggiatoa. Beggiatoa live in the restricted interface between hydrogen sulphide presence and oxygenated water [51]. O. mucofloris must therefore be in contact with toxic sulphide concentrations. Photographer: Helena Wiklund, Department of Zoology, Göteborg University, Sweden. doi:10.1371/journal.pone.0035975.g001
Figure 2. Confocal laser scanning microscopy (CLSM) of the trunk of *Osedax mucofloris* females. A: Dorso-lateral view of a complete specimen, lateral ciliary bands occupies half the length of the trunk. B: Dorsal view of the anterior part of the trunk, elliptical shaped cilia bundles are directed anteriorly from the lateral part of the trunk. C: Depth coded z-stack, the elliptical shaped cilia bundles constituting the lateral ciliary band are formed by ciliary tufts. D: Close-up of transverse section of a trunk. E: Transverse section of a trunk, note the muscularized dorsal blood vessel. F: Single z-stack image of the trunk musculature, longitudinal muscles beneath circular and diagonal muscles. Abbreviations: ciliary tufts (ct), circular muscles (cm), diagonal muscles (dm), elliptical ciliary bundles (ecb), lateral ciliary band (lcb), longitudinal muscles (lm), muscular gap (mg), palp (p), root structure (r), torn ovisac (to), trunk (t), dorsal blood vessel (dbv). doi:10.1371/journal.pone.0035975.g002
one running more laterally beneath the ciliary band. This is supported by similar findings in Osedax “yellow-collar” (Figure 4E).

Musculature (F-actin, phalloidin staining). The longitudinal muscles run along the entire length of the trunk (Figure 2A), originating posteriorly at the trunk basis, and inserting anteriorly at the base of the four palps (Figure 3D). In one individual, it was possible to detect clustering of longitudinal muscles into 14–16 bundles of 20 muscle strands in the posterior part of the trunk, anterior to the root structure (Figure 4A). Along, and around the entire trunk, the muscles are distributed in a dense cylindrical formation with an average of six strands per 50 μm (Figures 2D, E, 3D). The musculature is slightly separated internally to the oviduct (possibly by the nerve cords), as well as randomly along the trunk, creating minor gaps most likely for mucus gland exits or nerves (Figures 2A, 3D). Anteriorly, a gap in the musculature is found ventrally at the position of the brain, as well as at each of the four insertion points of the palps. At each of these four points, the longitudinal musculature divides into two bundles (of each 20–30 strands), encircling the insertion point of the palp (Figure 3D). The longitudinal palp muscles originate at the base and run along the entire length of the palps to their tips, separated by a smaller frontal and a larger abfrontal gap (Figures 3C, D, 5A). No longitudinal musculature was detected in the pinnules.

Around the ovisac and anterior root structure the longitudinal musculature divides, and together with the circular muscles, creates a mesh-like structure (Figure 4A). The musculature extends posteriorly along the roots in a cylindrical formation, supporting the tissue penetrating the bone.

Thin circular muscles are found peripheral to the longitudinal muscles along the entire trunk, with 24 strands per 50 μm (Figure 2D). The circular muscles are most dominant in the posterior part of the trunk and continue into the root structure (Figure 4A). Notably, much thicker diagonal muscle strands were found beneath the circular musculature, but peripheral to the longitudinal muscles (Figure 2F). Attached at the mid-dorsal line, the strands run diagonally around the trunk in an anterior direction and attach at the mid-ventral line. The diagonal muscles are distributed along the entire length of the trunk, lying further...
apart (35–70 μm) in the posterior end than along the rest of the trunk (5–10 μm).

The circular musculature of the palps encircles the longitudinal musculature as a cylinder with ~18 muscle strands per 50 μm and is evenly distributed along the entire palp (Figure 3C). Fine circular muscles enclose each vessel of the pinnular loop (Figure 4C, D); they were likewise visible in the semi-thin sections on the outside of the pinnular loop and along the midline of each pinnule (Figure 5F, arrow tips), with a spacing corresponding to those shown with phalloidin staining (Figure 4C, D).

**Blood vascular system**

The trunk of *Osedax mucofloris* encloses two major longitudinal blood vessels (Figures 2E, 6A, B). The dorsal vessel is highly muscularized with circular musculature throughout its length (Figure 2D, E). No lateral connecting vessels between the major longitudinal vessels or epidermal capillaries were found in the trunk. From the trunk, the two blood vessels continue posteriorly into the root structure and increase in diameter (Figure 6A, B). When studied under the light microscope, the muscularized dorsal vessel is visible as a defined tube along the trunk, and continues into the anterior root structure, curling up in the centre anterior to the ovisac. The curling configuration is most likely caused by contraction of the basal part of the trunk. The exact further path of the vessel was difficult to determine. The Confocal Laser Scanning Microscope (CLSM) studies also showed the continuation of the dorsal blood vessel around the ovisac, revealing cylindrical musculature at the ovisac, corresponding in diameter to the musculature of the dorsal blood vessel of the trunk (Figure 4B).

The ventral blood vessel also continues into the root structure, but as a narrower and less defined vessel, the path of which was even more difficult to determine, than that of the dorsal vessel. Blood vessels of different sizes, as well as multiple obvious capillaries within the root tissue were observed (Figure 6B–E). Larger vessels extend out from the area of the ovisac and divide into thinner vessels (diameter: 7–30 μm). Capillaries were detected in the periphery of the root tissue (Figure 6E), with distances between the detected capillaries ranging from 65–220 μm.

Anteriorly, each palp encloses a pair of blood vessels created by invaginations of the inner lamina of the basement membrane of the epidermis (Figures 5F, G, 7C). Imaging of live specimens

**Figure 4. CLSM of the root system and pinnules of *Osedax mucofloris* females.** A: Single z-stack image of the ‘trunk-root system’ connection, note the bundles of longitudinal muscles. B: Musculature located by the ovisac, assumed to be the posterior end of the longitudinal dorsal blood vessel. C: Single z-stack image of pinnules, circular musculature encircling the pinnular loop, note the distal perikaryon and sensory cilia. D: Single z-stack image showing a longitudinal section of the pinnule in C, note the internal nerve. E: Depth coded z-stack, pinnule nerves in *Osedax* ‘yellow-collar’. Abbreviations: cilia (ci), circular muscles (cm), lateral ciliary band (lcb), longitudinal muscles (lm), nerve (n), perikaryon (pe). doi:10.1371/journal.pone.0035975.g004
confirmed the presence of two blood vessels, running along the entire length of the palps.

The pinnules are largely filled by a blood cavity lined with a membrane, which fuses in between the two blood cavities along most of the pinnule length, thereby creating the pinnular loop (Figures 5B–G, 7C). The palps of the histological sections were 300 μm in diameter at the base and the palp epidermis was 50–75 μm thick. The palp diameter and the thickness of the epidermis are important factors in the function of the palps as respiratory organs.

Figure 5. Diagram of a transverse section of the basal part of palps (A), DIC light micrographs of benzidine stained palps (B–D) and transverse 1.2 μm sections of O. mucofloris palps stained with toluidine blue (E–G). A: Diagram of a transverse section at the basal part of the palp region. Circular musculature (continued green lines) encircles the longitudinal musculature (green broken lines) in a cylinder formation. Two gaps separate the longitudinal muscle bands. Black lines illustrate the motile lateral ciliary bands and two main palp nerves run along each palp as shown (blue dots). B: Pinnular loop filled with blood. C: Pinnular loops, broken lines and arrows indicate the assumed direction of blood flow. D: Midsection of palp, longitudinal blood vessels and pinnular loops visible. E: Transverse section of a pinnule, the pinnular loop enclosed by a membrane fusing in the centre. F: Transverse section of the distal part of a palp, arrow tips shows circular musculature. G: transverse section of the distal part of a palp, the two longitudinal blood vessels obvious. Abbreviations: circular muscles (cm), epidermis (ep), lateral ciliary band (lcb), left dorsal palp nerve (ldpn), left ventral palp nerve (lvpn), membrane fusion (mf), musculature (m), palp blood vessel (pbv), pinnule (pin), pinnular loop (pl), right dorsal palp nerve (rdpn), right ventral palp nerve (rvpn). doi:10.1371/journal.pone.0035975.g005
Pinnules vary in diameter along their length and along the palp, with a median diameter of 40–60 μm for the pinnules and 20–40 μm for each blood vessel. The diffusion distance across the pinnule epithelium was measured on the semi-thin sections to be 1–2 μm, and for the epidermis of the distal part of the palps, the diffusion distance was measured to be ≈30 μm.

**Oxygen consumption**

When corrected for background respiration, the measured O2 consumption (MO2) of *O. mucofloris* ranged almost across a factor of ten from 220±93 μg O2 g⁻¹ h⁻¹ to 2053±1950 μg O2 g⁻¹ h⁻¹ (Table 1), depending on the approach used for measurement. MO2 measured on *O. mucofloris* inhabiting sectioned bone pieces (B1–B3) vs. MO2 measured *O. mucofloris* inhabiting bones in cuvettes (C1, C2), resulted in two distinctly different ranges of MO2. The MO2 of C1 and C2 was 220±93 μg O2 g⁻¹ h⁻¹ and 238±29 μg O2 g⁻¹ h⁻¹, respectively, while the MO2 of B1–B3 range from 976±201 μg O2 g⁻¹ h⁻¹ to 2053±1950 μg O2 g⁻¹ h⁻¹. Methods and ranges are commented further in the discussion.

**Oxygen levels around Osedax**

Micro sensor measurements of O2 concentrations in proximity to the bone interface were conducted, through agar-filled holes, on *Osedax*-colonized bone fragments in cuvettes. The obtained profiles of O2 concentration from the aerated seawater, and inwards showed steep O2 gradients towards both the bone and tissue surface (Figure 8A, B). Anoxic conditions or very low O2 levels were found at the bone surface, within the bone, and in proximity to the embedded root tissue of *O. mucofloris* (Table 2). The average
surfaces, into the overlying O₂-rich water in order to uptake O₂. *Osedax mucofloris* extend its palps and pinnules, with large respiratory tube showed a decrease in O₂ concentration in the microenvironment surrounding the palps, tightly fitting to the base of the trunk and the surface of the bone, preventing any water exchange to the ovisac and roots from the bone surface. The longitudinal musculature is moreover able to stretch the extensive branchial structures (palps) into the aerated water in order to increase the O₂ uptake.

The well-developed longitudinal musculature of the trunk serves to retract the trunk and palps into the tube and bone, presumably as protection from predators. As no regular retraction patterns of trunk musculature were detected, retraction into the tube is not considered a significant mode of ventilation for required O₂. Furthermore, the tube only surrounds the trunk, tightly fitting to the surface of the bone, preventing any water exchange to the ovisac and roots from the bone surface. The longitudinal musculature is moreover able to stretch the extensive branchial structures (palps) into the aerated water in order to increase the O₂ uptake.

The circular trunk musculature in *O. mucofloris* is weakly developed, as found in several other annelids [26,27]. The diagonal musculature revealed in the present study, may compensate for the weak circular musculature by having a similar supportive function. Diagonal musculature has most likely been mistaken for circular musculature in several annelids [26,27], including previous studies of *Osedax* [15,21].

Our study demonstrates the respiratory challenges faced by *Osedax*, as no oxygen was measured in the bone matrix as well as in the immediate vicinity of root structures and ovisac. Furthermore, we found no ventilating structures such as ciliary bands on the root epidermis, nor dense capillaries beneath the epidermis. Therefore, the epidermis of the embedded root structure is unlikely to facilitate ventilation or local O₂ supply to the *Osedax* root system. The O₂ necessary to maintain the metabolism and heterotrophic endosymbionts of the roots may instead primarily be transported via the blood vascular system from uptake at the aerated palps (Figure 7A). However, the microvillar root epidermis in *Osedax* does constitute a large surface area to volume ratio, which besides uptake of organic compounds may also facilitate uptake of e.g., hydrogen sulphide in the bone matrix. This is the case for the ‘root’ surface of the vestimentiferan *Lamellibrachia*, where hydrogen sulphide is absorbed and then transported via the circulatory system to the chemoautotrophic endosymbionts in the trophosome [16,17,28].

Previous studies of other close relatives of *Osedax* such as *Vestimentifera* and *Sabellidae* have shown anterior branchial structures to be of vital importance when tube-bound tissue is not site for O₂ uptake (Figure 7A–C). Uptake of O₂ over the trunk surface is possible, but a thicker epidermis, short and seemingly immotile ciliary bands and no obvious respiratory structures suggest that the trunk is a minor site of O₂ uptake. Oxygen does, to some extent, diffuse from the surrounding water into the mucus tube, thereby supplying the dwarf males.

### Discussion

#### Ventilation and branchial structures

The presence of highly vasculated palps and pinnules, the former densely ciliated showed that the anterior crown is the main...

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**Table 1. Weight specific O₂ consumption (MO₂) of *Osedax mucofloris*.**

<table>
<thead>
<tr>
<th>Numbers of individuals</th>
<th>Fixed weight (g)</th>
<th>MO₂ (\mu\text{g O}_2 \text{g}^{-1} \text{h}^{-1})</th>
<th>SD*</th>
<th>N‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>4</td>
<td>0.081</td>
<td>976</td>
<td>201</td>
</tr>
<tr>
<td>B2</td>
<td>4</td>
<td>0.044</td>
<td>1292</td>
<td>650</td>
</tr>
<tr>
<td>B3</td>
<td>1</td>
<td>0.017</td>
<td>2053</td>
<td>1950</td>
</tr>
<tr>
<td>C1</td>
<td>2</td>
<td>0.054</td>
<td>220</td>
<td>93</td>
</tr>
<tr>
<td>C2</td>
<td>3</td>
<td>0.11</td>
<td>238</td>
<td>29</td>
</tr>
</tbody>
</table>

MO₂ was determined in sea water at 100% atmospheric saturation and carried out on *O. mucofloris* inhabiting sectioned cow bone (B1–B3) and cow bone in cuvettes (C1–C2).

* Standard deviation.
‡ Numbers of measuring sequences on which MO₂ is based.

doi:10.1371/journal.pone.0035975.t001

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**Figure 7. Schematic illustration of O₂ distribution in the internal and external environments of *Osedax mucofloris*.**

A: *Osedax mucofloris* extends its palps and pinnules, with large respiratory surfaces, into the overlying O₂-rich water in order to uptake O₂. B: Schematic illustration of assumed blood flow in palp and pinnules, longitudinal section. Blue vessels carrying venous blood through afferent vessels, red vessels carrying arterial blood through efferent vessels. C: Schematic illustration of assumed blood flow in palp and pinnules, transverse section. Likewise blue vessels carries venous blood through afferent vessels, red vessels carries arterial blood through efferent vessels. Note that the palp blood vessels are created by an invagination of the basement membrane. Green indicates musculature.

doi:10.1371/journal.pone.0035975.g007

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O₂ flux at the bone and tissue interface was 0.028±0.0024 nmol O₂ cm\(^{-2}\) s\(^{-1}\) \((n=5)\) and 0.029±0.0040 nmol O₂ cm\(^{-2}\) s\(^{-1}\) \((n=3)\), respectively.

Micro sensor measurements of O₂ distribution in one mucus tube showed a ~50% decrease in the O₂ concentration in the centre of the mucus tube wall as compared to outside the tube (Table 3). Direct measurements within the tube were not possible due to disturbance by the worm. Measurements of the O₂ microenvironment surrounding the palps showed a strong decrease in O₂ concentrations, when a palp approached the microelectrode measuring tip. At the base and middle of the palp, the O₂ levels were almost zero showing the O₂ uptake to be high (Figure 7A). However, the microvillar root epidermis in *Osedax* does constitute a large surface area to volume ratio, which besides uptake of organic compounds may also facilitate uptake of e.g., hydrogen sulphide in the bone matrix. This is the case for the ‘root’ surface of the vestimentiferan *Lamellibrachia*, where hydrogen sulphide is absorbed and then transported via the circulatory system to the chemoautotrophic endosymbionts in the trophosome [16,17,28].

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Likewise, the anterior branchial structures of *O. mucofloris* seem morphologically adapted to facilitate efficient O₂ uptake from the surrounding water. As with the branchial plume of Vestimentifera [31,32], the anterior palps and pinnules of *O. mucofloris* have a large surface area to volume ratio and short diffusion distances. Furthermore, O₂ uptake is optimized by the two ventilating ciliary bands on each palp, a feature also seen in other Siboglinidae and in Sabellida in general [20,31,33].

A rough estimate was made of the surface area of the anterior crown, calculated from palp length given by Glover et al. [12], and the dimensions of palps and pinnules of *O. mucofloris* found in the present study. This results in a weight specific branchial surface area (SBSA) of $\sim 22 \text{ cm}^2 \text{ g}^{-1}$ fixed mass, which is similar to the SBSA of *Riftia pachyptila* and higher than the SBSA of fish, crabs and other annelids [31]. This is especially obvious when compared to the SBSA of *e.g.*, *Arenicola marina* (4.00 cm$^2$ g$^{-1}$) [34], although this species can also take up O₂ across its general epidermis.

![Experimental setups of micro sensor measurements and O₂ profiles towards bone and tissue surfaces](image)

**Figure 8.** Experimental setups of micro sensor measurements and O₂ profiles towards bone and tissue surfaces. A: Depth profile of O₂ towards bone surface, blue: agar, red: cuvette wall, green: bone surface. B: Depth prof of O₂ towards tissue surface, blue: agar, red: cuvette wall, green: tissue surface. C: Schematic drawing of the micro sensor measuring path through the cuvette wall. D: Placement of measuring site on WB1, note the blackened areas indicating presence of ferrous sulphide. E: Close-up of measuring sites on WB1.

doi:10.1371/journal.pone.0035975.g008

The estimated diffusion distance in *O. mucofloris* (pinnule epidermis 1–2 μm, palp epidermis $\sim 30$ μm) is furthermore comparable to what has been found for *R. pachyptila* (pinnules $\sim 2.00$ μm; branchial filaments $\sim 25$ μm) and *Ridgeia pesciaria* (pinnules $\sim 1.00$ μm; branchial filaments $\sim 17$ μm) [31,32].

Interestingly, the present study showed that each pinnule is equipped with a distal sensory cell and external sensory cilia. These structures may sense disturbance in the water to avoid predators, as *Osedax* need not sense food items or reproductive indicators in the water [1,8,35]. Additionally, sensing of currents may also be beneficial in order to orientate the palps e.g., for optimal uptake of dissolved O₂. *Riftia pachyptila* does not have sensory structures on the pinnules, but does have long, separate sensory filaments that lack ciliary bands and pinnules. These structures, with unknown function, are placed between pinnulated filaments [33].
Blood vascular system

The quantity and complexity of capillaries in the root structure of *O. mucosporis* reflects the high O2 demand of *Osedax*, presumably for the metabolism of its heterotrophic endosymbionts and production and development of eggs. Similar capillaries are visible on the exposed ovisac of *O. frankpressi* ([figure 2F in [1]]) and *O. roseus* ([figure 4E in [21]]), while the present study shows the presence of capillaries supplying the more distally placed root tissue and bacteriocytes. However, the extent of capillaries does not match the extensive capillary network of the trophosome in other Siboglinidae ([20,33]). This is in accordance with the original description [1] mentioning the lack of a discrete trophosome, the *Osedax* trophosome instead being diffuse and beneath the epidermis of the embedded tissue.

The present study supports the basic histological findings with regard to the circulatory system of the palps in *O. roseus* ([21]). The increased level of detail in the present study suggests similarities with some previous siboglinid studies ([31,32,36]). The pinnular loop is most likely lined by an extension of the outer lamella of the basement membrane, which is in accordance with the findings of Norrevang ([36]). No indication of a capillary plexus between the two elements of the loop was observed, as otherwise seen in *Riftia pachyptila* and *Ridgeia piscesae* ([31–33]). In accordance with Norrevang’s ([36]) observations of blood flow in *Siboglamum*, the blood of *Osedax* flows through an afferent vessel into the palp, through the pinnular loops and back through an efferent palp vessel ([Figures 7B–C]). This circulatory system carries oxygenated blood to the trunk and root system. In the present study of *O. mucosporis* and previous studies of *Osedax* ([1,21]) the main trunk blood vessel has been shown to be muscularized, and is now regarded as dorsal ([Rouse & Worsaae, unpublished]). This is in accordance with other annelids ([37] and siboglinids ([20,33]), where the muscularized dorsal vessel, possibly assisted by a heart, create blood flow in an anterior direction in the dorsal trunk vessel and posterior in the ventral trunk vessel. Rouse et al. [1] reported an anterior lying dorsal heart in the original description of the genus, not noted in any descriptions since and would now appear to be an error ([Rouse, pers. obs.]).

The main blood flow within the palp vessels may be generated by the circular body wall musculature of the palp. However, the thin circular musculature of the pinnules surrounding each branch of the looped blood vessel ([Figure 4C, D]) most likely assists local blood flow, as suggested for the tentacular vessels of *Riftia pachyptila* ([22]). Musculature in anterior appendages has been found in several Vestimentifera, in the form of sphincter muscles located in the branchial lamella and filament vessels ([31]).

As with the trunk of *O. mucosporis*, the anterior vestimentum of Vestimentifera lacks branching vessels between the ventral and the muscularized dorsal vessel ([33]). The resemblance between the blood vascular systems adds new evidence to the hypothesis that the trunk of *Osedax* and the vestimentum of Vestimentifera are homologous regions ([21]).

**Table 2.** O2 concentration measured by micro sensors at the bone or tissue surface of *Osedax mucosporis* inhabiting whale bone in two cuvettes, WB1 and WB2.

<table>
<thead>
<tr>
<th>Bone surface</th>
<th>O2 concentration (µmol l−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>0.00</td>
</tr>
<tr>
<td>Site 2</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 1 Site 4</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 1 Site 5</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 1 Site 6</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 2 Site 1</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 2 Site 2</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 2 Site 5</td>
<td>3.44</td>
</tr>
<tr>
<td>Tissue surface</td>
<td></td>
</tr>
<tr>
<td>WB 1 Site 3</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 2 Site 3</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 2 Site 4</td>
<td>0.67</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0035975.t002

**Blood vascular system**

**Oxygen consumption**

The present study shows that *Osedax mucosporis* has a higher weight specific O2 consumption (MO2) than other resting annelids ([Figure 9]) ([18]). This may reflect an elevated demand of the embedded tissue due to presence of heterotrophic aerobic endosymbionts, which have a higher metabolism than regular tissue. The measured MO2 actually corresponds to that found for *Riftia pachyptila* ([22,23]) ([Figure 9]), possessing a vast amount of chemoautotrophic bacteria in their trophosome. Furthermore, a high MO2 may also reflect oxidative sulphide detoxification. The high MO2 corresponds well with the large SBSA and elaborate branchial structures, which are both usually associated with animals exhibiting a high O2 demand. This correlation is also found in *Arenicola marina* with smaller SBSA and lower MO2 (red square, [Figure 9]).

Our data show that *O. mucosporis* inhabiting bone in cuvettes ([purple and yellow dots, Figure 9]) have a lower MO2 than *O. mucosporis* inhabiting sectioned cow bone (green, blue, red dots, [Figure 9]). Along with high standard deviations, this highlights the difficulties of measuring O2 consumption on these embedded worms. The variations are most likely caused by the difference in blind respiration measurements and the large biological activity present on decaying bone, which was difficult to quantify. Future studies should thus be carried out to more precisely determine the
from Cammen [18], the regression line (log R = 1.682+0.850·log W) calculated from measurements of resting nonventilating annelids only.

**Dots:** Previous measured O₂ consumption of *R. pachyptila*. No sulphide present in water when measuring: Red, blue [22] and green [23]; sulphide present in water during measurement: Purple [23]. **Red Square:** O₂ consumption of resting *Arenicola marina* [52].

doi:10.1371/journal.pone.0035975.g009

MO₂ of *O. mucosilosis* and the contribution of other O₂-consuming surfaces.

**Oseodax** adaptations to the bone environment

The bone matrix exhibited strong O₂ depletion with anoxic or low O₂ levels at its surface and with areal O₂ consumption rates comparable to *in situ* diffusive O₂ uptake measured in sediments proximate to whale falls [38]. The respiratory and circulatory system of *Oseodax* is well suited to these environmental challenges. The elaborate branchial structures of the palp and pinnules of *Oseodax mucofloris* facilitate a high O₂ uptake from the surrounding seawater that, in combination with the blood vascular system, apparently enables sufficient oxygenation of the embedded tissue and endosymbionts. A similar, well-developed respiratory system is also found in other members of Siboglinidae (for review see [39]), also exhibiting high surface area to volume ratio in the branchial structures in particular. Based on these morphological and functional similarities among several siboglinids it seems possible that a well-developed anterior branchial structure may be a plesiomorphic condition among siboglinids and possibly a prerequisite for housing aerobic symbionts while dwelling in anoxic habitats. This respiratory system may therefore very well be a pre-adaptation present in the common ancestors of *Oseodax* and other siboglinids, responsible for the successful colonization and evolution of *Oseodax* in the unique bone environment.

An extended root surface (and high area to volume ratio) is likewise found in both *Lamellibrachia* (e.g., [16]) and *Oseodax* (yet branched). However, the main function has so far been interpreted as very different. Whereas the *Oseodax* root surface is suggested to mainly facilitate uptake of organic compounds (with less focus on the possible congruent uptake of sulphide) [9], the root of *Lamellibrachia* is found to be the main respiratory surface of hydrogen sulphide necessary for the chemosynthesis of the symbionts [16,17,20].

The measured O₂ depleted bone environment as well as ferrous sulphide precipitations (Figure 8E) supports the suggested exposure of *Oseodax* to sulphide. Furthermore the large root surface in the related *Lamellibrachia* is highly efficient in hydrogen sulphide uptake [17]. Since the main trophic source of *Oseodax* compared to other siboglinids (including *Lamellibrachia*) seems to be heterotrophic bacteria rather than sulphide-detoxifying chemoautotrophic bacteria [6], *Oseodax* may instead possess physiological adaptations to detoxify sulphide.

Though beyond the scope of the present paper, microanalytical approaches such as microsensors [40,41] and functional imaging techniques [42] could yield a more complete mapping of the chemical microenvironment of *Oseodax*, including the exact levels of sulphide exposure and spatio-temporal dynamics of O₂ in the root system. It would also be interesting to know whether *Oseodax* have sulphide-binding properties of their haemoglobin as found in *Vestimentifera* [43,44], as they might use them to transport toxic sulphides from the area surrounding the root structure to e.g., the palp, somehow releasing the toxic compounds to oxygenated seawater or detoxifying them.

**Materials and Methods**

**Sampling and aquarium setup**

In January 2009, 3 replicate experimental sampling devices, using cow and whale bone, for recruitment of female *O. mucosilosis* (Figure 10) were placed at 125 m depth off the coast of Tjärnö, Sweden (58°52.976N; 11°05.715E) in close vicinity to a minke whale carcass sunk in October 2003 [13]. One device for morphological and reproductive studies was successfully retrieved in May 2009. A second device was retrieved in November 2009 for *in vivo* studies (respirometry and microsensor analysis) and additional morphological studies. Additionally, a piece of cetacean vertebra was collected from a scavenging minke whale located in close vicinity to the sampling device.

**Species-specific adaptations of the bone environment**

The experimental sampling devices were placed at 125 m depth off the coast of Tjärnö, Sweden (58°52.976N; 11°05.715E) in close vicinity to a minke whale carcass sunk in October 2003 [13].

doi:10.1371/journal.pone.0035975.g010
bone deposited at 123 m in the same area in May 2008 was retrieved in January 2009 and used for preliminary investigations and for designing experimental setups. In January 2009, the bottom water had a salinity of 34.6‰, a temperature of 8.4°C, and an O₂ content of 78.7% atmospheric saturation.

Placement and retrieval were carried out with a remotely operated vehicle (ROV, Sperre Subihfter, Norway) operated from the R/V *Lofthela* (Sven Lovén Centre for Marine Science, University of Gothenburg, Sweden). Upon retrieval, the sampling device was placed in a cooling box with ambient aerated water and transported to the Marine Biological Section, University of Copenhagen within 8 hours. In the laboratory bones were kept in aerated seawater under *in situ* conditions, in aquariums (Vol 60 litre) with aeration, protein skimmers and a recirculating pumping system. The *O. mucofloris* "yellow-collar" shown as comparison in Figure 4 was sampled in November 2009 in Monterey Bay, California at 363 m from a Grey whale skeleton and stained in accordance to the protocol described below. All necessary permits were obtained for the described whale fall experiment; received from Karin Pettersson at the County Administrative Board Vastra Gotalands Lan, Oct. 2003. The field studies did not involve endangered or protected species.

**Fixation**

Specimens were carefully dissected from the bone and fixed for immunohistochemistry, benzidine staining and histology. Prior to dissection, *O. mucofloris* were anesthetized for 5–10 minutes in a 1:1 solution of seawater and MgCl₂ (isotonic to seawater). Anaesthetized animals were gently dissected with scalpels and fixed at 4°C over night in 4% paraformaldehyde in 0.15 M phosphate-buffered saline (PBS) with 5% sucrose, pH 7.4. Subsequently, the animals were rinsed 4–6 times for 30 min in PBS with 5% sucrose and were then stored at 4°C in PBS with 0.05% sodium azide (NaN₃).

**Immunohistochemistry**

Four specimens were stained following the protocol of Worsaae & Rouse [45]. First staining included the primary antibodies monoclonal mouse anti-acetylated α-tubulin (Sigma T6793, 1:200) & polyclonal rabbit anti-serotonin (Sigma: S5545; 1:100/1:400) or monoclonal mouse anti-acetylated α-tubulin & Anti-FMRFamide (ImmuNoStar: 20091, 1:100). This was complimented by secondary antibodies; anti-mouse CY5 [Jackson ImmunoResearch: 115-175-062, 1:400] and anti-rabbit TRITC (Sigma T3268, 1:200/1:400). Hereafter specimens were incubated for 60 min in phalloidin conjugated with FITC or Alexa Flour 488 (Sigma F3282 or Invitrogen A12379, 0.17 or 0.33 μmol l⁻¹ phalloidin in PBS). Specimens were mounted in 100% Vectashield® containing DAPI (Vector Laboratories inc., California, USA) and stored at −18°C. The specificity of primary antibody binding versus e.g., autofluorescence was tested by omitting one of the primary antibodies, but otherwise treating specimens as described.

Specimens were studied using a Leica TCS SP5 confocal laser scanning microscope (CLSM) (University of Copenhagen, Faculty of Health Science, courtesy of M. Givskov and T. Bjarnsholt). Leica LASAF computer software or Imaris® x64 6.0.0 (Bitplane AG, Zurich, Switzerland) was used to produce projections of z-stacks of CLSM images, while further analyses of z-stack series were performed with Imaris® x64 6.0.0. Computed 2D images of muscles, nerve and cilia with relation to respiration and palpb morphology were further optimized with Adobe Photoshop CS3 and Adobe Illustrator (Adobe System Incorporated) for presentation.

**Histology**

One specimen was embedded in epon and used for histological analysis. Semi-thick 1.2 μm sections were cut on a microtome (EM UC6, Leica, Wetzlar Germany) with a diamond knife (Diatome; Biel, Switzerland). A small amount of Pattex contact adhesive (Pattex Compact; Henkel KGAA, Düsseldorf, Germany) was diluted with a few drops of xylene in an Eppendorf tube and applied to the side of the epon block to make serial sectioning of ribbons possible following the protocol of Henry [46] and Ruthensteiner [47]. Bands of ~20 sections were stained with toluidine blue and mounted in Entellan® (Electron Microscopy Sciences, Pennsylvania, USA). Sections were studied and photographed using light microscopy (BX50 microscope; Dp71 camera; CellF software; Olympus, Japan).

**Benzidine staining of haemoglobin**

Using a modified version of the benzidine staining method by Knox [48], haemoglobin was stained in four fixed specimens. A 100% saturated benzidine solution was prepared by adding benzidine to distilled water. The solution was stirred for two hours. Fixed specimens were rinsed in running tap water in the same time period. Specimens were subsequently incubated in the filtered benzidine solution for 1 hour, also under stirring. Next, 5% hydrogen peroxide was added drop by drop until blood vessels turned dark blue. Specimens were either mounted in glycerol directly or dehydrated in a series of alcohol acidified with drops of 0.1% acetic acid, where after tissues were cleared in xylene and mounted in D.P.X between two cover slips. Specimens were analyzed and photographed under a light microscope (BX50 microscope; Dp71 camera; CellF software; Olympus, Japan).

**Oxygen consumption measurements**

O₂ consumption was measured in seawater kept at ~100% atmospheric saturation using intermittent respirometry in accordance with Vismann and Hagerman [49]. The experimental setup was placed in a constant temperature room at 6°C. Each experiment encompassed 3–8 measuring sequences consisting of a flushing period of 10–30 min and a measuring period of 30–45 min. The set-up had a chamber flushing rate of 30 ml min⁻¹ and a shunt water flow of 8 ml min⁻¹ past the O₂ electrode (E5046, Radiometer Medical ApS, Bronshoj, Denmark).

The O₂ electrode was connected to a blood/gas monitor (PHM73, Radiometer Medical ApS, Bronshoj, Denmark) and measuring signals were acquired continuously on a PC via data acquisition software (Labtech Notebook Pro version 12.1, Laboratory Technologies Corporation, USA). Prior to each experiment, the O₂ electrode was calibrated in 0% O₂ solution (saturated solution of sodium sulphite in borax) and 100% atmospheric O₂ solution. The O₂ consumption (MO₂, μg O₂ g⁻¹ h⁻¹) was calculated according to the equation:

\[
MO_2 = \left( \frac{pO_2}{100 \times wW} \right) \times \beta \times v
\]

where \( pO_2 \) = the O₂ partial pressure at full saturation (kPa), \( wW \) = wet weight of fixed specimen rinsed for mucus tube (g) (issue fixed in 4% paraformaldehyde and stored in PBS is assumed to have similar weight as unfixed tissue), \( \alpha \) = slope of regression line for O₂ decrease (% h⁻¹), \( \beta \) = O₂ solubility (μg O₂ l⁻¹ 1% KPa⁻¹) and \( v \) = volume of the respiration chamber (l).

O₂ consumption was measured on *O. mucofloris* inhabiting three sectioned cow bone pieces (B1, B2 and B3) and bones in two cuvettes (C1 and C2). Measurements were either initiated directly
after the annelids protracted subsequent to the disturbance of being moved (C1, C2, B1) or after one night of acclimatization (B2, B3). All measurements were corrected for background respiration. For B2 and B3 the background respiration was measured using the same water and bones (after dissection and 48 hours of acclimation to restore biological activity). For C1, C2 and B1 the background respiration was the mean value of measurements using new water, bones in two cuvettes and three sectioned bone pieces without O. mucofloris. Different respiration chambers were used for sliced bone pieces (volume: 186 ml) and cuvettes (volume: 51 ml).

**Microscale O₂ measurements**

O₂ concentration profiles were measured in vertical steps of 100 µm with Clark-type O₂ microelectrodes with a guard cathode [50] (OX-10 Unisense A/S, Aarhus, Denmark) mounted on a manually operated micromanipulator (Märzhäuser, Wetzlar, Germany) and connected to a picoammeter (PA2000, Unisense A/S, Aarhus, Denmark) and a strip chart recorder (BD25, Kipp & Zonen, Delft, Netherlands). The microelectrodes had a measuring tip diameter of 10 µm, a stirring sensitivity of <1–2% and a 90% response time of <1 second. Linear calibration of the electrode was done from electrode readings in seawater at 100% atmospheric saturation and in anoxic seawater (by addition of sodium dithionate). The O₂ concentration ([O₂], µmol O₂ 1⁻¹) at each measuring position was calculated according to the equation;

\[
[O_2] = \frac{C_m}{C_{sat}}
\]

Where \(C_m\) = O₂ reading at depth n (µA), \(x_0\) = O₂ reading at 100% atmospheric O₂ saturation (µA) and \(C_{sat}\) = O₂ concentration at 100% atmospheric O₂ saturation (µmol O₂ 1⁻¹). We measured O₂ concentration profiles from the mixed aerated seawater, across the diffusive boundary layer (DBL) and towards the surface of O. mucofloris roots in minke whale bone kept in cuvettes (WB1 and WB2) (Table 2). Prior to these measurements, manually drilled holes (diameter: ~340 µm) on the side of the plastic cuvettes (Figure 8D, E) were covered with agar (1.5% w/w in seawater) and the cuvettes were left to acclimatize for 2–3 days in order to restore the environment prior to disturbance. The O₂ concentration was measured through the holes of the cuvette wall towards the enclosed bone surface and further into the bone (Figure 8C). Measurements are given for seven sites on WB1 and five sites on WB2.

The area specific O₂ flux (J, nmol O₂ cm⁻² sec⁻¹) at the bone surface was calculated from linear parts of the O₂ concentration profiles in the DBL and agar plug according to Fick’s first law:

\[
J = D_0 \frac{dC}{dz}
\]

Where \(dC/dz\) = the change in O₂ concentration (dC, nmol O₂ cm⁻²) with distance (dz, cm), \(D_0\) = the molecular diffusion coefficient of O₂ (cm² sec⁻¹). We applied a diffusion coefficient of 1.3382 × 10⁻⁵ cm² sec⁻¹, corrected for experimental temperature and salinity.

Additional microscale O₂ measurements were performed through the mucus tube wall and at the surface of the palps of the individial on WB1. On the mucus tube, the O₂ concentration was measured from outside the tube and tube wall and the wall inside the mucus wall. Undisturbed measurements could not be obtained within the centre hole of the tube due to contractions of the worm. On the palps, the O₂ concentration was measured by keeping the electrode measuring-tip at a fixed position, while the palps approached the tip position as the annelid protracted after being left undisturbed.

**Acknowledgments**

We would like to thank Tom Fenchel for guidance and lending of equipment for benzidine staining and live video recordings. We also thank Per Juel Hansen for access to his Olympus BX50 microscope, Dp71 camera and Cell³ software. Special thanks are directed to Michael Givskov and Thomas Bjarnsholt for the many hours made available to us on their Leica TCS SP5 confocal laser scanning microscope at the Faculty of Health Science, University of Copenhagen. In addition we thank Tomas Lundblad for ship and ROV operations and Helena Wiklund for assistance at the Sven Lovén Centre for Marine Sciences – Tjärnö.

**Author Contributions**

Conceived and designed the experiments: RSH BV MK MM KW. Performed the experiments: RSH MK MM VC KW BV. Analyzed the data: RSH MM VC KW. Contributed reagents/materials/analysis tools: KW MK BV AG GR TD. Wrote the paper: RSH KW BV.

**References**


