Sponge-associated bacteria mineralize arsenic and barium on intracellular vesicles

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Arsenic and barium are ubiquitous environmental toxins that accumulate in higher trophic-level organisms. Whereas metazoans have detoxifying organs to cope with toxic metals, sponges lack organs but harbour a symbiotic microbiome performing various functions. Here we examine the potential roles of microorganisms in arsenic and barium cycles in the sponge Theonella swinhoei, known to accumulate high levels of these metals. We show that a single sponge symbiotic bacterium, Entotheonella sp., constitutes the arsenic- and barium-accumulating entity within the host. These bacteria mineralize both arsenic and barium on intracellular vesicles. Our results indicate that Entotheonella sp. may act as a detoxifying organ for its host.
Microorganisms greatly influence arsenic\(^1\) and barium\(^2\) geochemical cycles. Of the two, arsenic is extremely toxic to most life forms, except for a few arsenic-respiring bacteria\(^1\) and eukaryotic hyperaccumulators\(^3\). Most soluble forms of arsenic\(^4\), as well as the barium ion\(^5\), are potent bioavailable toxins. However, their bioavailability decreases when assimilated into organic forms, or by mineralization\(^6,7\).

Soluble barium concentrations in surface seawater are typically 10 µg L\(^{-1}\) and present in two states: most is found as a divalent barium ion and a small amount as undissociated barite (BaSO\(_4\))\(^8\). Barite is primarily detected as particulate matter and is especially enriched in areas of high microplankton productivity\(^9\). Arsenic is a common trace element (\(~2\) µg L\(^{-1}\)) in marine environments. In the oxygenated seawater where *Theonella swinhoei* is found, arsenate is the dominant form of arsenic, followed by arsenite and a minute amount of methylated arsenic\(^10\). Most marine organisms accumulate arsenic to some extent, usually as organoarsenicals\(^11\).

Although both arsenic and barium can be detected in all tissues, their distribution is not homogenous. Toxie element concentrations is highest in tissues and organs dedicated to detoxification and excretion\(^12,13\).

Sponges are ancient metazoans\(^8\), of paramount significance to benthic communities\(^9\). Inhabiting all types of marine ecosystems, sponges contribute to global carbon\(^10\), nitrogen\(^11\) and silicon\(^12\) cycles. Sponges are filter feeders, filtering seawater up to 50,000 times their body volume daily\(^13\), resulting in high exposure to trace elements. Several studies have shown that sponges have the tendency, differing by species, to accumulate trace elements\(^14-17\).

Sponge-associated bacteria are known to contribute to important elemental cycling in sponges, namely carbon, nitrogen and sulfur\(^18\), but their role in trace element cycling is rarely studied\(^18,19\).

Recent trace element analyses of Red Sea (Gulf of Aqaba) demosponges single out *Theonella swinhoei*, a common Indo-Pacific sponge\(^20\), as a hyperaccumulator of arsenic (As) and barium (Ba)\(^14\). The concentrations of arsenic (8,600 µg g\(^{-1}\)) and barium (13,400 µg g\(^{-1}\)) are the highest concentrations recorded in any organism from an uncontaminated environment.

Symbiotic bacteria comprise approximately half the volume of *T. swinhoei*\(^21\). These include both phototrophic and heterotrophic bacteria\(^21\). The phototrophic cyanobacteria are located in a thin layer, close to the surface of the sponge, giving the host its characteristic colour. The heterotrophic bacteria reside mostly in the sponge’s inner mesohyl part. While the majority of sponge-associated bacteria are unicellular\(^21\), the most abundant bacterium is filamentous and identified as *Entotheonella* sp.\(^21,22\).

Moreover, *Entotheonella* sp. is considered a ‘talented producer’, synthesizing many of the bioactive compounds found in this sponge\(^22\).

Because sponges lack organs and tissues, there is no apparent localization for the storage of arsenic and barium. Following the initial discovery of arsenic and barium accumulation, the sponge was separated into enriched fractions of either sponge cells or bacterial cells, revealing arsenic was localized to the bacterial fraction. Barium concentrations were also shown to be high in these fractions (although the differences were not statistically significant)\(^18\). Culturing trials recovered numerous arsenic-tolerant, sponge-associated bacteria growing on media enriched with as much as 100 mM arsenate\(^18\).

Here we show that a population of a single symbiotic bacterium, *Entotheonella* sp., appears to drive arsenic and barium accumulation in this sponge. We further demonstrate that the bacterium can mineralize both elements on intracellular membrane vesicles.

**Results**

*Entotheonella* sp. accumulates arsenic and barium. Arsenic and barium have the highest atomic weight, among elements found in the sponge\(^14\). Thus, their accumulation can be detected using a scanning electron microscope (SEM) with backscatter detection mode (backscattered electron). Our preliminary results indicated that arsenic, and to some extent barium, were accumulated by the sponge-associated bacteria\(^18\). Surprisingly, when examining the sponge using SEM (n = 4), we discovered that a single bacterium, identified as *Entotheonella* sp.\(^22\) (Fig. 1a), had the highest electron density (Fig. 1b). Using energy-dispersive X-ray spectrometry (EDS) we analysed their elemental composition and found that *Entotheonella* sp. contained both arsenic (6.07% weight ratio) and barium (11.7% weight ratio). The filamentous bacterium *Entotheonella* sp. was previously described in *T. swinhoei* as trichomes or chains\(^21,22\), with 4 to 20 cells. However, no analysis has been made to date of the electron-dense material observed in its cells, which from our analysis we now know to be rich in barium and arsenic. Next, we used inductively coupled plasma mass spectrometry (ICP-MS) to quantify arsenic and barium in four, easily separated, cell-enriched fractions: sponge cells (F\(_{EC}\)), *Entotheonella* sp. (F\(_{ENTO}\)) and unicellular bacteria (F\(_{BAC}\) from the inner layer. The fourth fraction was taken from the outer layer and contained *Entotheonella* sp. and cyanobacteria (F\(_{EC}\)). The three fractions enriched from the inner layer (F\(_{EC}\), F\(_{ENTO}\) and F\(_{BAC}\) were tested against each other (Supplementary Table 1). As the two samples containing *Entotheonella* sp. (F\(_{ENTO}\) and F\(_{EC}\)) were not independent, they were compared separately (Supplementary Table 2). We found the highest concentration of barium in F\(_{ENTO}\) (26,420 ± 6,450 µg g\(^{-1}\) cells dry weight, n = 5; Fig. 1c). Permutated analysis of variance (ANOVA) followed by Tukey’s *post hoc* grouping showed F\(_{ENTO}\) barium concentration to be significantly higher than F\(_{BAC}\) (Supplementary Table 1). The difference between F\(_{ENTO}\) and F\(_{SC}\) was also tested by an exact permutation test, which showed they were significantly different (P = 0.024). All cell fractions contained a high arsenic concentration compared to other marine taxa\(^3\), with fractions containing *Entotheonella* sp. (F\(_{ENTO}\) and F\(_{EC}\)) displaying the highest concentration (F\(_{ENTO}\): 12,072 ± 3,740 µg g\(^{-1}\) cells dry weight, n = 5; F\(_{EC}\): 12,142 ± 3,406 µg g\(^{-1}\) cells dry weight, n = 5; Fig. 1d). Permutated ANOVA followed by Tukey’s *post hoc* grouping showed arsenic concentration in F\(_{ENTO}\) was significantly higher than those of the other inner fractions (Supplementary Table 1). We thus deduced that *T. swinhoei* either actively retains arsenic\(^14\) or passively uptakes and sequesters at a greater rate than it can excrete\(^24\).

Our examination revealed that *Entotheonella* sp. comprises 3.25% (s.e. = 0.0987) of the sponge volume (see calculation in Methods), and has a volume/weight ratio of 0.478. From this, we calculated that a 100 ml sponge would include 1.55 g of *Entotheonella* sp., containing 7,110 g of water-soluble arsenic (Supplementary Table 2). This is equivalent to the amount of arsenic found in 2,370 l of seawater (according to the recorded concentration of 3 µg l\(^{-1}\); ref. 18). At maximum pumping rate\(^25\), such a specimen can filter 288 l of seawater daily, reaching a maximum arsenic exposure of 864 µg. Thus, we propose that *Entotheonella* sp. acts as the accumulating and detoxifying entity in the sponge holobiont.

*Entotheonella* sp. mineralizes barium and arsenic. Biominer-alization is usually described as either biologically controlled mineralization (BCM) or biologically induced mineralization (BIM), although intermediate forms are known\(^26-28\). BIM generally occurs extracellularly when metabolic byproducts
react with chemicals in the environment. Minerals formed this way have poor crystallinity and are often unspecific. Intracellular BIM can occur in some bacteria, even up to the point of cell lysis\textsuperscript{29,30}, but this occurs mostly in environments enriched with metal ions. These ions are mainly associated with sulfide, and precipitation is considered to be a detoxification mechanism\textsuperscript{28}. BCM also exist in bacteria, such as magnetotactic bacteria, though is far less common than BIM. In BCM, minerals are deposited intracellularly on or within organic matrices or vesicles. The intravesicular conditions are controlled by the bacteria and are not affected by the environment. The minerals formed by BCM are well-ordered and specific.

A close look at Entotheonella sp. filaments from T. swinhoei shows that they contain multiple spherical granules inside their cells (Fig. 2a). The sphere volume is quite homogenous, averaging 0.11 ± 0.007 µm\textsuperscript{3} with a median volume of 0.08 µm\textsuperscript{3} (n = 121; Supplementary Fig. 1). Observation of thin sections of Entotheonella sp. under transmission electron microscopy (TEM) revealed that mineralization nucleates from the membrane of vesicles (Fig. 2b), thickening inwards (Fig. 2c).

To verify that the vesicle membranes are lipid-based, live Entotheonella sp. cells (n = 5) were stained with the fluorescent membrane stain, 3,3\textsuperscript{-}dihexyloxacarbocyanine iodide (DiOC\textsubscript{6}) (Fig. 3a). Focused ion beam (FIB) cross-sections of the bacterium (n = 1) were prepared for SEM-EDS analysis. High-resolution imaging of the cross-sections shows that the mineral ‘wall’ is highly porous (Fig. 3b). We hypothesize that the porous nature of the sphere wall may facilitate material exchange between the core of the vesicle and the cytoplasm, via the membrane. Analysis of the elemental composition of the mineral wall and the vesicle core was conducted (Fig. 3c). Barium was significantly higher in sphere wall than in the core (two-sample permutation test using Welch’s t-test, two-sided test, n = 3, t = −3.6213, P value < 2.2e−16). Arsenic however was similar between wall and core. The core contained more organic matter, as indicated by a substantially higher carbon presence (two-sample permutation test using Welch’s t-test, one-sided test, n = 3, t = 4.4772, P value = 0.0471). Nitrogen content in the core was also significantly elevated (two-sample permutation test using Welch’s t-test, two-sided test, n = 3, t = 3.8697, P value = 0.0474), which might indicate the potential presence of proteins.

To determine the biomineral phase composition in Entotheonella sp., we analysed spheres using high-resolution synchrotron X-ray powder diffraction (XRD) in freeze-dried samples (n = 5). We found that the crystalline fraction contains several minerals.

Barite was the major phase, with arsenates and phosphates probably minor phases (Fig. 4a). Crystalline barite presence within spheres was also confirmed by high-resolution TEM examination of FIB-prepared thin section (Fig. 4b). The fast Fourier transform of the TEM image obtained from a magnification close to an individual atomic lattice layer exhibits a single crystal electron diffraction from \([-2 0 0]\) zone axis of barite. Entotheonella sp. is thus the only known prokaryote capable of intracellular barite mineralization.

The detected barite was polycrystalline, and a precise structural determination of the minor phases could not be achieved due to their poor crystallinity and the high organic matter content present in the freeze-dried samples. Aiming to improve the quality of the crystals and to eliminate the organic phase, we further subjected the samples to mild thermal annealing (250 °C for 2h). The diffractogram collected after heating allowed us to detect crystalline calcium arsenate (Ca\textsubscript{3}(AsO\textsubscript{4})\textsubscript{2}) and calcium sulfide phosphate (Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}S) as minor phases (Fig. 4c).
Quantitative phase analysis performed using the Rietveld method enabled the precise quantification of each crystalline phase (weight fractions) comprising the investigated samples: BaSO\(_4\) accounted for 87% of the crystalline weight, Ca\(_3\)(AsO\(_4\))\(_2\) for 7% and Ca\(_{10}\)(PO\(_4\))\(_6\)S for 6%.

The presence of barite and calcium arsenate were further confirmed on unprocessed ‘intact’ flash-frozen Entotheonella sp. samples by synchrotron X-ray microprobe analyses (X-ray fluorescence mapping, X-ray absorption spectroscopy and XRD) performed at 95\(^\circ\)K. The distribution of arsenic in the filaments was evident by micro-focused X-ray fluorescence (\(\mu\)XRF) mapping (Fig. 5a and inset). All arsenic K edge X-ray absorption near edge structure (XANES) spectra of Entotheonella sp. showed As(V) as the dominant valence when compared to relevant standards (Fig. 5b). Generally, the white line increases in height, shifts towards higher energy and decreases in full-width at half-maximum as the oxidation state increases. Least-square linear combination fitting (LCF) of 39 filament spectra (Fig. 5b) to a large library of 64 arsenic reference compounds from the ALS BL 10.3.2 database was performed. Top best LCF fits using 1, 2 or 3 components consistently required non-sulfur As(III), calcium arsenates (mostly pharmacolite) and sodium arsenate and best fits were obtained using combinations of these three components. \(\mu\)XRD performed on some of the filaments’ XANES locations showed the presence of pharmacolite (Supplementary Fig. 3).

The three-component LCF performed on 39 filaments indicated an average (rounded to full digits) of 18% As(III), 32% pharmacolite and 50% sodium arsenate. However, a precise determination of compound proportion could not be achieved for several reasons: the identity of the non-sulfur As(III) compound in unknown, the spectral fine structure of sodium arsenate varies with pH\(^{13}\) and concentration\(^{34}\) and fine spectral features vary with the degree of crystallinity. Compound abundance depended on location, as further evidenced by As(III)/As(V) chemical mapping, which exhibited heterogeneous distribution (Fig. 5c). LCF of Ba L\(_3\) edge XANES data on the filaments (Fig. 6a) best matched barite\(^{35}\) whose presence was further confirmed by \(\mu\)XRD (Fig. 6b). These combined results provide strong evidence of intracellular mineralization of barium (in the form of barite) and intracellular crystalline arsenic.

To determine whether the crystalline phases of barium and arsenic could explain all the measured elemental concentrations, we compared the atomic ratios of barium, sulfur and arsenic. Water-soluble element concentration (barium: 541 \(\mu\)g g\(^{-1}\) cells; sulfur: 1,084 \(\mu\)g g\(^{-1}\) cells; arsenic: 4,577 \(\mu\)g g\(^{-1}\) cells) was subtracted from the total elemental concentration and normalized by atomic weight. For crystalline barite (BaSO\(_4\)) to explain all insoluble barium the Ba:S atomic ratio should be \(\leq 1\). While our measurements showed the ratio to be \(> 1\) (average = 2.14 ± 0.84, \(n = 5\)), this is not significantly different from a ratio of 1.
(paired T-test, $t = 1.4212$, d.f. = 4, $P$ value = 0.2283). Our analysis thus showed that the majority of insoluble barium can be explained by crystalline barite. This was further confirmed by Ba XANES and μXRD data (Fig. 6). Examination of crystalline arsenic is slightly more complicated. To do so, we calculated the weight ratio of crystalline barium and arsenic. Barium comprises 58.8% of barite molecular weight, and thus constitutes 51.2% of total crystalline weight. Arsenic comprises 37.7% of Ca$_3$(AsO$_4$)$_2$ molecular weight, and thus constitutes 2.6% of total crystalline weight. From the Ba:As ratio (20:1), we calculated that 1.290 µg g$^{-1}$ (or 17.2%) of the insoluble arsenic is crystalline. The poor crystallinity of arsenic and spatial variation of calcium arsenate and As(III) in the XANES fit prevents a clear identification of the amorphous arsenic solid. However, since the average ratio of calcium arsenate by XANES analysis is 32% ($\sim$ 3,850 µg g$^{-1}$ cells), we can say with confidence that some of the calcium arsenate is amorphous. The identity of the As(III) compound is unknown and may either be soluble or solid.

Altogether, the results indicate that Entotheonella sp. controls mineral formation within its cells to a relatively high degree. This phenomenon exhibits many of the characteristics of BCM, with the exception of the ability to mineralize finely ordered crystalline material. Thus, we conclude that Entotheonella sp. possesses an intermediate form of BCM, with some similarities to magnetotactic-like bacteria.

Bioavailability of arsenic in Entotheonella sp. We tested the presence of arsenate (as sodium arsenate) and a compound with As(III) using the XANES analysis (Fig. 5). Since most soluble arsenic species are extremely toxic, we identified and quantified them in the enriched cell fractions from the sponge (Table 1). We separated arsenic species using ion exchange chromatography and identified them against known standards (arsenite: As(V), arsenate: As(V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), arsenobetaine (AsB) and arsenocholine (AsC)). As(V) and MMA were detected in all arsenic species are extremely toxic, we identified and quantified them in the enriched cell fractions from the sponge (Table 1). We separated arsenic species using ion exchange chromatography and identified them against known standards (arsenite: As(V), arsenate: As(V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), arsenobetaine (AsB) and arsenocholine (AsC)). As(V) and MMA were detected in all arsenic species.

The dominant arsenic species within each fraction was tested by a permuted ANOVA, followed by a post hoc Tukey’s test. We found that the arsenic concentration, as hypothesized previously, was significantly higher than all other arsenic species in all cell fractions, except in $F_{\text{bac}}$ ($F_{\text{bac}}$: 892.4 ± 284.4 µg g$^{-1}$ cells, $F = 9.797$, $P$ value = 3.37e$^{-5}$, $F_{\text{ento}}$: 4,498 ± 983.4 µg g$^{-1}$ cells, $F = 20.7$, $P$ value = 5.2e$^{-8}$, $F_{\text{ec}}$: 4,301 ± 1,228 µg g$^{-1}$ cells, $F = 12.06$, $P$ value = 6.5e$^{-6}$, $F_{\text{bac}}$: 747 ± 485.7 µg g$^{-1}$ cells, $F = 2.48$, $P$ value = 0.0719).

Upon testing differences in arsenic concentration among fractions, we found that $F_{\text{ento}}$ had a significantly higher arsenic concentration than all other cell fractions (permuted ANOVA, followed by post hoc Tukey’s test, $F = 10.55$, $P$ value = 0.00227). Bacteria are reported to tolerate as much as 1 M of arsenate (or 75,000 µg g$^{-1}$ arsenic) by means of continuous excretion and maintenance of low intracellular concentrations. Our analysis indicates that Entotheonella sp. accumulates, rather than excretes, arsenate. Moreover, a statistical test (exact permutation test estimated by 999 Monte Carlo replications) showed that arsenate concentrations in Entotheonella sp. (normalized by atomic mass) are significantly higher than those of water-soluble sulfur (912 ± 205 µg g$^{-1}$ cells, $P$ value = 0.024, confidence interval (CI) = 0.00877, 0.04534) and phosphorous (641 ± 169 µg g$^{-1}$ cells, $P$ value = 0.01, CI = 0.00135, 0.02511), both of which are essential elements. Even with such high arsenate concentrations, Entotheonella sp. remains viable, as indicated by 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFDA/SE) vital staining (Supplementary Fig. 2). If arsenic is essential to the metabolism of Entotheonella sp., we can reasonably expect to detect high arsenate concentrations within the cell. If Entotheonella sp. does not utilize arsenic metabolically, it should then possess highly efficient detoxification mechanisms. Mineralization of arsenic is one such mechanism, but with 4,577 µg g$^{-1}$ water-soluble arsenate, other mechanisms must also exist. We hypothesize that the soluble arsenic is localized within the core of the vesicles since arsenate does not pass through lipid membranes.

Potential pathway for element accumulation in Entotheonella. To achieve mineralization, Entotheonella sp. needs to transport and concentrate the relevant elements at the site of mineralization. Entotheonella sp. possesses an outer sheath, which surrounds the entire filament, and two sets of membranes (Fig. 7a). The mineralized vesicles are only found in the inner
space. In the marine environment, both arsenate\(^3\) and barium ions\(^6\) are charged, so passive transport across an outer wall and two layers of lipid membranes is an unlikely explanation for the accumulation\(^39\). In bacteria, arsenate may enter cells via phosphate transporters\(^5\), and barium ions via K\(^+\) and Ca\(^{2+}\) channels\(^40\). Such unspecific transport mechanisms are also an unlikely accumulation path, since the aforementioned transporters favour their intended molecule. *Entotheonella* sp. may have yet undiscovered specific transporters for these ions. A third potential mechanism for ion transport and accumulation is pinocytosis of seawater\(^41\). Such a process is rare in bacteria, but there is some evidence of an endocytosis-like process in the genus *Gemmata*\(^42\).

Observation of *Entotheonella* sp. reveals that the space between the two membranes contains many small vesicles (Fig. 7b). Interestingly, some vesicles contain a single vesicle while others have a double membrane (Fig. 7b, green arrow). We hypothesize that these vesicles may be involved in the transport of arsenic and barium between the immediate surroundings and the inner volume of *Entotheonella* sp. Arsenic may be linked to the

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**Figure 4 | XRD and TEM analysis of spheres inside *Entotheonella* sp.** (a) Rietveld refinement plot of preheated freeze-dried *Entotheonella* sp. reveals the resulted fit for the observed (blue dots) and calculated (black line) diffraction patterns and the difference between them (red line). Black notches indicate the positions of the diffraction peaks of crystalline barite (BaSO\(_4\)), the major phase in the sample. Diffraction peaks highlighted by arrows in the difference curve are compatible with an arsenate or phosphate phase. These minor phases are not clearly visible in the observed diffraction pattern as they overlap with the diffraction peaks of barite. (b) HRTEM micrograph of a portion of the freeze-dried *Entotheonella* sp. Insert shows higher magnification image revealing the lattice, and with its Fast Fourier Transform (FFT). (c) Rietveld refinement plot of a freeze-dried *Entotheonella* sp. after thermal annealing, revealing minor phases of sodium chloride (NaCl), calcium arsenate (Ca\(_3\)(AsO\(_4\))\(_2\)) and calcium sulfide phosphate (Ca\(_{10}\)(PO\(_4\))\(_6\)S). Observed (Obs.), calculated (Calc.) and difference curves are presented in the same schematics as in a.
membranes themselves as As(III), while barium ions would likely be in the bulk liquid.

Another noteworthy aspect of the accumulation process is the merging of discrete mineralized spheres (Supplementary Fig. 4a). These spheres are discrete in the majority of cell units in Entotheonella sp. filaments, but the merging of some spheres was also observed, in some cases affecting all mineralized material ($n = 9$). Further, all cells in which full sphere merging occurred, seemed to have lost their inner membrane (Supplementary Fig. 4b).

**Discussion**

Here we describe a host–bacterium relationship established around the accumulation and detoxification of harmful trace elements. Previously, we concluded that arsenic and barium were coaccumulated, based on statistical analyses that showed grouping of the aforementioned elements\(^4\). Here we demonstrate that both elements are localized to a specific symbiotic bacterium, *Entotheonella* sp. The combined results of previous\(^4\) and present research provides evidence supporting a linkage between arsenic and barium cycles in *T. swinhoei*, driven largely by *Entotheonella* sp. In past measurements of arsenic in Japanese sponges, a *Theonella* sp. was found to have the highest arsenic concentration among analysed species, with 157 $\mu$g g\(^{-1}\) (only water-soluble arsenic was measured)\(^3\). While this is much lower than the concentration we found in Red Sea *T. swinhoei*, it is still relatively high for sponges. Interestingly, the concentration of AsB in the Japanese *Theonella* sp. equals the concentration of AsB in F\(_{EC}\). It is compelling to speculate that at some point in the *T. swinhoei* evolution and dispersion towards the Red Sea, *Entotheonella* sp. evolved in a way that significantly affected the arsenic cycle of the sponge.

Multicellular organisms can detoxify arsenic by accumulating and precipitating the element in designated excretion and detoxification tissues. *T. swinhoei* does not possess differentiated tissues but our results suggest an alternative detoxification mechanism. *Entotheonella* sp., residing within the sponge’s mesohyl, can accumulate ten times more water-soluble arsenic than the sponge’s daily exposure to soluble arsenic, which it then mineralizes. Therefore, we propose that *Entotheonella* sp. may function, to some extent, as a detoxifying organ for *T. swinhoei*. Excretion of *Entotheonella* sp. by the sponge is unlikely, as past measurements have shown very few bacterial cells in this sponge’s excurrent\(^25\).

Other microorganisms reported to accumulate arsenic have a much lower arsenic concentration\(^34\) or maintain a high concentration due to elevated arsenic in the environment\(^35\). Total arsenic concentration in *Entotheonella* sp. (12,000 $\mu$g g\(^{-1}\) cells) is among the highest measured in any known organism\(^3\) or surficial deposit\(^46\). Furthermore, we show that *Entotheonella* sp. can form intracellular crystalline arsenic, and mineralize barium intracellularly. The only other arsenic biogenic mineral so far described is otrimpem\(^38\), thus biominalerization of calcium arsenate by *Entotheonella* sp. adds to the ever growing list of biominerals. Another prokaryote (*Desulfothermococcus auripigmentum*) precipitates arsenic intracellularly\(^47\), but amorphously, localized near the cell membrane and eventually excreted. Our analyses indicate that *Entotheonella* sp. mineralizes arsenic and barium solely intracellularly, without excretion.

Still, some questions remain unanswered. The origin of the membranes on which the biominalerization occurs remains to be elucidated. Whether they result from invagination of the plasma membrane or constitute a separate synthesized membrane\(^48\) is unknown. The exact mechanism of element accumulation and transport to the site of mineralization is another subject for which future research may provide answers. However, it is clear that *Entotheonella* sp. possesses a unique bacterial system for element accumulation and mineralization. The formation of the biominerals might contribute to detoxification, but they could also play other, yet unknown functions.

**Methods**

**Sample collection.** We collected samples of *T. swinhoei* ($n = 12$) from the Red Sea (34°55’02”/29°60’05”), by SCUBA diving at 15–30 m depth.
Sponges were identified by visual and tactile characteristics underwater. In the lab we further inspected the inner tissue visually. Final identification was based on the presence of Entotheonella sp. and typical microdesma spicules under light microscope. We processed the samples immediately after collection at the Interuniversity Institute for Marine Sciences in Eilat, Israel. Work was performed under sterile conditions in a laminar flow hood. Before processing we thoroughly rinsed the sponges with sterile calcium-magnesium-free artificial seawater (CMF-ASW-NaCl 26.22 g, KCl 0.67 g, Na2SO4 4.62 g, NaHCO3 0.21 g, Na EDTA 0.37 g, ddH2O 1 L set to pH 8) to wash off epibionts and transient bacteria.

Methods for cell separation and subsequent quantitative analysis, XRD, staining, X-ray microprobe analysis and cryogenic scanning electron microscopy (cryo-SEM) are described below.

Figure 6 | X-ray microprobe analysis of barium in Entotheonella sp. at 95 °K. (a) Ba L₃ edge XANES on a filament (spot3) along with LCF fit (dotted line) to 100% barite (residuals in dashed line, norm. sum-sq = 3.54e⁻⁴). (b) Indexing of the XRD pattern recorded at 17 keV at that location confirms the presence of crystalline barite. I- peaks from Ice crystals; Cu- peaks from copper grid.
SEM-EDS sample preparation and analysis. We cut small pieces of sponge samples for energy dispersive X-ray spectrometry with SEM (SEM-EDS) using sterile scalpel blades. We included the cyanobacteria-rich outer layer and the dense endoderm. We fixed the samples in 2.5% glutaraldehyde (in CMF-ASW) and kept them in the dark at 4 °C until used. Fixed sponge samples were dehydrated by an ethanol gradient series (50% EtOH for 30 min, 70% EtOH 8 h, 100% EtOH 30 min), followed by critical point drying and coating with carbon. Imaging and elemental analysis were performed using a High-Resolution SEM (HR-SEM: ULTRA Plus; Zeiss, Oberkochen, Germany) equipped with INCA (Oxford Instruments, England) EDS. SEM images were captured at different acceleration voltages using an Everhart Thornley secondary electron detector, enabling investigation of the surface topography and an energy selective backscatter detector. 

### Table 1: Identification and quantification (μg g⁻¹ ± s.e.) of soluble arsenic species in cell fractions.

| As species | F_SC | F_ENTO | F_EC | F_BAC |
|------------|------|--------|------|-------|
| As(III)    | 0 (± 0) | 0 (± 0) | 7.9 (± 2.8) | 0.6 (± 1.3) |
| As(V)      | 892 (± 284) | 4,498 (± 983) | 4,301 (± 1,228) | 747 (± 549) |
| MMA        | 9.5 (± 3.4) | 78.9 (± 14.1) | 42.6 (± 11.3) | 9.8 (± 5.1) |
| DMA        | 0.2 (± 0.2) | 0 (± 0) | 2.4 (± 1.7) | 0.8 (± 0.8) |
| AsB        | 1.4 (± 0.4) | 0 (± 0) | 136 (± 12) | 2.6 (± 5.3) |

ANOVA, analysis of variance; AsB, arsenobetaine; As(III), arsenite; As(V), arsenate; DMA, dimethylarsinic acid; F_SC, bacteria-enriched fraction; F_ENTO, Entotheonella sp.-enriched fraction; F_EC, sponge cell-enriched fraction; F_BAC, Entotheonella sp. and Cyanobacteria-enriched fraction; F_SC, F_ENTO, F_EC were excluded from among permuted ANOVA analysis, failing the independence assumption. F_EC was tested only for within difference in As species. 

Denotes significant difference for arsenic species tested within a given cell fraction. Testing was conducted by permuted ANOVA test and Tukey’s HSD post hoc grouping. 

**Figure 7**: Membrane system of Entotheonella sp. (a) Cryo-SEM micrograph of freeze-fractured Entotheonella sp. filament. Red arrows mark the filament sheath. Yellow arrows mark the outer membrane. White arrows mark the internal membrane harbouring the mineralized spheres. Scale bar, 1 μm. (b) TEM micrograph of thin sections of Entotheonella sp. showing vesicles in the space between the two membranes. Yellow arrow marks the outer membrane. White arrow marks the internal membrane. Black arrow marks a single membrane vesicle. Green arrow marks a double membrane vesicle. Scale bar, 500 nm. 

from the area observed and a depth of 2 μm (the diameter of Entotheonella sp. filaments). The surface area of Entotheonella sp. in a given image was measured using the ICY software and the volume, assuming a cylinder shape. To compute the weight-to-volume ratio of Entotheonella sp., we collected a 0.25 ml bacterial pellet by centrifugation and measured its weight after lyophilization.

Quantitative elements analysis. Form each cell fraction, we aliquoted a 600 mg dry weight sample and sent it to Brooks Applied Labs (WA, USA) for elemental...
Total elemental extraction for analysis. Elements were extracted by nitric acid (HNO₃) digestion, following the EPA method 3050B with 50–100 mg (dry weight) of the cell fraction. Samples were placed in polypropylene centrifuge tubes and heated in concentrated HNO₃ to 95 °C for 15 min on a hot-block apparatus. Once cooled, additional HNO₃ was added and the samples were reheated to 95 °C for 30 min, during which the formation of brown fumes was observed. This step was repeated until no fume formation was detected, and ended with 2 h incubation at 95 °C. Once acid digestion was completed, H₂O₂ (30%) was added to the solution in aliquots and gassing was monitored until it subsided to a minimum, or sample appearance remained unchanged. An additional 2 h of incubation was allowed to reduce solution volume before it was cooled to room temperature. Before analysis, samples were diluted in ddH₂O and filtered to remove any particulate matter.

Water-soluble arsenic forms. Aliquots of each water-methanol extract were injected onto an anion exchange column and mobilized by an alkaline (pH > 7) gradient, following a described protocol52. Retention time for each detected arsenic species in the samples was compared to those of known standards for species identification: (1) arsenate (As(V)); (2) arsenite (As(III)); (3) monomethylarsonic acid (MMA); (4) dimethylarsinic acid (DMA); (5) AsB; (6) arsonochelate (AsC). All other arsenic species identified were defined as unknown arsenic species. 

Separation and detection of arsenic species for analysis. Aliquots of each water-methanol extract were injected onto an anion exchange column and mobilized by an alkaline (pH > 7) gradient, following a described protocol52.

Quantitative analysis of elements and element species. Quantification concentration was done using ICP-MS. All samples analyses were preceded by a digestion reacted solution volume before it was cooled to room temperature. Before analysis, samples were diluted in ddH₂O and filtered to remove any particulate matter. The analysis was performed using the GSAS-II software53.

X-ray microprobe. Entotheonella sp. samples for hard X-ray microprobe analyses under cryogenic conditions were flash frozen onto carbon-coated copper 200-mesh grids at the EM unit (Weizmann Institute of Science, Israel)54. Grids were glow discharged for 45 s before application of sample. Cells in CMF-ASW were resuspended until reaching homogenous suspension and then 5 µl were placed on the grid. Grids were blotted (5 s) and plunged into liquid ethane using a Leica EM-CPG automated plunger (Leica Microsystems) and stored in liquid nitrogen until measurements. Arsenic and barium bearing standard compounds were either mounted on carbon-coated copper 200-mesh grids TEM Cu grids or on SiN₃ windows (SiM pore Inc.). Three Entotheonella sp. sample grids with a minimum of two regions per grid were analysed. μXRF mapping, μXRD and X-ray absorption spectroscopy (XAS) measurements were conducted at ALS bending magnet beamline 23.3.5 (XRF) with the storage ring operating at 504 nm (50 K). All data were recorded at 95 °K using a custom cryogenic setup (Instec Inc.) that allows for the transfer of frozen samples following procedures described in details elsewhere55. Radiation damage was not observed under these conditions. μXRF elemental distribution maps and μXRF spectra on each pixel were collected at 12.3 K. False elemental maps at 15 K also showed 15 K. For each μXRF spectrum, the relative K-edge to better detect Ca, P and S. Beam spot size was 3 × 3 µm² with pixel size 1 × 1 µm² or 2 × 2 µm² and counting times 80–120 ms per pixel. Fluorescence emission counts were recorded using a 7-element Ge solid-state detector (Canberra) and XIA electronics. Arsenic chemical maps were taken in multienergy per line mode, at 11.830 (pre-edge background), 11.868 (As(V) sulfides) and 11.896 (As(III) sulfides), 11.871 (arsenate), 11.895 (arsenate) and 11.979 eV (postedge, total As). These emissions were chosen to investigate the distribution of As(III), As(V) and sulfides, but do not allow to distinguish sodium arsenite from calcium arsenate. Chemical maps shown were deadtime corrected and fitted using As(OH)₃ as a proxy for As(OH)₃ and phosphomolybdate as a proxy for As(OH)₄.

X-ray microprobe. Entotheonella sp. samples for hard X-ray microprobe analyses under cryogenic conditions were flash frozen onto carbon-coated copper 200-mesh grids at the EM unit (Weizmann Institute of Science, Israel)54. Grids were glow discharged for 45 s before application of sample. Cells in CMF-ASW were resuspended until reaching homogenous suspension and then 5 µl were placed on the grid. Grids were blotted (5 s) and plunged into liquid ethane using a Leica EM-CPG automated plunger (Leica Microsystems) and stored in liquid nitrogen until measurements. Arsenic and barium bearing standard compounds were either mounted on carbon-coated copper 200-mesh grids TEM Cu grids or on SiN₃ windows (SiM pore Inc.). Three Entotheonella sp. sample grids with a minimum of two regions per grid were analysed. μXRF mapping, μXRD and X-ray absorption spectroscopy (XAS) measurements were conducted at ALS bending magnet beamline 23.3.5 (XRF) with the storage ring operating at 504 nm (50 K). All data were recorded at 95 °K using a custom cryogenic setup (Instec Inc.) that allows for the transfer of frozen samples following procedures described in details elsewhere55. Radiation damage was not observed under these conditions. μXRF elemental distribution maps and μXRF spectra on each pixel were collected at 12.3 K. False elemental maps at 15 K also showed 15 K. For each μXRF spectrum, the relative K-edge to better detect Ca, P and S. Beam spot size was 3 × 3 µm² with pixel size 1 × 1 µm² or 2 × 2 µm² and counting times 80–120 ms per pixel. Fluorescence emission counts were recorded using a 7-element Ge solid-state detector (Canberra) and XIA electronics. Arsenic chemical maps were taken in multienergy per line mode, at 11.830 (pre-edge background), 11.868 (As(V) sulfides) and 11.896 (As(III) sulfides), 11.871 (arsenate), 11.895 (arsenate) and 11.979 eV (postedge, total As). These emissions were chosen to investigate the distribution of As(III), As(V) and sulfides, but do not allow to distinguish sodium arsenite from calcium arsenate. Chemical maps shown were deadtime corrected and fitted using As(OH)₃ as a proxy for As(OH)₃ and phosphomolybdate as a proxy for As(OH)₄.

Arsenic K edge μXAS spectra were recorded in fluorescence mode by continuously scanning the Si (1 1 1) monochromator (Quick XAS mode) from 11,774 to 12,580 eV, with 0.5 eV steps in the XANES region. Spectra were calibrated using the white line of a Na₂HAsO₄ powder standard at 11,875 eV, recorded at room temperature in transmission mode. All data were processed using the LabVIEW custom BL 10.3.2 software and standard procedures described elsewhere56. All reference and sample XANES spectra were carefully postedge normalized up to 12,178 eV. Least-squares linear combination fitting of the XANES spectra56 was performed using a large spectral database of arsenic compounds (64 compounds) from ALS beamline 10.3.2 and procedures described elsewhere56.

Arsenic standards were used: sodium arsenite at pH 8.2 (aqueous 1 M, Na₂HAsO₄; Sigma; CAS no. 10048-95-0), calcium arsenate (powder, As₂Ca₃O₈; Alpha Chemicals; CAS no. 7778-44-1), phosphomolybdate (crushed mineral, CaH₂AsO₄·2(H₂O), provided by the Mineral Collection of the Earth and Planetary Science Department, UC Berkeley, and barium arsenate (powder, Ba₃As₂O₈·H₂O, provided by the lab and made from a BaCl₂ solution (Sigma; 0.1 M; CAS no. 10361-37-2) and sodium arsenite (Sigma). As(V) was dissolved in ddH₂O to a concentration of 0.1 M. Equal volumes of the solutions were mixed and precipitation followed. The precipitate was collected by centrifugation and freeze dried. Barium and sodium arsenite were recorded at 95 °K in fluorescence mode; phosphomolybdate and calcium arsenate were recorded at 95 °K in fluorescence mode. As(OH)₃ was recorded at room temperature in transmission mode.
Microdiffraction patterns were collected in transmission mode with a CCD camera (Bruker APEX2) at 17kV (Δ = 0.729 Å) using a beam spot size of 12 × 6 μm² and exposure time of 240 s. Calibration of the camera distance was obtained using an alumina (α-Al₂O₃) powder standard and Fit2D software. Fit2D was also used to obtain one-dimensional XRD profiles from the radial integration of 2D patterns. These data were then indexed using Jade 9 software (Materials Data Inc.) and the ICDD PDF-4+ and MIRCURYST crystallographic databases.

**Vitality staining of Entotheonella sp.** We stained Entotheonella sp. filaments with CFDA-SE® to assess their viability. We pelleted cells from CMF-ASW and resuspended them in PBS, adding CFDA-SE (50 mM in dimethylsulphoxide) to a final concentration of 100 μM. We followed this by stirring the suspended cells and cycling the temperature between 25°C and 37°C (15 min each) for 2.5 h. After temperature treatment we harvested the cells by centrifugation, washed and incubated them in medium with 5 μM Al₂O₃ (1 g 1⁻¹ yeast extract in ASW, pH = 8.2) at 15°C with shaking for 48 h. Following incubation, we again harvested cells by centrifugation, washed them in PBS and mounted them on glass slides in 70% glycerol CMF-ASW. We observed the cells with a confocal microscope (Zeiss) for fluorescence at an excitation wavelength of 492 nm and emission of 517 nm. The fluorescence image was overlaid on a differential interference contrast (DIC) image to assist localization of the stain in the filaments.

**Cryo-SEM of Entotheonella sp.** Sample preparation and observation was conducted at the Electron Microscopy Unit at the Weizmann Institute of Science. We transferred a live sample of Entotheonella sp. in CMF-ASW to the EM unit within 24 h of sampling. There we preserved the sample by high-pressure freezing (HPM 10; BAL-TEC, USA). Before observation, we fractured the samples in a freeze fracture device (BAF 60; BAL-TEC, USA). We observed Entotheonella sp. in an Ultra 55 fully digital field emission scanning electron microscope (Zeiss) with a Leica cryo stage (AG, Liechtenstein, Germany), exposing the filaments by light heat milling.

**Statistical analysis.** Sample size for quantitative analysis (total element concentration and arsenic species) was decided after a preliminary test with minimal sample size (n = 3). Following the analysis, separation methods were further optimized so the original samples were not included analysis. Statistical analysis was performed using R statistics with RStudio IDE (RStudio, USA). Significant values for all tests were regarded for a post hoc comparison.

**Data availability.** The authors declare that all relevant data supporting the findings of this study are available within the article and its Supplementary Information Files, or from the corresponding author on request.

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57. Two-dimensional detector software: from real detector to idealised image or reproducible research.

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61. Author contributions M.I. supervised the study. R.K., B.M. and M.I. designed the study with inputs from all authors. R.K., A.L. and M.I. collected and processed samples for experiments. R.K., B.M. and A.L. analysed the ICP-MS and ICP-ICP-MS data. A.L. and R.K. ran statistical tests. I.P., R.K. and B.P. analysed SEM-EDS data. I.P., B.P. and A.L. performed TEM experiments, with contributions from R.K. R.K. and A.L. performed cryo-SEM and confocal microscopy analysis. D.L. and B.P. performed XRD experiments and data analysis. S.F. performed X-ray microprobe experiments and data analysis. R.K. and B.M. wrote the manuscript. All authors commented on and edited the manuscript.

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