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INVERTEBRATE MICROBIOLOGY

The Bacterial Community Associated with the Marine Polychaete *Ophelina* sp.1 (Annelida: Opheliidae) Is Altered by Copper and Zinc Contamination in Sediments

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Abstract Tolerant species of polychaete worms can survive in polluted environments using various resistance mechanisms. One aspect of resistance not often studied in polychaetes is their association with symbiotic bacteria, some of which have resistance to metals and may help the organism to survive. We used "next generation" 454 sequencing of bacterial 16S rRNA sequences associated with polychaetes from a copper- and zinc-polluted harbor and from a reference site to determine bacterial community structure. We found changes in the bacteria at the polluted site, including increases in the abundance of bacteria from the order Alteromonadales. These changes in the bacteria associated with polychaetes may be relatively easy to detect and could be a useful indicator of metal pollution.

Introduction

Polychaete worms are good indicators of anthropogenic disturbance [15] because they exhibit a wide range of responses [50, 66]. Genomic and proteomic analyses are being used to understand mechanisms of tolerance [57, 79]. While there has also been some suggestion that mucous secretion by polychaetes confers resistance [42, 70], not much is known about other forms of resistance. One aspect of resistance not often studied in polychaetes is their symbiotic bacteria. Certain species of symbiotic bacteria coexisting with other marine invertebrates in polluted environments are metal-resistant and may help the host to survive [65, 78].

Hydrothermal vents are naturally high in toxic compounds, including heavy metals [10, 62] yet marine communities thrive, often because of their association with bacteria [18, 37]. Polychaetes associated with hydrothermal vents have evolved relationships with bacteria that provide the worms with both energy and protection against metal toxicity [1, 45]. The energy requirements of the giant tube worm, *Riftia pachyptila*, are derived entirely from chemolithoautotrophic bacteria living in specialized organs within the worm [8, 20, 58]. The bacteria present on the polychaete epidermis are thought to confer resistance to the worms [1, 14, 16, 76]. Bacteria isolated from the epidermis of hydrothermal vent polychaetes are resistant to cadmium, zinc, arsenate, and silver and are tolerant to high copper levels [31]. Although the time for adaptation is much shorter, polychaetes in anthropogenically polluted environments may also be able to form relationships with metal-resistant bacteria.

Studies of bacteria associated with polychaetes typically rely on culturing [31]. These studies provide valuable information but are potentially compromised by missing the large number of uncultivable bacteria. Cloning and "Sanger sequencing" of the 16S rRNA gene provides a greater understanding of uncultivable bacterial diversity [24, 27], but this technique is also limited to identification of only the most predominant bacteria in a sample. Next generation sequencing techniques, such as pyrosequencing, allow for

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extensive sequencing of bacterial communities and may resolve some of the problems previously associated with determining bacterial diversity [69]. This technique has been successfully applied to the investigation of free-living bacterial populations in the open ocean [51] and deep-sea hydrothermal vents [5], as well as to bacteria associated with marine organisms [34, 71]. We are, however, not aware of pyrosequencing studies on the bacteria associated with polychaetes.

We predicted that the composition of the bacterial community associated with polychaetes from metal-impacted sites would differ to that of polychaetes at non-polluted reference sites. We aimed to determine whether changes in the composition of the community involved metal-resistant bacteria and whether the bacterial assemblage associated with polychaetes could be used as an indicator of metal pollution.

Materials and Methods

Study Sites

Cullen Bay Marina, hereafter referred to as Cullen Bay, is a constructed lock in Darwin Harbour, Australia (Fig. 1). It has been subjected to metal contamination from intensive recreational use and also as the result of a pest eradication program. During the wet season of 1998–1999, the Black Striped Mussel (*Mytilopsis sallei*) was found to have invaded Cullen Bay and steps were taken to ensure it did not become established [21]. Sodium hypochlorite and copper sulfate were added to Cullen Bay over several weeks to sterilize the water, eradicate the mussel and ensure it did not spread to other parts of northern Australia [21]. Cullen Bay still contains high levels of metals, especially copper [44], and is a useful site to test for the effects of metals on the bacteria associated with polychaetes. A reference site was chosen at Dinah Beach on the eastern side of Darwin. The Dinah Beach site was located on the subtidal area of a mudflat in front of extensive mangrove forests and was open to the waters of Darwin Harbour. Although this reference site was close to the city, previous chemical studies have shown Darwin Harbour to be relatively unpolluted [43].

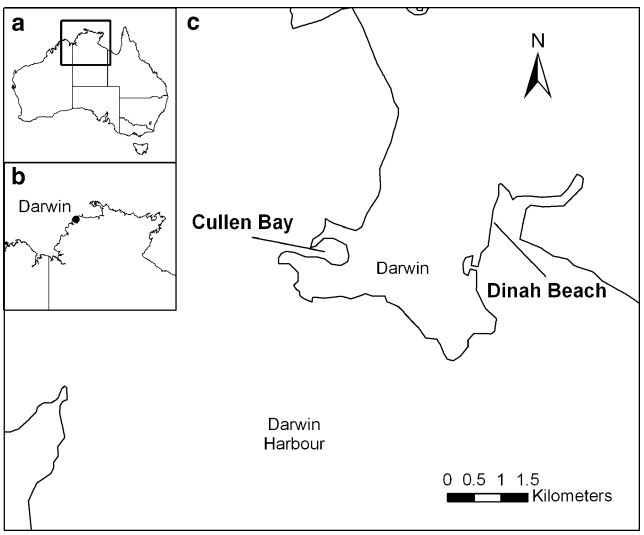

Figure 1 Map of the study sites, Darwin, Australia

Cullen Bay has been recolonised by many organisms since the copper treatment, including opheliid polychaetes [59]. Opheliids are subsurface burrowers in sandy or muddy sediments [64] and are generally considered to be non-selective deposit feeders [19]. These polychaetes are likely to be exposed to metals as they indiscriminately ingest sediment and they are constantly in contact with the sediment, which is primarily where metals accumulate [67]. *Ophelina* sp.1 was chosen as an appropriate polychaete to test for changes in its associated bacterial community in the presence of elevated metal levels.

Chemical Analysis

Cullen Bay was sampled on 14 April 2010 and Dinah Beach on 22 April 2010. Three replicate 2-L sediment samples were collected from both sites using a Van Veen grab, placed in plastic zip-lock bags and transported back to the laboratory on ice. The sediment samples were separated into two grain size fractions (>63 and ≤63 μm) using a 63-μm sieve. The concentrations of Fe, Mn, Co, Ni, Cu, Zn, As, Cd, Pb, and U were analyzed from the fraction ≤63 μm. The elements were analyzed after a concentrated nitric and perchloric acid digestion at 100°C for 30 min, 130°C for 30 min, and 200°C for 30 min, by inductively coupled plasma—mass spectrometry (ICP-MS, Agilent 7500 ce).

The concentration of total organic carbon was also analyzed from the ≤63-μm sediment fractions. The sediments were first reacted with concentrated hydrochloric acid to remove inorganic carbonates, then combusted in a LECO furnace at 1,400°C in the presence of strongly oxidizing iron/tungsten chips. The evolved carbon was then measured using infrared detection.

Sample Collection and Treatment Types

The polychaete *Ophelina* sp.1 was collected from Cullen Bay and Dinah Beach at the same time as the chemistry samples, also using a Van Veen grab. The sediment was sorted by hand and individuals of *Ophelina* sp.1 were

removed and divided into two groups. In the first group, the specimens were washed in sterile seawater to remove all traces of sediment. This "washed polychaete" treatment was expected to remove bacteria that were not closely associated with the polychaete epidermis, leaving only tightly associated epidermal bacteria or bacteria in the gut. In the second group, the specimens were left covered in sediment in the expectation that this would include loosely associated bacteria. At the same time, an additional sediment sample was collected to examine the sediment bacterial community at both the sites. In summary, the three treatment types analyzed at each site were washed polychaetes, unwashed polychaetes and sediment samples. The polychaetes and sediments were transported to the laboratory at ambient temperature and the DNA was extracted immediately.

DNA Extraction and Pyrosequencing

The bacterial DNA from both the washed and unwashed polychaetes was extracted using the MoBio UltraClean Microbial DNA Isolation Kit (Geneworks, SA, Australia), according to the manufacturers instructions, except whole polychaetes (approximately 20 mm in length) were added directly to the MicroBead tube with the MicroBead solution. The Bacterial DNA from 10 g of each of the sediments was extracted using the MoBio PowerMax Soil DNA Isolation kit, Mega Preps (Geneworks, SA, Australia), according to the manufacturer's instructions.

The bacterial 16S rRNA hypervariable V6-region was amplified from the polychaete and sediment samples using the A-967F forward primer: CAACGCGAAGAACCTTACC and the B-1046R reverse primer: CGACAGCCATGCANCACCT [69]. Each site and treatment type were amplified with a unique barcode attached to the primer to ensure the samples could be distinguished. PCR reactions were compiled using the Roche FastStart High Fidelity PCR System, dNTPack (Roche Diagnostics, NSW, Australia). Each PCR reaction (four replicates per treatment) comprised 1-μl template DNA, 5 μl of 10× buffer with 18 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 10 μl of 4 μM forward and reverse primers, 0.5 μl of FastStart High Fidelity Enzyme Blend, and 32.5 μl of dH_2O for a total volume of 50 μl. After denaturation (92°C for 2 min), PCR products were amplified for 30 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 1 min, then a final extension of 72°C for 10 min. The four replicate PCR reactions were combined to create a single bacterial library for each treatment type. The pooled PCR products were then purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR products were quantified after separation on a 2% agarose gel and comparison to a Low Mass Ladder (Invitrogen, CA, USA). The purified, quantified amplicons were sequenced using a Roche GS FLX (454) sequencer at the Australian Genome Research Facility in Brisbane, QLD, Australia.

Data Analysis

Each read from the sequencer was analyzed for quality using the Ribosomal Database Project's initial pipeline process (www.pyro.cme.msu.edu). Sequences were removed when they were <70 nucleotides in length, contained one or more Ns or if they returned a quality score <20. The trimmed sequences were then analyzed using Mothur [63]. The sequences were aligned using *align.seqs* with the SILVA database [53]. Sequences that didn't align to the V6 region were removed using *screen.seqs* and common gaps were removed using *filter.seqs*. Chimeras were removed from the alignment using *chimera.slayer* and the SILVA database as a template [53]. Sequences were clustered into operational taxonomic units (OTUs) using *dist.seqs* and the average method in *cluster* with a 3% distance cut-off criterion. OTUs were classified using *classify.seqs* at the 0.03 level using the SILVA database [53]. The sequences of interesting OTUs with ambiguous classification were further analyzed using the Ribosomal Database Project's classifier (www.rdp.cme.msu.edu/classifier) and the SINA alignment service in SILVA (www.arb-silva.de/aligner/). Sequences with a consensus classification using all three methods were assigned to that classification. Rarefaction curves and the Venn diagrams were calculated at the 0.03 level using *rarefaction.single* and *venn* in Mothur [63]. To calculate the Chao1 richness estimate and Shannon Index, the samples were first normalized down to the sample with the fewest sequences using *normalize.shared*, then calculated using *summary.single* in Mothur [63]. The reported abundances for each sample are all relative abundances obtained by dividing the number of sequences for the OTU by the total number of sequences. The multi-dimensional scaling plot (MDS) and the similarity percentages (SIMPER) table were calculated according to Clarke and Warwick [13] using PRIMER (Plymouth Routines in Multivariate Ecological Research, version 6). The MDS and PRIMER plots were calculated on standardized, square-root transformed sequence data so that samples with different sequence counts could be compared, and the influence of highly abundant OTUs was reduced [13]

Data Availability

All V6 sequence data are available in the NCBI Sequence Read Archive under the accession number SRP007196.

Results

Chemical Analysis of the Sediments

Cullen Bay sediments contained approximately 8 times more copper and 3 times more zinc than Dinah Beach sediments (Table 1). The copper and zinc concentrations at Cullen Bay were above interim sediment quality guideline low (ISQG low) values and have the potential to cause adverse biological effects [3]. The copper and zinc concentrations at Dinah Beach were below the ISQG low values, indicating that this site was an appropriate reference site. The concentration of nickel was also higher than the ISQG low value at both the sites, although this concentration is similar to natural background levels for northern Australia [43].

Bacterial Community Structure

Of 104,653 total bacterial V6 amplicons sequenced, 84,925 reads passed the quality tests and clustered into 10,595 unique OTUs at a 3% distance threshold (Table 2). The sediment samples contained the highest number of OTUs, followed by the unwashed polychaetes, then the washed polychaetes (Table 2). In addition, the sediment samples were the most diverse and were estimated to have the highest number of species (Table 2). Our study captured approximately 50% of the bacterial diversity in the polychaete treatments and sediment samples, as shown by richness estimates and rarefaction curves (Table 2; Fig. 2). The treatment types contained a similar number of OTUs at both Cullen Bay and Dinah Beach.

Many of the OTUs were unique to a specific site and treatment type (Fig. 3). The washed polychaete treatment and the surrounding sediment had few OTUs in common, indicating that the sediment was effectively removed from the polychaetes by the washing method. In addition, many OTUs associated with the unwashed polychaete treatment were not found in either the sediments or on washed polychaetes, suggesting that the washing step removed a cohort of potentially symbiotic bacteria. OTUs that were unique to washed polychaetes were likely to be closely associated with the polychaetes, including symbiotic bacteria and gut fauna. Many of these 'close associates' were members of the phylum Verrucomicrobia, which accounted for three of the top 10 most abundant polychaete OTUs (Appendix).

At the phylum level, the vast majority of OTUs were assigned to the Proteobacteria. This included over 70% of sediment bacteria sequences, over 80% of unwashed polychaete sequences and over 90% of the washed polychaete sequences. At the class level, at least 30% of the sequences in each sample were classified as Gammaproteobacteria. This number increased in the polychaete treatments, while the sediments contained higher abundances of Deltaproteobacteria and more of the sequences were unclassified.

Over 30% of the total number of sequences were assigned to the top 10 most abundant OTUs and this number increased to 58% for the top 100 most abundant OTUs and 79% for the top 1,000 most abundant OTUs (Fig. 4). This indicates that most of the diversity can be attributed to rare species. The top 20 most abundant OTUs across all samples comprised a diverse range of bacteria which showed marked differences in abundances across the treatment types (Table 3). The bacteria associated with the unwashed polychaete in Cullen Bay were dominated by a bacterium of the order Alteromonadales, which was much more abundant on Cullen Bay polychaetes than Dinah Beach polychaetes and was completely absent from the sediments. Another member of the Alteromonadale—Idiomarinaceae—was also more abundant at Cullen Bay. A bacterium in the order Oceanospirillales and sulfur-oxidizing bacteria were more prevalent on the polychaetes at Dinah Beach compared with those at Cullen Bay. In general, the composition of the bacteria in the sediments at both sites was similar.

The abundance and diversity of OTUs from the three treatments and the two sites were compared using multivariate ordinations (Fig. 5). The washed polychaete treatment from both sites and the sediment samples from both sites contained similar bacterial communities. There were, however, differences between the sites in the bacteria associated with unwashed polychaetes. The main contrib-

Table 1 Metal concentrations and total organic carbon (TOC) content at the two study sites, Cullen Bay and Dinah Beach, and ISQG low trigger values for sediment quality [3]

	Fe	Mn	Co	Ni	Cu	Zn	As	Cd	Pb	U	TOC (%)
Cullen Bay	33,200±1,110	278±2	10.7±0.1	22.6±0.1	174±8.6	293±9.6	19.5±0.9	0.11±0.01	21.7±1.1	2.52±0.19	0.94±0.02
Dinah Beach	45,000±3,320	204±7.8	8.9±0.2	22.4±1	21.5±0.7	93.8±7.3	18.6±1.3	0.12±0.02	23.4±1.2	2.06±0.04	1.10±0.07
ISQG low trigger values	N/A	N/A	N/A	21	65	200	20	1.5	50	N/A	N/A

Concentrations are given in mg/kg (dry weight) for the metals and % (dry weight) for TOC. The standard error is included

Table 2 Sequencing results, diversity and richness estimate for the bacteria associated with washed and unwashed polychaetes and for bacteria in the sediment

Site Treatment	Cullen Bay Washed	Dinah Beach Washed	Cullen Bay Unwashed	Dinah Beach Unwashed	Cullen Bay Sediment	Dinah Beach Sediment
Total number of sequences	16,280	11,617	18,515	20,689	15,761	21,791
Number of sequences passed quality tests	13,464	9,532	15,377	16,311	12,505	17,736
Total OTUs	776	416	2,716	2,667	3,605	4,714
Shannon Index	0.61	0.51	0.81	0.82	0.89	0.83
Chao1 richness estimator	1,227	803	4,572	4,460	7,239	7,671

OTUs, Shannon Index, and Chao1 were calculated at 3% difference

utors to these differences were again bacteria of the order Alteromonadales, including Idiomarinaceae, which were more abundant at Cullen Bay (Table 4). The other important contributors were more abundant at Dinah Beach, and included sulfur-oxidizers and bacteria in the order Rhodospirillales. The metals that were best correlated with the bacterial changes associated with Cullen Bay unwashed polychaetes were zinc and copper; arsenic changes were correlated with differences in the bacteria associated with Dinah Beach worms (Fig. 5).

Discussion

The structure of the bacterial community associated with the polychaete *Ophelina* sp.1 in Cullen Bay differed from that at the reference site at Dinah Beach. Although the bacterial communities at both sites had similar diversity indices, there was a change in community membership in the bacteria associated with *Ophelina* sp.1 at Cullen Bay. This change coincided with higher concentrations of copper and zinc in Cullen Bay sediments compared with Dinah Beach sediments. *Ophelina* sp.1 is a subsurface deposit-feeding polychaete [19, 64] and is, therefore, likely to make contact with these metals. Copper concentrations were high in Cullen Bay due to a pest eradication program using copper sulfate in 1999 [21] and zinc concentrations were also high, probably due to commercial and recreational boating activity [4, 68]. Since the copper and zinc concentrations in Cullen Bay exceeded ISQG low sediment quality guidelines [3], any measureable effects on the polychaete *Ophelina* sp.1 and its associated bacteria compared with the reference site, can be considered within this context.

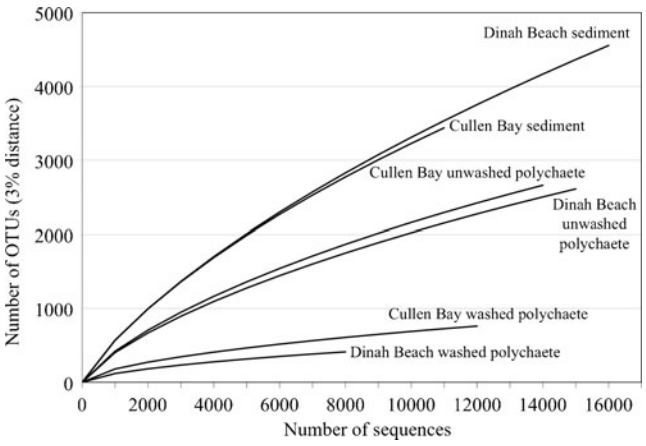

Figure 2 Rarefaction curves for bacterial V6 sequences for both sites and each of the three treatment types. OTUs were calculated by clustering sequences with a 3% distance threshold

A large difference in the bacteria loosely associated with *Ophelina* sp.1 at Cullen Bay and at the reference site was that bacteria of the order Alteromonadales were more abundant on Cullen Bay polychaetes. Members of this order have also been found to increase in abundance at sites affected by urbanization and eutrophication [39, 80] and some species are metal-resistant [12, 30, 65]. Furthermore, Alteromonadales are associated with deep-sea polychaetes and show tolerance to high copper levels [31]. Certain species of Alteromonadales isolated from deep-sea polychaetes produce an exopolysaccharide (EPS) which has metal-binding properties [76]. The EPS produced by some Alteromonadales bacteria is polyanionic, meaning it binds cations including Cu^{2+} and Zn^{2+} [26, 46, 54]. The Alteromonadales bacteria associated with *Ophelina* sp.1 in Cullen Bay may bind metals and reduce their toxicity, which could explain their abundance in this environment.

Another bacteria of the order Alteromonadales, which was classified to Idiomarinaceae, was also more abundant on Cullen Bay polychaetes. This bacterium was particularly abundant on washed Cullen Bay polychaetes, suggesting it has a close association with the polychaete epidermis or forms part of the polychaete's gut fauna. Some species within the Idiomarinaceae are metal-resistant [11], and are often in greater abundance at polluted sites [28, 55], and some also produce an anionic EPS that has a significant affinity for metals [41]. In addition, the *Idiomarina loihiensis* (Idiomarinaceae) genome sequence has been

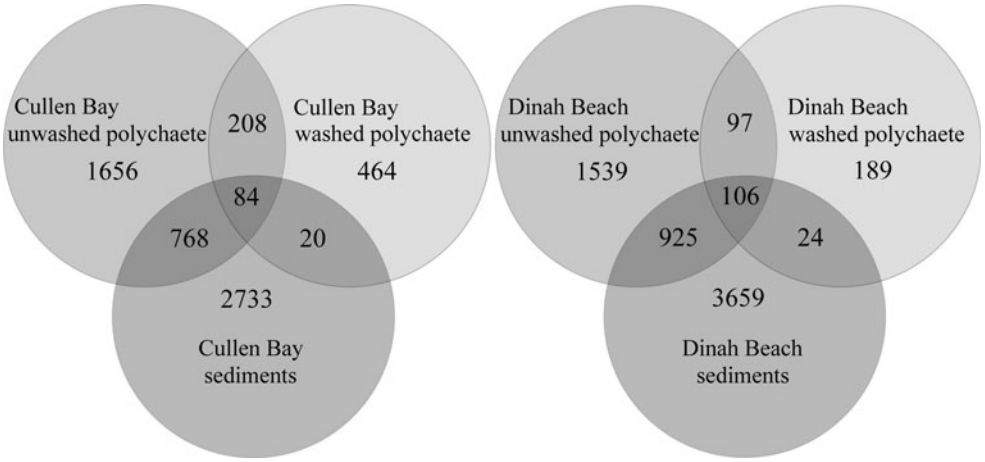

Figure 3 Venn diagrams of shared and unique OTUs from the washed polychaetes, unwashed polychaetes and the sediment samples at each site. The Venn diagrams were calculated by clustering sequences with a 3% distance threshold

found to have flexible adaptation mechanisms, including systems for the detoxification of metals [29]. The Idiomarinaceae associated with *Ophelina* sp.1 in Cullen Bay may also have metal-resistant mechanisms, which could detoxify metals on the polychaete epidermis or in the gut of the polychaete.

Dinah Beach contained many bacteria that were more abundant than at Cullen Bay, including bacteria of the order Oceanospirillales. The abundance of Oceanospirillales on the washed polychaete at Dinah Beach suggests that these bacteria are either tightly associated with the epidermis or are part of the polychaete gut fauna. Oceanospirillales are associated with the gut of bone eating polychaetes in the genus *Osedax* and are thought to metabolize carbon [25, 75]. The Oceanospirillales associated with *Ophelina* sp.1 may benefit from the slightly higher organic carbon levels at Dinah Beach, which may have resulted from inputs from the nearby mangroves [61]. Several sulfur-oxidizing bacterial species were also more abundant at Dinah Beach, which may benefit from increased sulfur concentrations also resulting from the mangroves [22, 35], although this was not measured. In summary, the bacteria that were more abundant at Dinah Beach may benefit from the more natural and nutrient-rich mangrove environment.

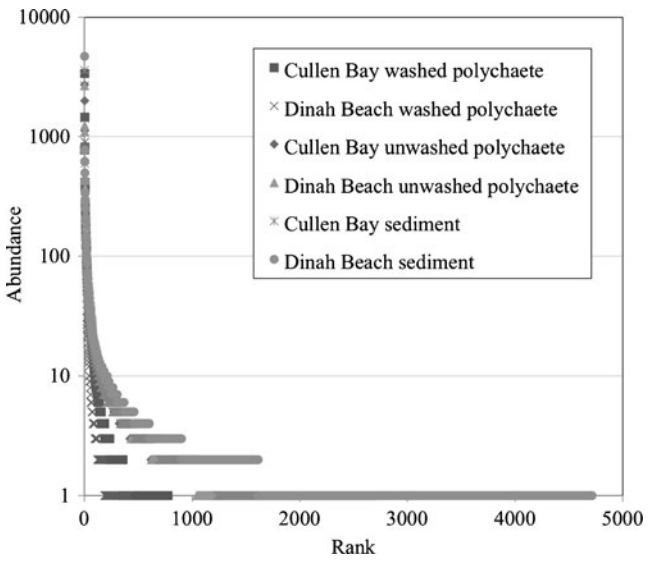

Figure 4 Rank abundance curves for the bacterial OTUs associated with the polychaete treatments and sediment samples from Cullen Bay and Dinah Beach. The abundance was log-transformed, and the OTUs were clustered with a 3% distance threshold

It is also possible that bacteria that were more abundant at Dinah Beach and less abundant at Cullen Bay may be sensitive to increases in copper and zinc concentrations. Copper and zinc are essential trace elements for living organisms but they are toxic at high concentrations [47, 48]. Copper catalyzes the production of hydroxyl radicals, which cause DNA damage [6, 32], and high copper levels also damage the iron-sulfur dehydratase protein family [38]. Elevated zinc concentrations inhibit the production of glutathione reductase, resulting in higher levels of reactive oxygen species and oxidative stress [56]. Copper is generally considered to be more toxic than zinc [33, 47] and two in combination may have synergistic effects [9, 17, 23]. Furthermore, copper and zinc reduce the biomass of bacteria that are symbiotic with aquatic organisms [73, 78]. The copper concentration in Cullen Bay was over 170 ppm, and the zinc concentration was more than 290 ppm, which is well above the ISQG low values of 65 ppm for copper and 200 ppm for zinc [3]. Therefore, it is possible that the high concentration of these metals was responsible for the decreased abundance of some bacteria at Cullen Bay.

Three treatment types were analyzed in this study—bacteria associated with washed polychaetes, unwashed polychaetes and in the sediment. Results for the unwashed polychaete treatment were expected to ascertain which bacteria were associated with the polychaete epidermis or mucus layer, and this is where the biggest difference

Table 3 Relative abundance of the top 20 most abundant OTUs, classified to the lowest possible level

OTU rank	Identification	Cullen Bay Washed	Dinah Beach Washed	Cullen Bay Unwashed	Dinah Beach Unwashed	Cullen Bay Sediment	Dinah Beach Sediment
1	Gammaproteobacteria	27.18	17.51	7.73	7.99	0.00	0.00
2	Oceanospirillales	0.22	32.85	1.08	0.52	0.00	0.01
3	Gammaproteobacteria	0.04	11.06	0.10	7.07	0.00	0.00
4	Alteromonadales	0.04	0.04	13.80	0.68	0.00	0.00
5	Idiomarinaceae	11.70	3.07	5.84	2.77	0.06	0.11
6	Gammaproteobacteria	0.06	0.16	2.12	1.44	6.72	4.57
7	Oceanisphaera	3.04	0.27	2.87	2.08	0.00	0.01
8	Alphaproteobacteria	1.97	1.93	0.31	1.13	0.00	0.00
9	Alphaproteobacteria	6.56	3.49	1.08	4.14	0.00	0.00
10	Ectothiorhodospiraceae	0.09	0.12	1.19	2.10	5.06	3.69
11	Gammaproteobacteria	0.02	1.79	0.05	1.29	0.00	0.00
12	Enterobacteriaceae	2.03	1.77	1.22	1.04	0.01	0.01
13	Bacteria	3.35	0.85	0.00	0.01	0.00	0.00
14	Sulfur-oxidizing bacterium	0.02	0.00	0.66	2.40	2.58	2.96
15	Alphaproteobacteria	1.25	1.34	0.24	0.59	0.00	0.00
16	Cyanobacteria	0.25	0.26	0.58	0.49	0.48	1.70
17	Sulfur-oxidizing bacterium	0.00	0.00	0.24	0.61	1.54	1.21
18	Deltaproteobacteria	1.07	0.64	0.46	0.94	0.00	0.00
19	Endosymbiont	0.04	0.05	0.05	0.67	0.26	1.46
20	Desulfobulbus	0.02	0.00	0.18	0.60	1.07	1.25

between Cullen Bay and Dinah Beach was observed. The bacteria that were detected in the washed polychaete treatments were relatively similar at the two sites; this was surprising given that tightly associated bacteria were expected to be the niche involved in metal detoxification processes. However, others have reported that mucus secretion [42] and bacteria within the mucus [1] appear to have detoxifying properties. It is likely that our washing treatment removed most of the mucus from the worms; therefore, the large bacterial changes may have been related to bacteria within the mucus layer, and these bacteria may be involved in metal detoxification. The sediment bacterial community was relatively similar at both Cullen Bay and Dinah Beach, despite the large differences in copper and zinc concentrations in the sediments. The copper and zinc concentrations were much more influential on the bacterial community associated with *Ophelina* sp.1, suggesting that the associated bacteria are a more sensitive indicator of pollution.

The bacteria associated with polychaetes may be a useful indicator group because polychaetes are easy to find and collect in most marine systems [15]. In addition, changes in the bacteria associated with polychaetes can be directly

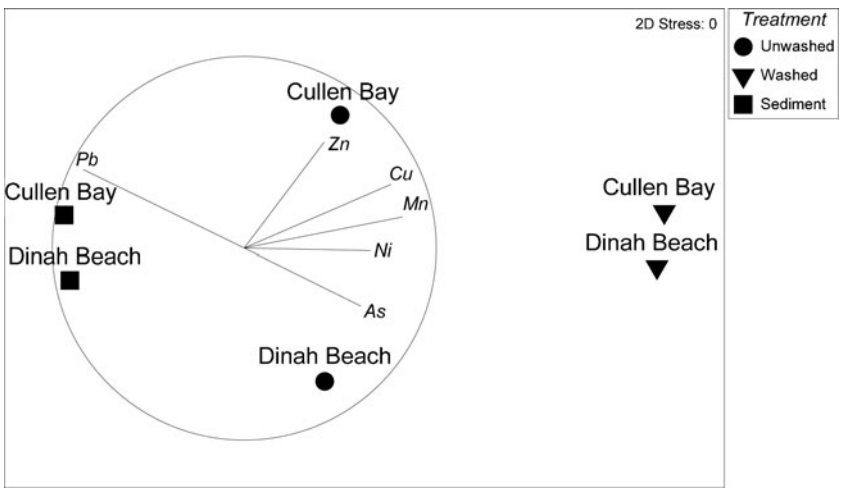

Figure 5 Multi-dimensional scaling (*MDS*) plot showing similarities among the bacterial communities associated with polychaetes and sediments. The metals that were correlated with the bacterial community patterns (Spearman's correlation, >0.6) were overlaid onto the plot. The MDS plot was calculated on standardized, square-root transformed OTU data using Bray Curtis similarity measures

Table 4 The 10 OTUs that contributed most to the differences between the Cullen Bay and Dinah Beach unwashed polychaetes

OTU rank	Classification	Cullen Bay Unwashed	Dinah Beach Unwashed	Contribution (%)
4	Alteromonadales	13.80	0.68	0.61
3	Gammaproteobacteria	0.10	7.07	0.50
9	Alphaproteobacteria	1.08	4.14	0.21
61	Rhodospirillales	0.00	0.86	0.20
11	Gammaproteobacteria	0.05	1.29	0.19
5	Idiomarinaceae	5.84	2.77	0.16
28	Chromatiales	0.85	0.06	0.16
22	Alphaproteobacteria	0.32	1.70	0.16
14	Sulfur-oxidizing bacterium	0.66	2.40	0.16
38	Sulfur-oxidizing symbiont	0.14	1.19	0.15

The relative abundance of each OTU is listed along with the contribution of the OTU to the differences between the two samples. Similarity percentages were calculated on standardized, square-root transformed OTU data

related to the surrounding conditions because many polychaetes are either sedentary or have specific habitat requirements, resulting in movement only at a local scale [40, 72]. Several molecular techniques could be developed for the rapid identification of certain bacteria associated with polychaetes. Techniques such as quantitative real-time PCR or DNA fingerprinting methods, such as denaturing gradient gel electrophoresis or terminal-restriction fragment length polymorphism, could be used to specifically target changes in the composition of indicative bacteria, such as the Alteromonadales or Idiomarinaceae in this study. These techniques may provide informative and rapid environmental impact assessments.

The symbiotic and gut bacteria associated with polychaetes in coastal environments have not been well studied. In the present study, OTUs that occurred only on polychaetes and were not detected in the sediment, were likely to be polychaete symbionts and/or gut fauna. Although, it should be noted that the sediment bacterial community was not exhaustively studied and, therefore, bacteria that were only detected on polychaetes may in fact be detected in the sediment with deeper sequencing. At Dinah Beach, 189 OTUs were unique to polychaetes and at Cullen Bay, 464 OTUs were unique to polychaetes. The Chao1 richness estimator for the washed polychaetes was 803 for Dinah Beach and 1,227 for Cullen Bay. As expected, the richness estimates are much higher in the current study using pyrosequencing compared with previous estimates of polychaete bacterial diversity, which used cloning and Sanger sequencing. In those studies, the mucous of hydrothermal vent worms was found to contain less than 50 phylotypes [1], and analysis of polychaete gut microbes found less than 25 species [36]. The richness estimates reported here are less than for some invertebrates such as sponges, which have chao1 estimates ranging from 1,500 to more than 4,500 estimated species [34, 77]. Other pyrosequencing studies of invertebrates have reported Chao1 estimates of less than 500 [2]. In this study, many of the bacteria species unique to polychaetes belonged to the Verrucomicrobia, which have previously been detected in the mucous of hydrothermal vent polychaetes [1], in the gut of sea cucumbers [60] and as nematode endosymbionts [74]. Members of the Verrucomicrobia are also ectosymbionts on ciliates and provide the host organism with a novel defensive mechanism [49]. Many of the potentially symbiotic bacteria identified in this study had recognized symbiotic lineages [7, 25, 75], and this study has increased our understanding of their abundance and diversity.

The resistance of polychaetes to the high metal levels around deep-sea hydrothermal vents has been attributed to bacterial symbionts [16, 52]. The resistance of *Ophelina* sp.1 to high copper and zinc in Cullen Bay may also be related to changes in their associated bacteria. This suggests that symbiotic relationships between polychaetes and bacteria can develop not only in naturally high metal environments but also in environments anthropogenically polluted with metals, in which the period of adaptation is much shorter. Resistance studies examining polychaetes in environments that are anthropogenically polluted with metals often overlook the potentially important associated bacteria, instead focusing on protein and gene-expression of the host. We recommend that studies examining resistance in polychaetes also examine the bacterial community for a complete picture of resistance.

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Appendix

Table 5 The 100 most abundant OTUs that were only detected in the washed polychaete treatments, classified to the lowest possible level

Identification	Relative abundance	Overall rank
Firmicutes	2.15	429
Opitutus	1.72	699
Actinobacteria	1.50	208
Bacteria	1.50	423
Bacteria	1.39	701
Bacteria	1.29	706
Verrucomicrobia	1.18	2,407
Brachymonas	0.97	671
Spartobacteria	0.97	796
Firmicutes	0.86	452
Bacteria	0.86	524
Bacteria	0.86	372
Bacteria	0.75	431
Bacteria	0.75	857
Verrucomicrobia	0.75	946
Microcella	0.75	1,393
Bacteria	0.75	469
Alphaproteobacteria	0.75	617
Firmicutes	0.75	473
Rhodobacter	0.64	1,352
Bacteria	0.54	1,177
Lactococcus	0.54	1,505
Cyanobacteria	0.54	1,993
Bacteria	0.54	2,450
Bacteria	0.54	2,780
Bacteria	0.43	828
Bacteria	0.43	842
Bacteria	0.43	849
Roseomonas	0.43	919
Bacteria	0.43	934
Gammaproteobacteria	0.43	1,113
Proteobacteria	0.43	1,300
Bacteria	0.43	1,480
Gammaproteobacteria	0.43	1,515
Proteobacteria	0.43	1,890
Enterobacteriales	0.43	10,106
Bacteria	0.43	1,074
Acetobacteraceae	0.43	1,057
Bacteria	0.43	1,419
Deferribacteres	0.43	2,052
Bacteria	0.43	4,922
Acinetobacter	0.43	7,860
Bacteria	0.32	1,003
Bacteria	0.32	1,106
Gammaproteobacteria	0.32	1,108
Bacteria	0.32	1,215
Bacteria	0.32	1,424
Oceanospirillales	0.32	1,794
Finegoldia	0.32	1,803
Bacteria	0.32	1,844
Alishewanella	0.32	1,908
Cyanobacteria	0.32	1,981
Burkholderiales	0.32	2,026
Sinobacteraceae	0.32	2,162
Alphaproteobacteria	0.32	2,181
Verrucomicrobia	0.32	2,508
Bacteria	0.32	2,793
Bacteria	0.32	2,884
Bacteria	0.32	4,804
Bacteria	0.32	6,741
Bacteria	0.32	7,111
Actinobacteria	0.32	8,650
Bacteria	0.32	1,002
Bacteria	0.32	1,288
Gammaproteobacteria	0.32	1,345
Gammaproteobacteria	0.32	2,648
Bacteria	0.32	2,732
Alphaproteobacteria	0.32	7,732
Deltaproteobacteria	0.32	6,050
Rhodobacteraceae	0.32	7,750
Bacteria	0.21	1,518
Acidobacteriaceae	0.21	1,599
Bacteria	0.21	1,602
Verrucomicrobia	0.21	1,666
Desulfovibrio	0.21	1,690
Verrucomicrobia	0.21	1,876
Gammaproteobacteria	0.21	1,883
Bacteria	0.21	1,954
Alphaproteobacteria	0.21	1,957
Proteobacteria	0.21	1,963
Bacteria	0.21	1,996
Bacteria	0.21	2,010
Bacteria	0.21	2,114
Bacteria	0.21	2,259
Erwinia	0.21	2,381
Bacteria	0.21	2,442
Desulfobacteraceae	0.21	2,445
Gammaproteobacteria	0.21	2,486
Proteobacteria	0.21	2,520
Acidimicrobiaceae	0.21	2,579
Bacteria	0.21	2,604
Bacteria	0.21	2,622
Betaproteobacteria	0.21	2,670
Bacteria	0.21	2,680

Table 5 (continued)

Identification	Relative abundance	Overall rank
Rhizobiales	0.21	2,774
Bacteria	0.21	2,778
Bacteria	0.21	2,818
Bacteria	0.21	2,821
Verrucomicrobia	0.21	2,912
Deltaproteobacteria	0.21	2,924

The relative abundance is the amount of sequences for each bacteria as a percentage of the total amount of sequences that were only detected in the washed polychaete treatments. The overall rank refers to the position of the OTU in the complete dataset

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