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Multiple approaches to assess the safety of artisanal marine food in a tropical estuary

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Abstract In this study, metal and metalloid concentrations and pathogens were measured in shellfish at different locations in a tropical estuary, including sites impacted by sewage and industry. Oyster, mangrove snails and mud snails did not exceed Australian and New Zealand Food Standards maximum levels for copper, lead or estimated inorganic arsenic at any site although copper concentrations in oysters and mud snails exceeded generally expected levels at some locations. Bacterial community composition in shellfish was species-specific regardless of location and different to the surrounding water and sediment. In the snails *Telescopium telescopium*, *Terebralia palustris* and *Nerita balteata*, some bacterial taxa differed between sites, but not in *Saccostrea cucullata* oysters. The abundance of potential human pathogens was very low and pathogen abundance or diversity was not associated with site classification, i.e. sewage impact, industry impact and reference.

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Introduction

Shellfish are abundant in the mangrove-fringed coastlines of northern Australia and are still collected and eaten by indigenous people. In urban areas faced with increased industrialisation, land clearing and population growth, there is concern that development is compromising the quality and safety of wild harvest shellfish for consumption. Shellfish ingest and accumulate metals and bacteria, including those that may be adverse to human health. Most of the animal is consumed and often eaten raw or lightly cooked.

Eating molluscs exposed to metal contaminants represents potential risk to humans particularly for metals such as mercury, cadmium, lead, copper and zinc (Bryan 1971, 1979). Maximum acceptable concentrations of metals in foods have been established in many countries. In Australia, Food Standards Australia New Zealand (FSANZ) has developed a code (standard 1.4.1) that lists maximum levels of specified metal and non-metal contaminants in nominated foods (FSANZ 2011). For molluscs, maximum levels exist for arsenic, lead and cadmium. Zinc and copper were included in the food standard prior to 2000, but were removed due to their low risk to human health, and are listed as generally expected levels or GELs as a benchmark (FSANZ 2001).

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Darwin Harbour is considered to be near pristine with a few areas of localised pollution (Fortune and Drewry 2011) although reports on metal contaminants in shellfish in Darwin Harbour are scarce. One study reported high lead concentrations in *Telescopium telescopium* (long bums) with cadmium, zinc and copper below recommended National Health Medical research council (NHMRC) limits (Peerzada et al. 1990). In a later study, copper concentrations in *T. telescopium* were high, above the expected level of 30 mg/kg (French 2013). In a separate study, oysters in Darwin Harbour had cadmium, zinc and copper concentrations below NHMRC guideline limits for those metals (Peerzada et al. 1993).

Pathogens that occur naturally in marine water or from sewage-contaminated areas can accumulate in molluscs (Metcalf et al. 1979; Rippey 1994; Martinez-Urtaza et al. 2003; Thompson et al. 2005; Miller et al. 2006; Cañigral et al. 2010; Keller et al. 2013; Bigoraj et al. 2014). This represents a potential risk to consumers and guidelines exist to minimise this risk for commercial use. In contrast to recreational water and commercial shellfish, there are no guidelines for monitoring the quality of wild shellfish harvested for non-commercial use. Little is known about pathogens in seawater or shellfish in Darwin Harbour although routine indicator monitoring (NHMRC 2008) has been used on Darwin beaches since 2010. As a result, several beaches have been closed for short periods (http://www.health.nt.gov.au/Environmental_Health/Beach_Water_Quality). Microbial source tracking further identified one sewage outfall as a source of faecal bacteria at adjacent beaches, but faecal indicator bacteria were also found to originate from urban rivers and creeks (Neave et al. 2014). Knowledge on the microbiological quality of marine foods from Darwin Harbour is exiguous (Dettrick and Schlusser, 2006). Many studies have also shown a poor relationship between faecal indicators and pathogens particularly viruses and naturally occurring pathogenic bacteria such as *Vibrio parahaemolyticus* (Muniain-Mujika et al. 2002; Marino et al. 2005; Clements et al. 2015) but there is reluctance for specific pathogen testing as the protocols are often complex, time-consuming and expensive. A more rapid and cheaper approach is the use of next-generation sequencing which can reveal overall microbial diversity including pathogenic taxa in a range of habitats (Luna et al. 2007; Bibby et al. 2010; Ye and Zhang 2011; Smith et al. 2012).

To address concerns on the quality and safety of wild harvested shellfish, we tested metal and metalloid levels in shellfish species from different parts of Darwin Harbour. We also assessed whole microbial populations in shellfish and attempted to identify changes in population structure and potential pathogens in those populations. Based on existing knowledge of metal and arsenic concentrations in shellfish and the ability of shellfish to accumulate bacteria during filtration and feeding, our specific hypotheses were (1) that shellfish from Darwin Harbour would not exceed FSANZ maximum levels for Cd, Pb, As or expected levels for Zn, but possibly exceed generally expected levels for Cu; (2) that shellfish have a cohort of core bacteria communities specific to the shellfish species regardless of location, (3) that each shellfish species also has a variable bacterial population, such as pathogens, dependent on the environment that vary with location and (3) shellfish from disturbed sites (e.g. impacted by sewage, industry) have different metal concentrations and different bacterial communities (including pathogens) compared to reference sites.

To test these hypotheses, four different shellfish species (one bivalve and three marine snails) were harvested from eight locations in Darwin Harbour with different impacts—sewage, industrial and reference. Arsenic, cadmium and lead concentrations were measured and compared to FSANZ standards for molluscs, and in addition, zinc and copper were also measured as these metals used to be included in the food standards guidelines. Microbial communities were assessed in biota by next-generation sequencing to identify core and variable bacteria, including pathogens. Statistical analyses were applied to identify significant differences in metal/metalloid concentrations and microbial community structures in shellfish between sites. Sediment and seawater collected and analysed (metals and microbes) from the same sites as the shellfish to understand the environment in which the shellfish are living and to test whether these matrices can be used as a reliable predictor in shellfish.

Methods

Study sites

Darwin is situated in northern Australia with a population of approximately 140,000 (Australian Bureau of Statistics;

http://www.abs.gov.au/ausstats/abs@.nsf/mf/3218.0). Six sites on the more developed eastern side of Darwin Harbour were selected for sampling based on proximity to human-derived contaminants including sewage outfalls, stormwater drains and urban runoff, light industry and the availability of target shellfish species (Fig. 1). Two sites on the western side of the harbour were chosen as reference sites due to minimal human disturbance. Table 1 lists the site names, coordinates and potential source of contamination.

Target shellfish species

Several shellfish species are harvested for food in the Darwin Harbour area and of these, four species were chosen based on their prevalence and occurrence at the time of year the study was conducted. The species chosen were *Terebralia palustris* or mud whelk (Mollusca: Gastropoda: Cerithioidea: Potamididae), *T. telescopium* or long bum (Mollusca: Gastropoda: Cerithioidea: Potamididae), *Nerita balteata* or nerites (Mollusca: Gastropoda: Neritoidea: Neritidae) and *Saccostrea cucullata* or rock oyster (Mollusca: Bivalvia: Ostredae). The snails *T. palustris* and *T. telescopium* live and feed on detritus in mangrove sediment. *N. balteata* is found on mangrove stems or aerial roots, eating algae. *S. cucullata* were found in more open rocky areas and are filter feeders.

Sampling

Samples were collected at low tide over 4 weeks in the early dry season (24 April to 18 May 2012) to minimise seasonal affects. At this time of year, rain

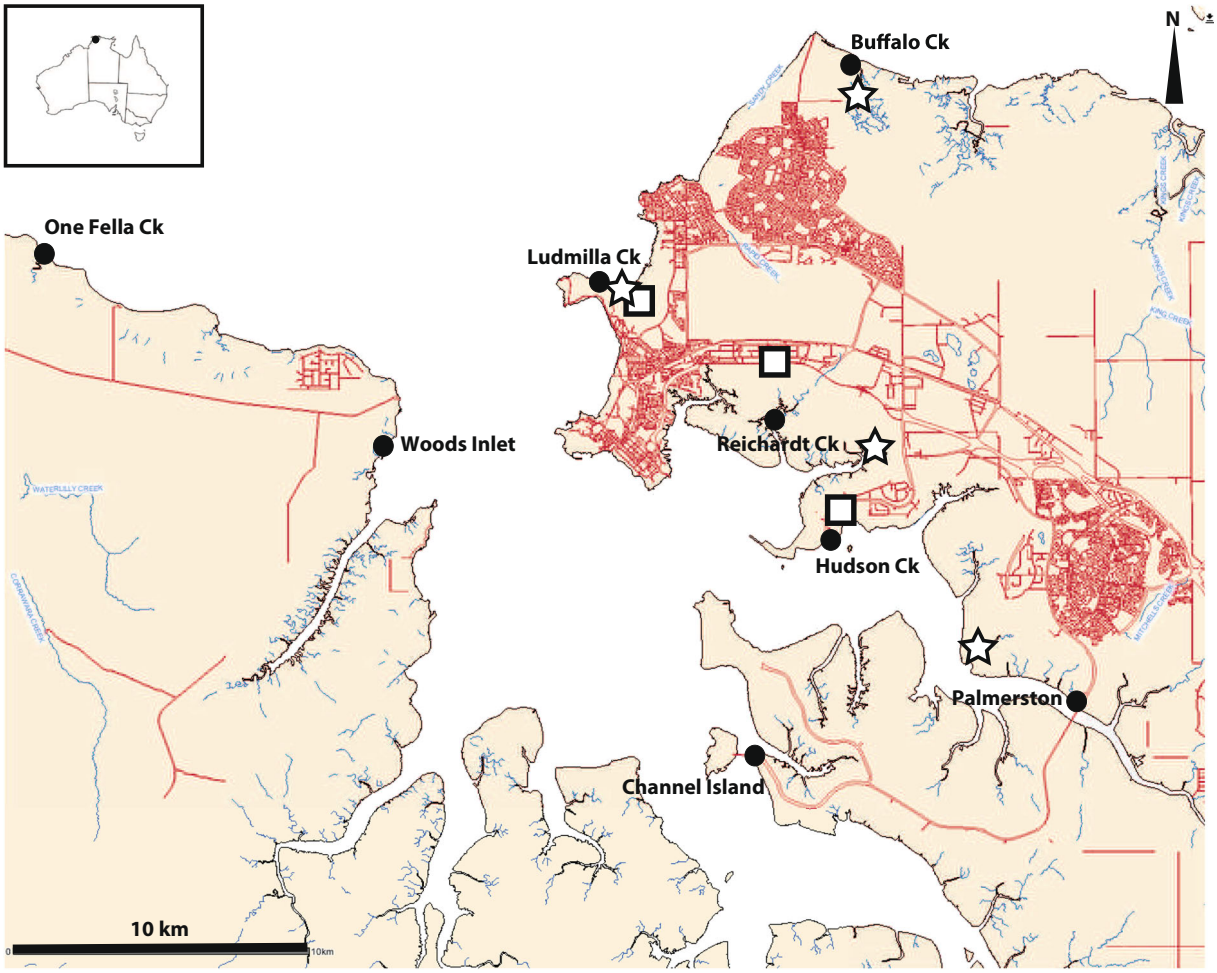

Fig. 1 Map of Darwin Harbour showing sampling sites and locations of sewage outfalls (*stars*) and industrial areas (*squares*). *Red lines* are roads and *blue lines* show creeks and rivers

Table 1 List of study site names, coordinates and level of impact

Site name	GPS coordinates	Potential source of contamination
Buffalo Creek	12° 20′ 21.8″ S, 130° 54′ 25.2″ E	Sewage, boating, urban runoff
Reichardt Creek	12° 26′ 56.9″ S, 130° 52′ 36.2″ E	Industrial, urban runoff, storm water
Hudson Creek	12° 28′ 53.0″ S, 130° 54′ 47.6″ E	Industrial, urban runoff, wharf loading
Ludmilla Creek	12° 24′ 34.2″ S, 130° 49′ 55.4″ E	Sewage, industrial
Palmerston ramp	12° 32′ 248″ S, 130° 58′57.5″ E	Boating, rubbish dump leaching
Channel Island	12° 33′ 33.3″ S, 130° 52′ 52.6″ E	Boat ramp, urban runoff, power station
Woods Inlet	12° 27′ 29.8″ S, 130° 45′ 40.0″ E	None
One Fella Creek	12° 24′ 50.3″ S, 130° 39′ 19.6″ E	None

becomes less frequent (Bureau of Meteorology; http://www.bom.gov.au/) and water temperatures are 1–2 °C lower (http://data.aims.gov.au/metadataviewer/uuid/cc202764-23d1-4043-bde2-09c53fb6a5df) compared to the wet season from October to March. All the sites including creeks were marine-dominated estuaries. In addition to the targeted shellfish, seawater (500 ml) and sediment (~20 g) were collected in triplicate for analysis. The water samples were obtained by inverting a 500-ml acid-washed Nalgene™ bottle approximately 20 cm below the water surface. Sediment was collected using plastic trowels. Ten of each shellfish species were collected in triplicate at each site (n = 10 × 3) taking animals of similar size where possible. Sediment and biota were stored in Whirl-Pak® bags. All samples were stored on ice and processed immediately on return to the laboratory.

Seawater

Seawater (50 ml) was filtered using a 0.45-μm syringe filter (Acrodisc Supor® membrane, Pall Australia, Cheltenham, Vic). Twelve millilitres were acidified with HNO_3 (UNIPURE®) and stored at room temperature for trace element analysis. The remaining filtered seawater samples were frozen at −20° for nutrient analysis. A further 400-ml seawater was filtered onto a 0.45-μm cellulose acetate membrane filter (Sartorium Stedim Biotech GmbH 37,070 Göttingen, Germany) with a 3-μm Supor® prefilter (Pall Australia) for DNA analysis. The filters containing the microbes were stored at −20 °C. DNA was extracted from the filters using the PowerWater® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA).

Sediment

Sediment was stored in plastic bags at −20 °C until all field trips were completed. The sediment was then wet sieved to keep the <2-mm fraction, oven dried and ground to a fine powder using an automated grinder. Sediment was digested with nitric + perchloric acid (1 + 4 ml) to obtain a near-total digest and stored at 4 °C for trace element analysis. This digestion method provides a conservative estimate of bio-available metal levels in sediment (Munksgaard and Parry 2002). Digest blanks, replicates and certified reference materials (NIST 1566 and 1575, IAEA 407) were included in each digestion batch. DNA was extracted from 10-g wet sediment for microbial analysis using the PowerMax® Soil DNA Isolation Kit (MO BIO).

Biota

Biota were stored frozen at −20 °C immediately after each field trip. After thawing in batches, animals were shucked, all soft tissue removed, washed with high pure water to remove shell pieces and sediment and pooled. For each replicate at each site, 4 individual *T. telescopium* and *T. palustris*, 6 periwinkle individuals and 10 *S. cucullata* were pooled and fresh weight recorded. Pooled specimens were freeze-dried, reweighed and ground to a powder using an automated grinder. Biota was digested with nitric acid + hydrogen peroxide (4 + 2 ml) in a microwave for trace element analysis. Digest blanks, replicates and certified reference materials (Dorm 3 (National Research Council Canada), AGAL-3 prawn (Australian Government Analytical Laboratories), NIST Oyster Tissue 1566b (National Institute of Standards and Technology) were

included in each digestion batch. For microbial community analysis, DNA was extracted from approximately 150 mg freeze-dried and ground material using the QIAamp DNA Blood Maxi Kit (QIAGEN Pty Ltd., Chadstone, Victoria, Australia) with additional steps to remove inhibitory substances (Reid et al. 2008).

Trace element analysis

Solutions of seawater, digested sediment and biota were analysed on an Agilent 7500ce inductively-coupled plasma mass spectrometer (ICP-MS). Replicate samples of selected seawater, sediment and biota samples were spiked with known amounts of trace elements during sample preparation for quality assessment. Elements measured were copper, zinc, arsenic, cadmium and lead. Aluminium was also measured in sediment samples for normalisation to account for different grain size in the sediment.

Nutrient analysis

Total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) in filtered (<0.45 μm) seawater were measured by flow injection analysis using an alkaline potassium persulphate method (Lachat method 31-1907-04-1-A for TDN and Lachat method 31-115-01-3B for TDP). Total Kjeldahl nitrogen (TKN) in sediment was measured using sulphuric acid digestion (Lachat method 13-107-06-2-D) and total phosphorus in sediment was measured by ICP-MS. Reference material was obtained from Choice Analytical Pty Ltd. (loam A) and Queensland Health Forensic and Scientific Services (ENCT Round 15 Bottle 2, 4, 6, and 8).

Microbial community analysis

Approximately 1 μg of DNA was dried and sent to MR DNA Molecular Research (Shallowater, Texas, USA) for 454 pyrosequencing. We selected primers 27F/519R which amplify the V1 region of the bacterial 16S ribosomal RNA (rRNA) gene and the option of 3000 reads per sample was chosen. The output sequence data was processed using a proprietary analysis pipeline (www.mrdnalab.com). Briefly, sequences were depleted of barcodes and primers, then short sequences <200 bp or ambiguous base calls or with homopolymer runs exceeding 6 bp were removed. Sequences were then 'de-noised' and chimeras removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences by clustering at 3% divergence (97% similarity) (Dowd et al. 2008a, b, 2011; Edgar 2010; Capone et al. 2011; Eren et al. 2011; Swanson et al. 2011). OTUs were then classified using BLASTn against a curated GreenGenes database (DeSantis et al. 2006) and compiled into each taxonomic level into both 'counts' and 'percentage' files. Pathogenic bacterial species were manually identified.

Data analysis

Trace element concentrations and microbial communities were analysed using the PRIMER v6 and PERMANOVA+ package (Primer-E Ltd., Plymouth, UK) to display and examine relationships in seawater, sediment and biota collected from different sites. Principal component analysis (PCA) was applied to normalised ($\log(X + 1)$) sediment and seawater trace element and nutrient concentrations to generate two dimensional plots displaying the relationships between sites. RELATE in (PRIMER v6) was used to determine the relationship between metal concentrations in sediment and seawater compared to biota.

For subsequent microbial community analyses, sites were designated as background, sewage or industrial based on the PCA of sediment trace element and nutrient concentrations normalised to an aluminium concentration of 10,000 mg/kg (Loring and Rantala 1992, Birch 2003). Microbial communities were analysed using the PRIMER v6 and PERMANOVA+ package (Primer-E Ltd., Plymouth, UK) to display and examine relationships between sites. PERMANOVA (Permutational ANOVA) analysis was performed on normalised and transformed ($\log(X + 1)$) resemblance matrices using unrestricted permutations of raw data with Monte Carlo sampling, to test for significant differences among sites. Taxa containing less than 10 counts were omitted from the analysis. If the overall test was significant, pairwise tests were done to determine which sites were significantly different. Rarefaction was performed using mothur (Schloss et al. 2009) and plotted using *R* and ggplot2 (Wickham 2009). SIMPER was applied to identify which microbes contributed most to differences between site groups for each shellfish species and the complex networks are shown visually using Cytoscape v3.2.0 only including taxa with >3% relative abundance (Smoot et al. 2011). To test if microbial abundance was significantly different across the

treatment groups, we used negative binomial modelling in DESeq2 (Love et al. 2014) as recommended by McMurdie and Holmes (2014). Obtained *P* values were adjusted for false discovery due to multiple testing using the Benjamini-Hochberg correction within Deseq2. Pathogens were identified manually based on a published list (Ye and Zhang 2011).

Results

Environmental chemistry

Seawater trace element and nutrient concentrations are shown in Supplement 1. Cadmium and lead concentrations were all below the detection limit and zinc concentrations were below the Australian and New Zealand Environment and Conservation Council (ANZECC 2000) trigger value for 95% species protection. The copper concentration at Reichardt Ck was 1.48 ppb and above the ANZECC trigger value of 1.3 ppb.

Total phosphorus concentrations in filtered seawater generally ranged from 8.04 to 27.2 μg/l. At 61.3 μg/l, however, total dissolved phosphorus at Buffalo Ck exceeded the ANZECC trigger value of 20 μg/l. Buffalo Ck also exceeded the ANZECC trigger value for total dissolved nitrogen concentrations in seawater, with Reichardt Ck and Ludmilla Ck also close to the ANZECC trigger value of 250 μg/l.

Concentrations of trace elements normalised against aluminium and nutrients in sediment are shown in Supplement 2. Concentrations of copper, zinc, cadmium and lead did not exceed ANZECC trigger values. Total arsenic concentrations could not be directly compared to ANZECC guidelines, which are for the inorganic form only, but values were highest at Reichardt Ck. Buffalo Ck had the highest phosphorus concentration of 889 mg/kg, compared to the lowest concentration of 77.0 mg/kg at Reichardt Ck. Total nitrogen in sediment was highest at Channel Island (0.21%).

The PCA plot (Fig. 2) shows the relationship between sites based on sediment trace elements and nutrients and these results informed the groupings into background (One Fella Ck, Palmerston and Channel Island); sewage affected (Ludmilla Ck and Buffalo Ck) and industrial (Reichardt Ck and Hudson Ck). The Woods Inlet site, originally chosen as a potential background site, had higher than expected concentrations of most of the elements measured when normalised to aluminium. While this reflects natural variability in Darwin Harbour, the Woods Inlet site was omitted from microbial analyses. Seawater analysis enabled us to check for substantial differences in chemical properties compared to expected background levels at the time of sampling but we did not use seawater characteristics to determine site groupings as water reflects short-term variation and therefore only a snapshot of the local conditions at the time of sampling.

Trace element concentrations

Trace element concentrations in biota are shown in Table 2. Element concentrations for all reference materials were within 82 to 125% of expected values. Concentrations of zinc, copper, arsenic, cadmium and lead recovered from spiked samples were within 86 to 119% of the expected range. Trace metals in all shellfish samples were below the FSANZ Guide (Standard 1.4.1 for Contaminants and Natural Toxicants) for cadmium and lead in molluscs. Total arsenic concentrations ranged from 1.46 to 6.07 mg/kg in all shellfish species from all sites but the proportion of inorganic arsenic (listed in the FSANZ guidelines) was not determined so a direct comparison could not be made. Higher than generally expected levels (GELs) of copper and zinc were measured in *S. cucullata* from Ludmilla Ck; however, these metals were not elevated in water or sediment at this site. This was also true for *S. cucullata* from Channel Island and Hudson Ck where zinc levels exceeded the GEL, even though zinc was not elevated in water or sediment at these sites. The only site that had slightly elevated zinc levels in seawater was Reichardt Ck but *S. cucullata* could not be found at this site. *T. palustris* from Channel Island also had greater than expected levels of copper but again this metal was not elevated in water or sediment at these sites.

RELATE analysis showed no significant relationships between As, Cd, Pb, Cu or Zn concentrations in biota and either filtered seawater (<0.45 um) or sediment (<2 mm fraction). The strongest relationship was between metal concentrations in *S. cucullata* with seawater (Spearman coefficient = 0.378, $P = 0.06$) and BEST showed that Cu, Zn and As explained most of the correlation (Spearman rank = 0.347, $P = 0.05$).

Microbiology data from biota, sediment and seawater

The number of sequence counts obtained for any one shellfish species varied between samples (Table 3) and

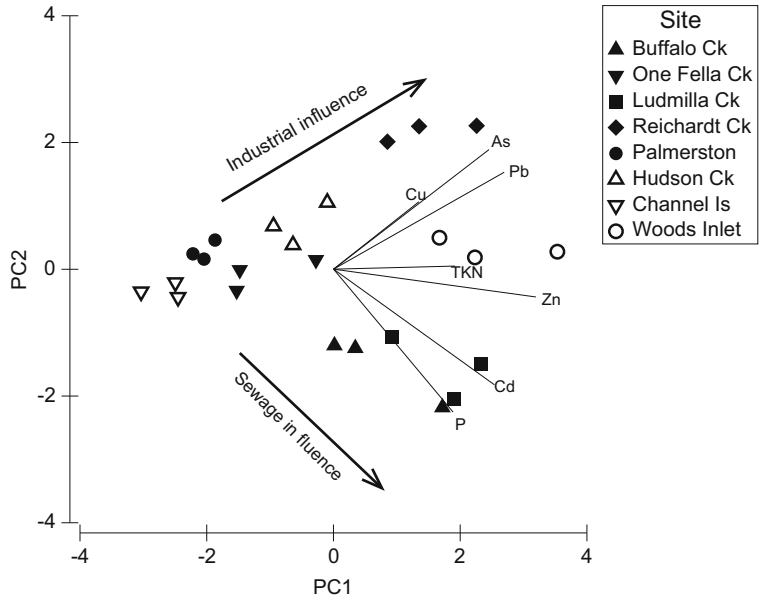

Fig. 2 PCA plot of sediment trace element and nutrient concentrations at eight sites in Darwin Harbour. Sites are grouped into clusters based on their proximity and surrounding land use. Axis PC1 describes 50.2% of the variation and axis PC2 describes 20.2% of the variation

we rejected samples with less than 1000 reads. The number of OTUs was generally lower in *S. cucullata* (range 141–1262, average 510) and *N. balteata* (range 94–981, average 553) compared to *T. telescopium* (range 243–2600, average 970) and *T. palustris* (range 265–1819, average 947) (Table 3). Sediment contained a greater diversity of bacteria (average 1730 ± 180) compared to seawater (average 860 ± 111).

Rarefaction curves (species richness for a given number of individual samples) generated in *R* showed that most of the diversity was captured with the sequencing depth for *S. cucullata*, *N. balteata* and seawater although this was not the case for some *T. palustris* and *T. telescopium* samples nor for sediment (Supplement 3).

Shannon's diversity indices (*H'*) for the shellfish, sediment and seawater across impacts are shown in Table 4. There were no statistically significant differences in indices between impact sites for any of the biota or sediment, but microbial communities in seawater were more diverse at the sewage impacted sites compared to the reference or industry sites.

Microbial community analysis

A multi-dimensional scaling (MDS) plot and PERMANOVA analysis showed distinct microbial communities in each of the biota species, seawater and sediment (Fig. 3; $P(MC) < 0.001$). For each of the shellfish species, PERMANOVA using unrestricted permutations with Monte Carlo simulations showed significant differences in microbial communities between reference and sewage sites in *T. palustris* ($P(MC) = 0.037$) and *S. cucullata* ($P(MC) = 0.042$). SIMPER analysis identified taxa that primarily discriminate between the impact and reference sites and taxa with >3% relative abundance were used in Cytoscape to visualise major differences between communities (Fig. 4).

The most dominant taxa in *T. telescopium* were *Mollicutes*, *Rhodobacteraceae*, *Planctomycea* and *Spirochaetes*, together contributing to nearly 30% of all sequences identified irrespective of site designation (Fig. 4). *Vibrio orientalis* and *Halobacillus trueperi* dominated samples from sewage sites while the alphaproteobacterium *Thalasosspira xiamenensis* and bacteria from the family *Rhodobacteraceae* were prevalent in *T. telescopium* samples from industry sites (Fig. 4). Several less abundant taxa contributed to the *T. palustris* microbiome with the halophilic bacterium *H. trueperi* contributing 9% to the total bacterial population, and *Flavobacteriaceae*, *Rhodobactereaceae*, *Mollicutes*, *V. orientalis* and *Intrasporangiaceae* each contributing from 8.0 to 4.8% each. *H. trueperi* was more prevalent in samples from sewage sites, members of the *Vibrionaceae* dominated *T. palustris* samples from industry sites while Mollicutes dominated samples from reference sites (Fig. 4).

Spirochaetes dominated *N. balteata* samples, contributing to 22% of all bacterial sequences.

Table 2 Concentrations of trace elements (mg/kg wet weight) in shellfish from Darwin Harbour

Classification	*T. telescopium*	As	Cd	Pb	Cu	Zn
Background	Woods Inlet	3.16 ± 0.83	0.26 ± 0.12	0.04 ± 0.02	13.8 ± 2.92	39.1 ± 17.0
	One Fella Ck	2.75 ± 0.60	0.21 ± 0.07	0.13 ± 0.08	13.5 ± 1.48	29.2 ± 8.98
	Channel Is	3.34 ± 1.82	0.29 ± 0.10	0.20 ± 0.17	35.8 ± 13.6	39.5 ± 14.7
	Palmerston	1.58 ± 0.14	0.09 ± 0.03	0.24 ± 0.06	26.0 ± 2.32	33.9 ± 8.69
Sewage	Ludmilla Ck	2.98 ± 0.88	0.09 ± 0.05	0.54 ± 0.14	14.8 ± 5.73	77.2 ± 41.2
	Buffalo Ck	1.99 ± 0.39	0.17 ± 0.02	0.27 ± 0.05	22.3 ± 2.69	32.9 ± 4.86
Industrial	Hudson Ck	2.72 ± 0.61	0.06 ± 0.02	0.24 ± 0.02	26.4 ± 4.96	30.2 ± 5.55
	Reichardt Ck	5.53 ± 0.70	0.10 ± 0.01	0.33 ± 0.05	13.5 ± 2.55	43.0 ± 14.4
	T. palustris	As	Cd	Pb	Cu	Zn
Reference	Woods Inlet	2.81 ± 0.44	0.19 ± 0.05	0.27 ± 0.12	6.54 ± 1.67	40.6 ± 12.7
	One Fella Ck	2.34 ± 0.32	0.28 ± 0.02	0.10 ± 0.02	21.5 ± 4.48	17.4 ± 2.19
	Channel Is	2.54 ± 1.10	0.25 ± 0.07	0.23 ± 0.15	33.8 ± 13.7	39.5 ± 21.4
Sewage	Ludmilla Ck	1.76 ± 0.20	0.08 ± 0.02	0.16 ± 0.03	11.2 ± 2.09	60.1 ± 5.08
Industrial	Hudson Ck	1.89 ± 0.22	0.19 ± 0.04	0.22 ± 0.05	18.9 ± 3.12	42.2 ± 9.46
	N. balteata	As	Cd	Pb	Cu	Zn
Reference	Woods Inlet	3.33 ± 0.23	0.05 ± 0.01	0.10 ± 0.02	1.62 ± 0.28	11.6 ± 1.76
	One Fella Ck	3.52 ± 0.19	0.12 ± 0.02	0.16 ± 0.02	2.71 ± 0.61	14.2 ± 1.43
	Channel Is	2.01 ± 0.16	0.04 ± 0	0.07 ± 0.04	2.56 ± 0.21	10.8 ± 0.87
	Palmerston	2.66 ± 0.57	0.07 ± 0.01	0.14 ± 0.07	2.97 ± 0.65	17.2 ± 1.78
Sewage	Ludmilla Ck	2.80 ± 0.20	0.05 ± 0.01	0.16 ± 0.05	2.46 ± 0.03	16.8 ± 0.16
	Buffalo Ck	1.99 ± 0.46	0.06 ± 0.03	0.19 ± 0.08	2.11 ± 0.16	11.3 ± 0.01
Industrial	Hudson Ck	3.57 ± 0.21	0.08 ± 0	0.21 ± 0.05	4.11 ± 0.83	16.8 ± 0.52
	Reichardt Ck	4.52 ± 0.22	0.08 ± 0.01	0.32 ± 0.13	2.96 ± 0.20	14.7 ± 0.67
	S. cucullata	As	Cd	Pb	Cu	Zn
Reference	Woods Inlet	3.59 ± 0.74	0.97 ± 0.24	0.04 ± 0.01	23.9 ± 5.27	142 ± 39.3
	One Fella Ck	2.13 ± 0.06	0.74 ± 0.08	0.03 ± 0.01	19.8 ± 1.60	86.5 ± 14.5
	Channel Is	2.04 ± 0.29	0.42 ± 0.03	0.03 ± 0	24.1 ± 2.35	307 ± 30.2
	Palmerston	1.78 ± 0.19	0.32 ± 0.05	0.03 ± 0.01	14.8 ± 2.02	253 ± 34.5
Sewage	Ludmilla Ck	2.70 ± 0.51	0.43 ± 0.08	0.03 ± 0.01	103 ± 15.0	388 ± 89.2
Industrial	Hudson Ck	2.17 ± 0.23	0.34 ± 0.11	0.04 ± 0.01	26.9 ± 6.95	302 ± 69.9
	DL	0.03	0.002	0.001	0.03	0.10
FSANZ ML GEL (90th percentile)		1*	2	2	30	290

*maximum level is for inorganic arsenic

Mycoplasmatales and *Rhodobacteraceae* each contributed 10% to the relative bacterial abundance in *N. balteata*, with *Mollicutes* and *V. orientalis* adding around 7% each to the total bacterial population. Several taxa were restricted to only certain site types (Fig. 4). *Sphingomonas faeni* was only found in *N. balteata* from sewage sites. *Bartonellaceae* were present in *N. balteata* from both industry and sewage sites, but absent from reference sites. *Jannaschia* sp. belonging to the *Roseobacter* lineage of the *Alphaproteobacteria*, was present in *N. balteata* samples from reference and sewage sites, but not industry sites.

Spirochaetes also dominated *S. cucullata* samples making up 33% of all bacterial sequences. *Rhodobacteraceae* were present at a relative abundance of 11% and *Oceanospirillaceae* contributed 5% to the

Table 3 Sequence counts (reads) for shellfish samples. Blank cells indicate samples were not sent for 454 analysis

		T. telescopium		*T. palustris*		*N. balteata*		*S. cucullata*		Seawater		Sediment	
Site	Rep	Reads	OTUs	Reads	OTUs	Reads	OTUs	Reads	OTUs	Reads	OTUs	Reads	OTUs
Woods Inlet	1	17,579	243	13,958	1711	8871	526	3664	381	15,798	1101	4329	1481
	2	0		9032	1064	6748	681	311	141	10,722	931	4733	1444
	3	6164	297	16,317	1507	7910	967	5850	477				
One Fella Ck	1	0		4014	516	0	NTC	2716	288	11,278	738	3167	1441
	2	8816	679	3964	736	318	NTC	7134	772	12,621	755	5243	1748
	3	5517	677	3365	366	9923	535	10,831	879				
Channel Is	1	2197	680	1787	265	2659	317	2231	269	12,233	866	5619	1806
	2	5		1363	354	1052	142	5782	492	13,387	841	5517	1930
	3	1651	515	3121	638	2171	415	5619	270				
Palmerston	1	6138	969	NTC	NTC	7108	359	2870	214	17,612	825	5142	1718
	2	1910	565	NTC	NTC	6028	195	3745	351	11,209	670	5118	1960
	3	1921	369	NTC	NTC	5871	468	1802	166				
Ludmilla Ck	1	6874	1577	17,190	1349	18,573	843	14,739	841	8566	830	5831	1811
	2	9992	1887	8873	1408	13,578	699	11,985	966	7610	747	3567	1455
	3	3997	1069	6132	802	15,188	981	17,837	1262				
Buffalo Ck	1	18,788	1245	NTC	NTC	0	NTC	NTC	NTC	11,874	1029	5856	1926
	2	8249	1450	NTC	NTC	4820	149	NTC	NTC	8836	857	5030	1778
	3	15,141	275	NTC	NTC	3018	499	NTC	NTC				
Hudson Ck	1	4729	676	10,614	1017	19,940	739	8121	431	14,026	961	6867	1719
	2	5070	867	4124	648	10,348	728	9138	529	14,405	870	6145	1836
	3	8562	576	5925	1819	19,223	832	6430	454				
Reichardt Ck	1	3300	1122	NTC	NTC	11,492	890	NTC	NTC	12,407	923	5427	1890
	2	11,796	2023	NTC	NTC	2501	94	NTC	NTC	10,370	825	6008	1734
	3	14,716	2600	NTC	NTC	0	NTC	NTC	NTC				

NTC none to collect

bacterial community in *S. cucullata*. *Rhodobactereaceae* and its members *Rhodovulum* sp. and *Roseovarius nubinhibens* were prevalent at the reference and sewage sites but not at industry sites.

Table 4 Shannon's indices in biota, seawater and sediment at reference, sewage and industry sites. Asterisk indicates significant difference

	Reference	Sewage	Industry
T. telescopium	3.54 ± 0.29	3.59 ± 0.76	3.84 ± 0.21
T. palustris	3.45 ± 0.25	3.56 ± 0.26	3.69 ± 0.54
N. balteata	2.40 ± 0.47	2.47 ± 0.88	2.66 ± 0.53
S. cucullata	2.41 ± 0.57	3.10 ± 0.27	2.77 ± 0.44
Seawater	2.71 ± 0.17	3.29 ± 0.30*	2.73 ± 0.17
Sediment	3.92 ± 0.14	3.99 ± 0.06	3.96 ± 0.20

Sequences identified in seawater samples were dominated by the cyanobacterium *Prochlorococcus* (22%), the *Alphaproteobacteria Pelagibacter ubique* (10%) and *Nautella* (9%), and the gamma proteobacterium *Oceanospirillaceae* (8%) making up nearly 50% of the total sequences identified. Several less abundant taxa occurred in sediment with *Deltaproteobacteria* (14%), *Gammaproteobacteria* (*Ectothiorhodospiraceae* and Chromatiales) (10%), *Actinobacteria* (4%) and *Nitrospira* (4%) making up 32% of the total sequences. Sediment microbial communities shared many common taxa across sites (Fig. 4).

Pathogens

Seven bacterial species known to be human pathogens and faecal indicators in the shellfish were identified and

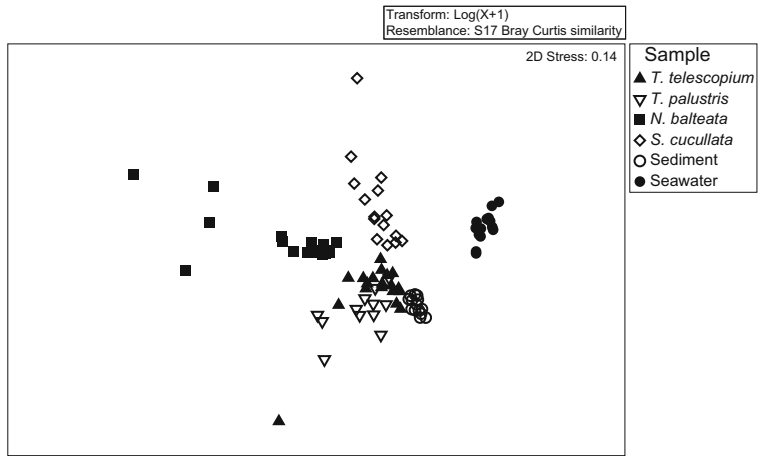

Fig. 3 MDS plot showing relationship of shellfish, sediment and seawater samples based on microbial communities (identified organisms)

they were *Aeromonas veronii*, *Clostridium perfringens*, *Enterococcus faecalis*, *Escherichia coli/Escherichia fergusonii*, *Francisella tularensis*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. In our data, *E. coli* was listed with *E. fergusonii* as these species share 100% sequence homology in the gene analysed (i.e. the sequence data could not distinguish between these two species). Members of the *Campylobacteraceae*, *Bacteroidia* and *Rickettsiaceae* were also present in shellfish but these were not further resolved. *Vibrio cholerae* was detected in seawater only, but at very low frequency. These potentially pathogenic bacteria represent <0.03% of the total bacteria in the shellfish samples with some shellfish specimens recording no pathogenic bacteria. *S. epidermidis* was the most abundant pathogenic species (76 counts) and *A. veronii* was the least abundant (3 counts) across all shellfish species. Pathogen diversity was very high between samples, within sites and between sites. There was no evidence that there were more pathogens at sewage or industry impacted sites. *E. coli* was detected in only one shellfish sample, a *T. palustris* from Channel Island. *E. coli* was detected in some seawater samples at very low frequency but was not detected in any of the sediment samples (results not shown). *E. faecalis* were identified in two *T. telescopium* samples from Buffalo Ck and one *S. cucullata* sample from Hudson Ck, but their abundance was low compared to the total bacterial population.

Differential taxa abundances in biota across sites (based on DESeq)

We measured 28 differentially abundant OTUs that had an adjusted (FDR corrected) P value <0.05. The results show that OTUs assigned to the species *Ralstonia pickettii* were more abundant in *T. telescopium* samples from reference sites, while *Planctomycetaceae*, *Oceanicola*, *Rhodobacteraceae*, *Erythrobacter piscidermidis*, *Phyllobacteriaceae*, *Planctomyces*, and *Burkholderiales* were more abundant in samples from industry sites (Fig. 5). Only OTUs assigned to *Planctomycetaceae* was more abundant in *T. telescopium* samples from sewage sites.

T. palustris samples at reference sites were characterised by significantly greater abundances of TG3 candidate division and the cyanobacterium *Fischerella* compared to either sewage or industry sites (Fig. 6). In addition, *Propionigenium*, *Mollicutes*, and *Colwelliaceae* were more abundant in samples from reference compared to industry sites. Taxa that were less abundant in *T. palustris* samples from reference sites were *Pelagibaca roseovarius* sp., *Rhodobacteraceae*, *Pelagibaca* sp., *Antarctobacter*, *Alteromonas*, *Rhodobacterales*, and *Erythrobacter aquimaris*. Additional taxa that were less abundant in *T. palustris* samples from reference compared to industry sites were *Planctomycetales*, *Prochlorococcus* spp. and *Oceanicola* sp. and taxa that were less abundant in samples from reference compared to sewage sites were *Celerinatantimonas diazotrophica*, *Oscillatoriales* and *Dinoroseobacter* (Fig. 6).

In *N. balteata* samples, only 1 taxon, *Flavobacteriaceae* was significantly more abundant in reference sites compared to sewage sites, with no differences observed with industry sites. No significant increases in abundance were identified in any of the oyster samples across sites.

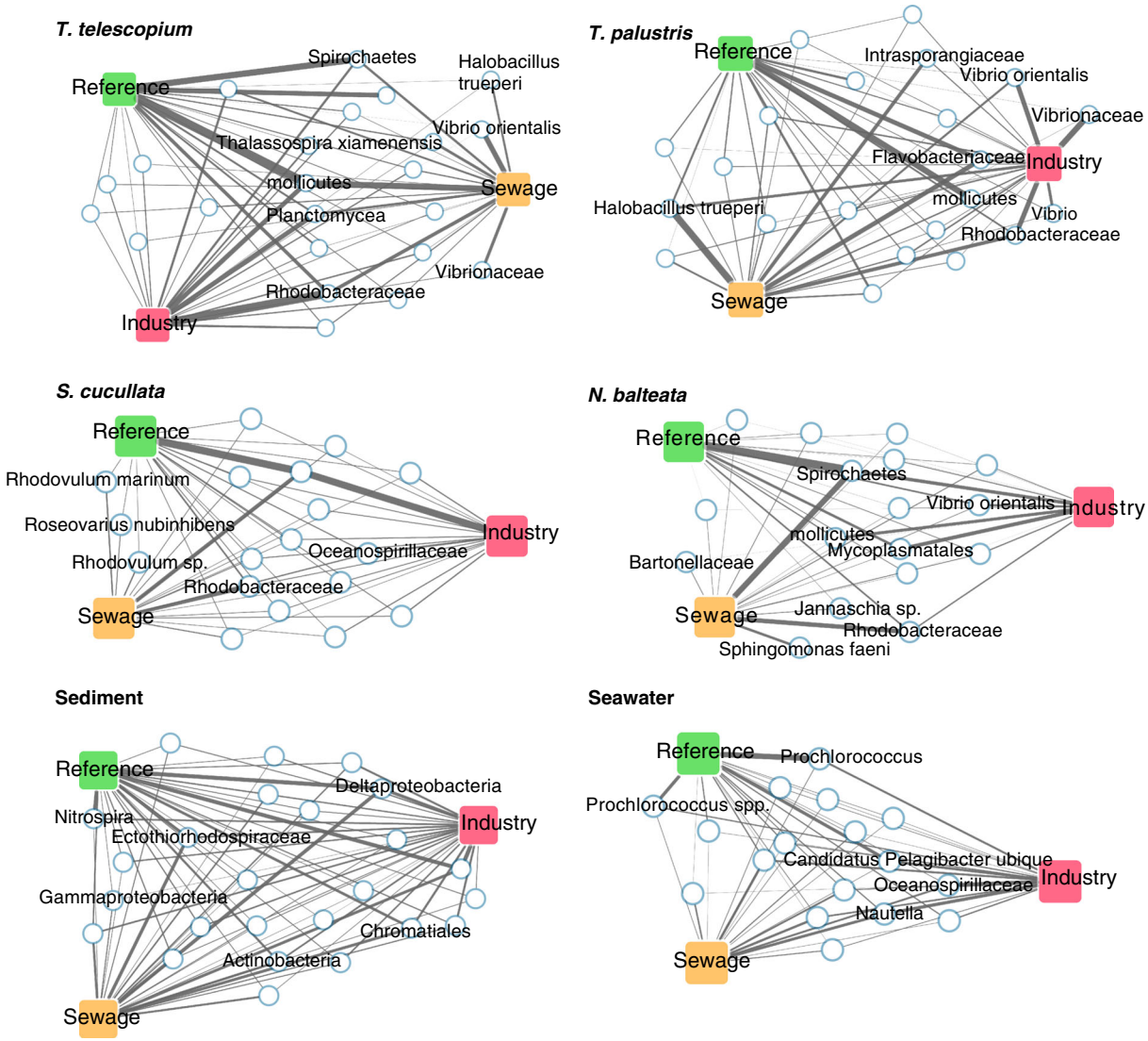

Fig. 4 Cytoscape network analysis of bacterial taxa in *T. telescopium*, *T. palustris*, *S. cucullata*, *N. balteata*, sediment and seawater samples from reference, industry and sewage sites. *Line thickness* corresponds to relative abundance and label size corresponds to their SIMPER contribution. Taxa with <3% total abundance were removed from the analysis

Discussion

Shellfish near sewage outfalls and industrial areas did not contain higher concentrations of metals/metalloids than shellfish from reference sites. *S. cucullata* were the best bio-accumulator of cadmium with respect to seawater, especially at the control sites, but concentrations did not exceed the FSANZ maximum levels. Concentrations did vary between sites, especially for Cu and Zn, but concentrations were high in shellfish at both reference and impacted sites and importantly, the levels of these metals in the environment at all these sites were extremely low. Similar results for *T. telescopium* were reported by French (2013), but in a separate study in a heavily polluted area, cadmium, mercury and zinc concentrations in sediment were significantly correlated with concentrations in mussels but not oysters (Rojas de Astudillo et al. 2005). In this study, a lack of correlation between metal/metalloid concentrations in seawater or sediment and shellfish means that routine environmental assessment must include biota because water and sediment alone are not sufficiently good surrogates for the safety of marine harvest. Although we do not

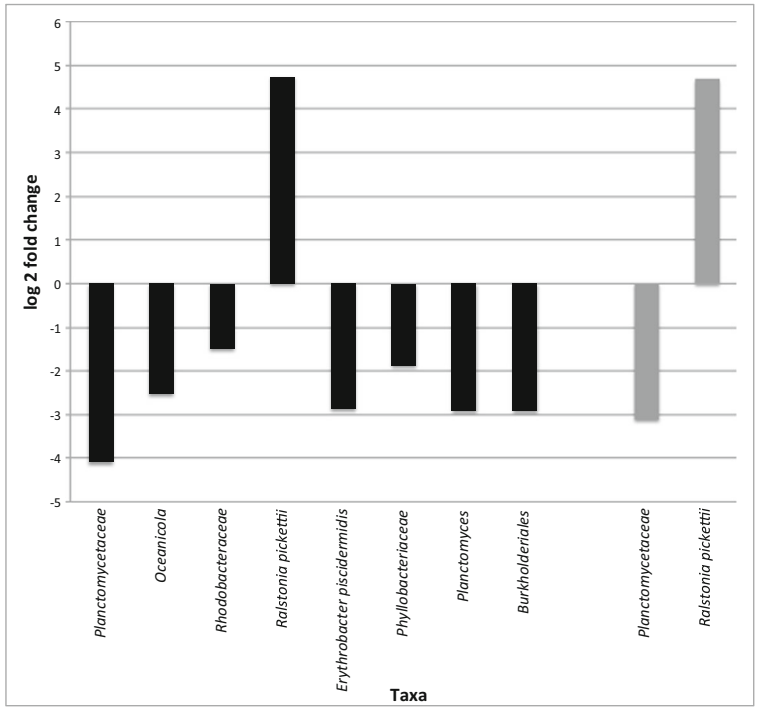

Fig. 5 Differentially abundant taxa in *T. telescopium* samples from reference sites compared to industry (*black squares*) and sewage sites (*gray squares*). The order of taxa from *left* to *right* is from greatest significance to least significance

know the mechanisms involved in metal accumulation by shellfish at these sites, it would be helpful to know if this is true at all times of the year, or if there are 'unsafe' times to collect and eat *S. cucullata* and other shellfish from these sites.

The percentage of inorganic arsenic in marine animals is variable ranging from 0.1 to 6.7% for molluscs and crustaceans (Sirot et al. 2009), 3% in mussels (Buchet et al. 1994) and 1.53–5.44% in clams and molluscs (Muñoz et al. 2000). If we assume that the shellfish in this study have an inorganic arsenic component of 7%, all the shellfish in this study are well below the FSANZ maximum level of 1 mg/kg. However, it is impossible to conclusively determine whether arsenic poses a health risk without first differentiating between the organic and inorganic proportions.

Next-generation sequencing enabled a huge diversity of shellfish-associated microbes to be captured, describing comprehensive flora in the studied species for the first time. Despite the great bacterial diversity observed, the mangrove snails had 4–6 dominant taxa, while *S. cucullata* and *N. balteata* had one dominant taxon each. Each shellfish species had a bacterial community that was distinct from the other species and from the surrounding seawater and sediment suggesting a permanent and consistent cohort of flora in the biota. These microbial communities may reflect different feeding guilds with the detritivore feeding mud snails supporting a greater diversity of bacteria compared to the algal-feeding mangrove snails and water filtering oysters. We do not know if these associations are stable over time, but consistent associations have been reported for other marine invertebrates from different phyla (see references in Harris 1993). Microbial studies of other oyster species also reported that only a few OTUs are dominant but the OTUs are different for different oyster species (Wegner et al. 2013 and references therein).

While particular shellfish species had distinctive microbiomes, individuals within each species also showed a substantial amount of bacterial community variation suggesting either an environmental influence on microbial community composition (e.g. what they are eating at the time) and/or an individual or genetic component (i.e. their particular physiology favours certain microbes). Bacteria taxa such as *Planctomyceae* and *Rhodobacteraceae* in the mud snails had significantly different abundances across sites and may be useful to signal environmental changes. However, they would need to be tested more extensively and cost-effective tests such as quantitative PCR would be required if they were to be included in routine surveillance programs. Interestingly, microbial community analysis of the water

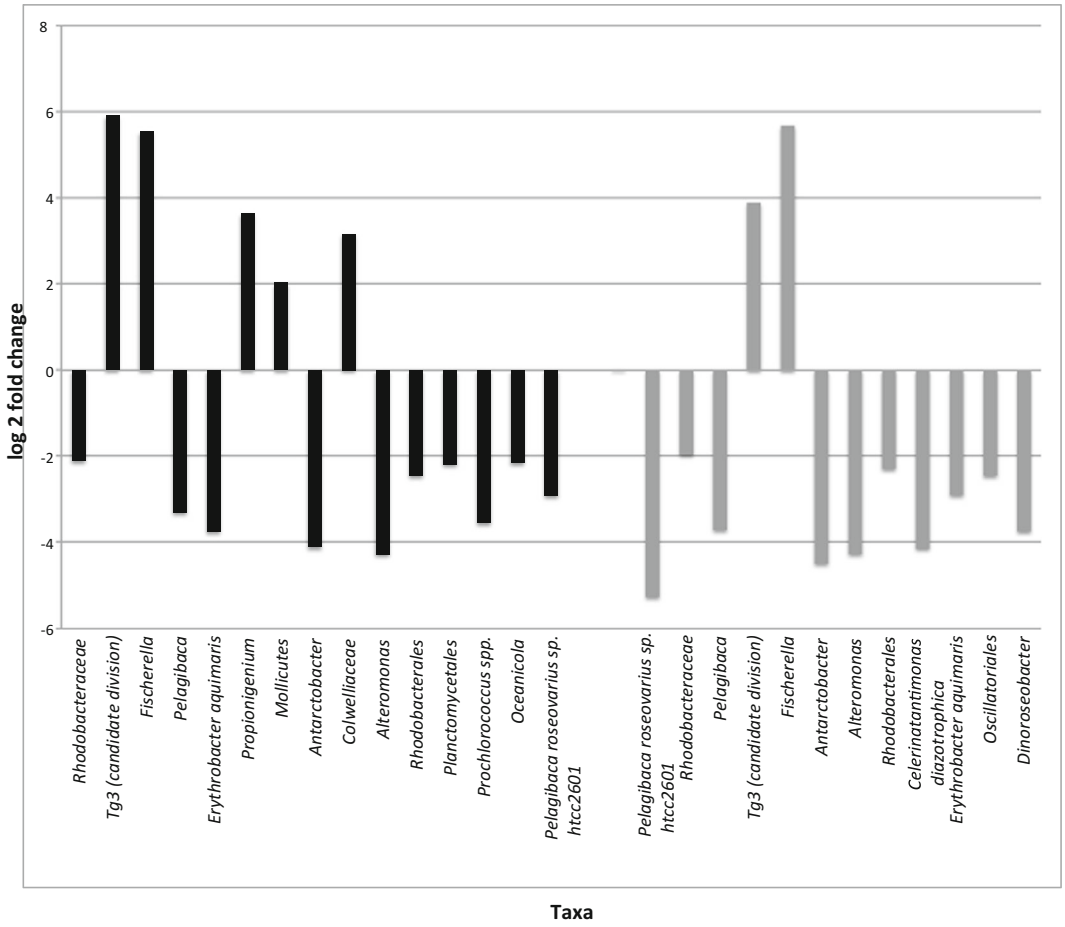

Fig. 6 Differentially abundant taxa in *T. palustris* samples from reference sites compared to industry (*black squares*) and sewage (*gray squares*) sites. The order of taxa from *left* to *right* is from greatest significance to least significance

filtering oysters did not identify taxa that could discriminate between background and impacted sites.

Pathogens were identified in several biota samples, but overall, counts were very low with no relationship to site. Even in wastewater samples, pathogens may represent only a small percentage of the total microbial population (Bibby et al. 2010; Ye and Zhang, 2011). Deeper sequencing, use of different primers or increased sequencing length may improve the detection and resolution of pathogens or new technologies such as the pathogen microarray (Li et al. 2015) could be helpful if adapted to shellfish.

Conclusions

In conclusion, this study showed that shellfish from Darwin Harbour did not exceed FSANZ maximum levels for Cd, Pb or estimated inorganic As. The lack of correlation between metal/metalloid concentrations in shellfish and their environment means that assessment of aquatic foods must include biota and not rely on water or sediment. Distinctive bacterial communities were found in each shellfish species regardless of location, but the abundance of several taxa such as *Rhodobacteraceae*, were significantly different between sites in *T. telescopium* and *T. palustris* samples, and in *N. balteata*, *Flavobacteriaceae* was significantly more abundant in reference sites compared to sewage sites. The abundance of potential human pathogens was low in all biota and environmental samples, and not related to site. While next-generation sequencing was not able to resolve pathogens, it provided a shortlist of pathogenic species to target by other methods such as microarray or qPCR. From a microbial and metal/metalloid analysis, we conclude that the shellfish analysed

in this study are safe to eat and the data serves as a baseline for further studies.

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