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Research article

Bacterial microbiomes from mucus and breath of southern resident killer whales (*Orcinus orca*)

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Opportunities to assess odontocete health are restricted due to their limited time at the surface, relatively quick movements and large geographic ranges. For endangered populations such as the southern resident killer whales (SKRWs) of the northeast Pacific Ocean, taking advantage of non-invasive samples such as expelled mucus and exhaled breath is appealing. Over the past 12 years, such samples were collected, providing a chance to analyse and assess their bacterial microbiomes using amplicon sequencing. Based on operational taxonomic units, microbiome communities from SRKW and transient killer whales showed little overlap between mucus, breath and seawater from SRKW habitats and six bacterial phyla were prominent in expelled mucus but not in seawater. Mollicutes and Fusobacteria were common and abundant in mucus, but not in breath or seawater, suggesting these bacterial classes may be normal constituents of the SRKW microbiome. Out of 134 bacterial families detected, 24 were unique to breath and mucus, including higher abundances of *Burkholderiaceae*, *Moraxellaceae* and *Chitinophagaceae*. Although there were multiple bacterial genera in breath or mucus that include pathogenic species (e.g. *Campylobacter*, *Hemophilus*, *Treponema*), the presence of these bacteria is not necessarily evidence of disease or infection. Future emphasis on genotyping mucus samples to the individual animal will allow further assessment in the context of that animal's history, including body condition index and prior contaminants burden. This study is the first to examine expelled mucus from cetaceans for microbiomes and demonstrates the value of analysing these types of non-invasive samples.

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Introduction

Although killer whales (*Orcinus orca*) have a worldwide distribution, their total numbers are unknown. Although killer whales are taxonomically a single species, distinct populations are differentiated on appearance, behaviour, prey preferences and habitat use patterns. In the eastern North Pacific Ocean along the continental USA and Canada, two sympatric ecotypes of killer whales have been described: resident killer whales that eat exclusively fish and transient killer whales that eat primarily marine mammals (Ford *et al.*, 1998). In spite of differences in dietary preference, resident and transient killer whales have significant overlap in habitat occupancy. A specific population of resident killer whales known as southern resident killer whales (SRKWs) has declined from 96–98 individuals in the mid-1990s to a current number of 73 individuals (Center for Whale Research; https://www.whaleresearch.com/orca-population). SRKWs were

listed as endangered under the US Endangered Species Act (ESA; National Marine Fisheries Service, 2005) and Canadian Species at Risk Act. Earlier and recent US status reviews identified three factors posing a significant risk to their future viability: reduced quantity and quality of prey, persistent anthropogenic contaminants that cause immune or reproductive dysfunction, and disturbance due to vessel traffic and associated noise (Krahn *et al*., 2004; National Marine Fisheries Service, 2008; National Marine Fisheries Service, 2016). Reduced prey quantity and quality can not only create metabolic deficiencies but also force shifts in habitat occupancy (Hanson *et al*., 2021) and, potentially, more energy expended for hunting. The risks from persistent contaminants are acute toxicity and sublethal effects, including increased susceptibility to infections or diseases through immunosuppression (Mongillo *et al*., 2016). Because killer whales employ sounds while foraging (Holt *et al*., 2019; Tennessen *et al*., 2019), underwater noise from marine vessel traffic can interfere with the acoustics of prey detection and pursuit (Houghton *et al*., 2015). These threats, alone or in combination, and their cumulative effects can reduce individual animal ability to respond to infection or pathogens through multiple immunological and physiological mechanisms (e.g. Mongillo *et al*., 2016), resulting in infectious disease as an immediate or proximate cause of death (Raverty *et al*., 2020). The coastal and inland waters occupied by SRKWs receive significant terrestrial and anthropogenic inputs from watersheds with high potential for biological pollution with pathogenic agents. For example, in March 2021 more than 85 marine water bodies in Washington State were listed as impaired (also called '303d listed') due to bacterial contamination by the State's Department of Ecology (https://ecology.wa.gov/Water-Shorelines/Water-quality/Water-improvement/Assessment-of-state-waters-303d; accessed 19 March 2021). As SRKWs transit and reside in these waters, they are exposed to these water-borne agents. In the most recent action plan for SRKWs developed by National Marine Fisheries Service, the priority actions for 2021–2025 include improved knowledge of SRKW health to provide better capabilities to assess and inform for recovery and emergency response (https://www.fisheries.noaa.gov/resource/document/species-spotlight-priority-actions-2021-2025-southern-resident-killer-whale).

Opportunities to investigate cause of death or even conduct physiological assessments of SRKWs rely primarily on opinions of expert field observers, necropsy findings and remote techniques such as photogrammetry (Fearnbach *et al*., 2018; Raverty *et al*., 2020). SRKWs and nearly all cetaceans spend limited time at the surface, move quickly and have large geographic ranges, making them elusive for studies of their behaviour, ecology and physiology (Mann and Karniski, 2017). Because invasive research techniques may pose hazards for SRKWs (e.g. L95 Expert Panel Report, September 2016; https://archive.fisheries.noaa.gov/wcr/publications/protected_species/marine_mammals/killer_whales/l95_expert_panel.pdf), exploiting non-invasive samples for these purposes becomes increasingly important. Killer whale faecal samples have been useful for assessing stress and pregnancy status through hormone analyses and for anthropogenic contaminants (Ayres *et al*., 2012; Lundin *et al*., 2016; Wasser *et al*., 2017), while exhaled breath, or 'blow', has been analysed for pathogens (Raverty *et al*., 2017). Respiratory infections and diseases are a common cause of mortality in marine mammals (Venn-Watson *et al*., 2012) and a life-threatening risk for diving marine mammals. The hyperbaric conditions of diving impose demands on respiratory physiology, causing compression and collapse of the lower tract to potentially interrupt gas exchange and avoid decompression sickness (Denk *et al*., 2020; Moore *et al*., 2011). Collection and analysis of exhaled breath from large cetaceans is relatively well developed (Apprill *et al*., 2017; Burgess *et al*., 2018; Hunt *et al*., 2013), but continues to pose challenges for smaller, free-ranging cetaceans (Raudino *et al*., 2019; Robinson and Nuuttila, 2020). Over an 11-year period, non-invasive samples of mucus and exhaled breath from SRKWs were collected, primarily from living animals. This collection provides a unique opportunity to evaluate the utility of mucus and breath for microbiome analysis. This analysis can be the basis for characterization of commensal bacterial communities, identification of potential opportunistic and pathogenic microorganisms and future discovery of biomarkers useful for health assessments.

Materials and methods

Field collection and sample handling

Samples were collected from both SRKWs and transient killer whales from 2009 to 2019 throughout much of the known range for SRKWs (i.e. outer US Pacific Northwest coast and into the Salish Sea). Expelled mucus was identified by following a focal group (Hanson *et al*., 2010). Samples were scooped from the water surface with a long-handled (4 m), fine-mesh net and either placed directly into a sterile 50-ml polypropylene tube or wiped from the net with sterile gauze and placed in a sterile plastic bag or tube. The bag or tube was kept cool and dark until transfer to long-term storage at −20°C. Dates of collection, pods in the focal group and general sampling locations are displayed in Supplementary Table 1.

Breath collection employed pole-mounted sterile Petri dishes covered with sterile 100-μm mesh nitex and required the pole operator to pass the dish through the exhaled plume. After sampling, dishes were enclosed in sterile Whirl-Pak bags and held on chiller packs in the dark until returning to shore. Within 12 hours of collection, the nitex mesh was aseptically separated from the dish and placed into a sterile 50-ml polypropylene tube. The petri dish was rinsed with 3–4 ml of RNALater™, transferred to the tube holding the nitex mesh, which was vigorously shaken and stored at 4°C or −20°C until long-term storage at −20°C.

All animal samples were collected under NOAA permits #781-1824, #16163 and #21348, and collection protocols

were reviewed and approved under IACUC protocols NWAK-18-01 and A/NW 2014-02 (NWFSC ESA/MMPA 5-year Marine Mammal Research Permit).

Seawater samples were collected within the known range of SRKWs in coastal waters of the USA from northern California to Washington and around the San Juan Islands during spring and summer months of 2016 and 2017 (Supplementary Table 2). Water samples (up to 1 l) were collected either from Niskin bottles or by submerging a clean bottle at the surface. Water was kept cool and in the dark until filtered through a 0.2-μm polyethersulfone filter. Filters were flash-frozen in liquid nitrogen until long-term storage at −80°C.

Sample processing

Mucus

Subsampling was performed in a sterile biological safety cabinet, and aseptic benchtop techniques were used to prevent environmental and cross contamination. DNA from mucus was extracted using TRI Reagent® (Molecular Research Center Inc, Cincinnati, OH, USA) following manufacturer's instructions. Briefly, ∼100 mg of sample and 1 ml of TRI Reagent® were vigorously shaken for 5 min, subjected to three cycles of freeze/thaw with liquid nitrogen, extracted with chloroform and back-extracted with buffer (1 M Tris base, 50 mM sodium citrate, 4 M guanidine thiocyanate). DNA was precipitated with isopropanol, resuspended in 1× TE (10 mM Tris pH 8.0, 1 mM EDTA) and measured with Qubit™ fluorometric quantitation (Invitrogen, Waltham, MA, USA).

Breath

Breath droplets and the RNALater™ were centrifuged from the nitex mesh and aseptically filtered through a 0.2-μm polyethersulfone filter in a sterile biological safety cabinet. The filter was treated with lysozyme (1 mg ml^{-1}) for at least 30 min at 37°C, then incubated overnight at 55°C with proteinase K (0.1 mg ml-1) and 1% sodium dodecyl sulfate. The lysate was sequentially extracted with phenol:chloroform and chloroform, and DNA was precipitated with isopropanol and subsequently handled as described for mucus. Aseptic benchtop techniques were used to minimize environmental and cross contamination.

Water

Water filters were thawed and immediately processed as described for the filtered breath samples above. Aseptic benchtop techniques were used to minimize environmental and cross contamination.

Library preparation, sequencing and bioinformatics

We prepared 16S ribosomal RNA amplicon libraries with dual indices using Nextera XT primers (forward primer: 5′—TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer: 5′—GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) according to the manufacturer's protocol with the following modifications. Working primer concentrations were 37.5 μM and contained 3.12 mg of purified bovine serum albumin μl^{-1}. The more concentrated working primers were used at 0.133 μl per reaction for the same final primer concentration prescribed in the manufacturer's protocol. The remaining reaction volume (12.23 μl) was template DNA. Both magnetic bead cleanups used twice the prescribed amount of bead to increase recovery. Samples were analysed using the Illumina MiSeq reagent kit v3 (600 cycles) on a MiSeq sequencer (Illumina, San Diego, CA, USA). Sequence reads were trimmed for quality using Trimmomatic (Bolger *et al.*, 2014) and paired ends were assembled using PANDAseq (Masella *et al.*, 2012). Additional sequence filtering removed sequences with lengths less than 400 base pairs (bp) and with homopolymers and ambiguous bases greater than 7 bp. Based on a mock community of equimolar amounts of genomic DNA from 14 known bacterial species, reads with a frequency of <21 in any one sample were discarded as sequencing errors. Highly similar sequences (≥97% identity) were grouped into operational taxonomic units (OTUs) using QIIME2 v.2019.4 (Bolyen *et al.*, 2019), and these OTUs were treated as the highest resolution taxon for community analyses. Bacterial taxonomic identifications were made by comparison against the Silva SSU database, release 132 (Quast *et al.*, 2013; Yilmaz *et al.*, 2014).

Community structure analyses and data management

Statistical and multivariate analyses used Primer version 7 (PRIMER-E, Auckland, New Zealand) and STATA SE 12.1 (StataCorp, College Station, TX, USA). OTUs and taxonomic counts were standardized by sample for relative abundance analyses, and transformed (log(x) + 1), when appropriate. Sequence and associated metadata are available in the Sequence Read Archive repository of the National Center for Biotechnology Information, under BioProject ID PRJNA752106.

Results

A total of 62 mucus, 13 breath and 25 seawater samples were analysed. Among the mucus samples 55 were collected from SRKWs and 7 were collected from transient whales. Twelve breath samples were from SRKWs and one was from a transient killer whale. Across all sample types, 919 OTUs were identified and the Silva database (release 132) was able to classify all OTUs to the Phylum level. As expected, fewer OTUs could be classified at lower taxonomic levels (918 to Class, 902 to Order, 749 to Family and 503 to Genus). Mucus samples produced an average of 196 OTUs (range, 66–350; $N = 62$), while breath samples produced an average

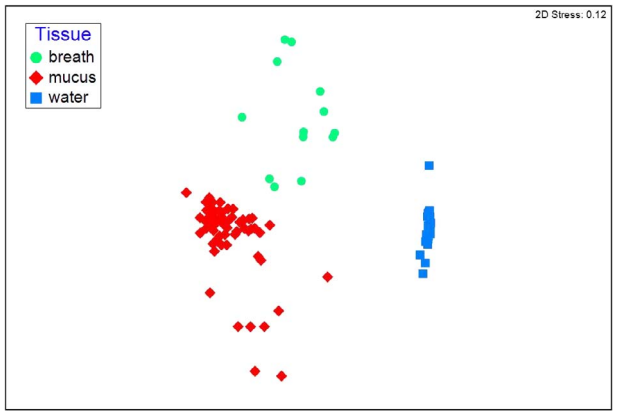

Figure 1: Non-metric multidimensional scaling plot of bacterial communities (based on OTUs) by sample type. Samples that are closer together are more similar in community structure. Calculated stress for the two dimensions is 0.12.

Table 1: Numbers of bacterial families unique to sample types and sample type combinations

Sample type or sample type combination	Number of Families
Mucus only	0
Breath only	6
Water only	9
Mucus and breath	24
Mucus and water	9
Breath and water	4
Mucus and breath and water	82

of 292 OTUs (range, 52–502; $N = 13$). In contrast, seawater samples averaged 393 OTUs (range, 317–510; $N = 27$). Although storage times varied widely, there was no evidence of a temporal trend in univariate diversity metrics among the mucus samples, which was the most abundant sample type (Supplementary Figure 1). Analysis of variance by sampling year for Shannon diversity index (H′), Margalef's index (*d*), and Pielou's evenness index (J) was not significant ($F \geq 1.15$; $P > 0.181$).

Bacterial communities were different among the sample sources, with most of the mucus samples exhibiting close similarity (Fig. 1). Analysis of similarity of mucus samples did not detect a significant difference between ecotypes (significance, >0.12) or locations (significance, >0.70). Breath sample communities were more varied, but still distinct from mucus, and both types of samples were strongly different from seawater communities (Fig. 1). Communities from each sample type strongly differentiated and exhibited very low similarity among sample types (<20%; Supplementary Figure 2). Although there was some tendency of subsets of samples collected in the same year to cluster, there was no gradient or clear pattern of clustering based on age of the sample (Supplementary Figure 2).

Taxonomic identification of communities revealed differential distributions among the sample types. At the phylum level, Proteobacteria and Bacteroidetes were common among all sample types with high relative abundance (Fig. 2). Communities from mucus samples contained the greatest diversity of phyla with high abundances (seven phyla). Four phyla that were prominent in mucus samples showed low representation in other sample types: Fusobacteria, Tenericutes, Patescibacteria and Spirochaetes. Episilonbacteraeota and Firmicutes were also prominent in mucus samples and moderately abundant in breath samples. The phylum WPS-2 (or Eremiobacterota) occurred nearly exclusively in breath samples. In contrast, Cyanobacteria and Verrucomicrobia were highly abundant in water but not the biological samples (Fig. 2). Furthermore, Euryarchaeota (kingdom Archaea) occurred in water samples, but were absent or very low abundance in killer whale samples (data not shown).

At the class level, Gammaproteobacteria (Proteobacteria) and Bacteroidia (Bacteroidetes) were common and highly abundant in mucus and breath (Fig. 3). Betaproteobacteria (Proteobacteria), Clostridia (Firmicutes) and Campylobacteria (Epsilonbacteraeota) were common and highly abundant in mucus, but less common and less abundant in breath. Mollicutes (Tenericutes) and Fusobacteriia (Fusobacteria) were distinctive for mucus, as these classes were absent or in very low abundance in breath and seawater. In comparison, Alphaproteobacteria (Proteobacteria), Actinobacteria (Actinobacteria), Oxyphotobacteria (Cyanobacteria) and Bacilli (Firmicutes) were more common and more abundant in breath than in mucus (Fig. 3).

Among the 134 families identified, 61% were present in all sample types, 28% were present in two sample types and 11% were present in only one sample type (Table 1). Although mucus contained no unique families, the greatest overlap in families was between mucus and breath.

Families comprising ≥5% of the average relative abundance for each sample type accounted for most of the total abundance (range, 63% to 93%; Supplementary Figure 3). *Burkholderiaceae* was abundant in both mucus and breath samples, and *Moraxellaceae* was common to mucus and breath communities (Table 2). Consistent with the observations for OTUs (Fig. 1), there was little overlap between families observed in seawater and in breath or mucus.

Cluster analysis and similarity profiling identified subsets of families that significantly co-occurred with each other for each sample type. There were 22 subsets in mucus samples (range, 2–7 families per subset; median, 2 families), 23 subsets in breath samples (range, 2–5 families per subset; median, 2 families) and 18 taxon subsets in water samples (range, 2–10 families per subset; median: 4 families; Supplementary Table 1). Common and high-abundance families (Table 2) were well represented in these

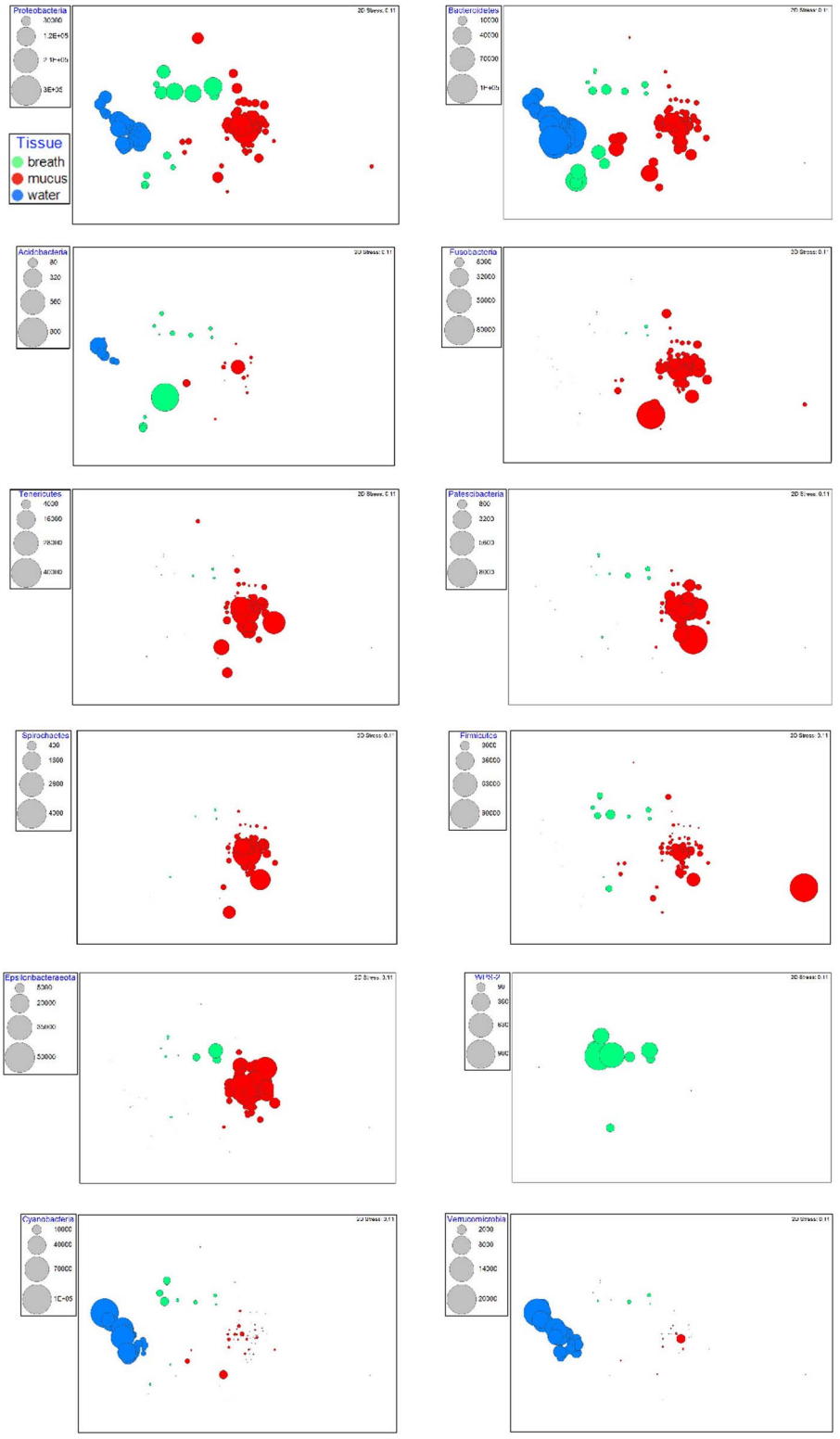

Figure 2: Non-metric multidimensional bubble plots of bacterial phyla for all samples with colour key by sample type. Bubble diameter shows relative abundance of each phylum by sample with respective key adjacent to each plot. Calculated stress for the two dimensions is 0.11.

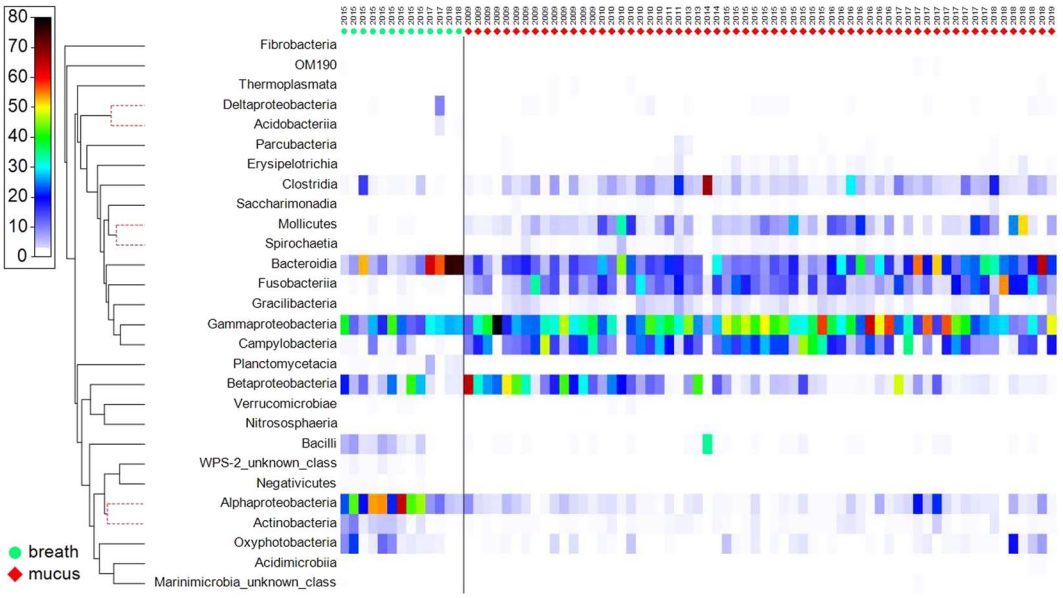

Figure 3: Shade plot of relative abundance of bacterial classes for mucus and breath samples. Raw abundances were standardized by sample before plotting, so that the shade key is the percentage of abundance. Samples are arranged by year of collection within each biological sample type. Bacterial classes are ordered by the cluster dendrogram on the left, where classes connected by red dashed branches are statistically indistinguishable by similarity profile permutational analysis ($P \leq 0.05$; cophenetic correlation, 0.82313).

Table 2: Common and abundant bacterial families by sample type

Breath	Mucus	Water
Burkholderiaceae	*Burkholderiaceae*	*Flavobacteriaceae*
Moraxellaceae	*Moraxellaceae*	*Rhodobacteraceae*
Chitinophagaceae	*Chitinophagaceae*	Chloroplast (Oxyphotobacteria)
Cyclobacteriaceae	*Arcobacteriaceae*	
Beijerinckiaceae	*Cardiobacteriaceae*	
Pseudoaltermonadaceae	*Leptotrichiaceae*	
Caulobacteraceae	*Saccharospirillaceae*	
Sphingomonadaceae	*Mycoplasmataceae*	

Listed families represented ≥5% of the average relative abundance for each sample type.

subsets for mucus and water samples. In mucus samples, *Saccharospirillaceae* and *Arcobacteraceae* occurred together, while *Mycoplasmataceae* and *Leptotrichiaceae* each co-occurred with *Campylobacteraceae*, *Porphyromonadaceae*, *Lachnospiraceae* and *Spirochaetaceae*. In water samples, *Flavobacteriaceae*, *Cryomorphaceae*, *Porticoccaceae* and *Rhodobacteraceae* occurred together. In contrast, none of the common and high-abundance families in breath samples co-occurred with other families (Supplementary Table 1).

At the genus level, there were many differences between breath and mucus samples. In breath samples, 20 bacterial genera represented ~75% of the average total relative abundance, with *Reichenbachiella*, *Ekhidna*, *Methylobacterium*, *Prevotella* and *Arcobacter* ranking among the top five (Supplementary Table 2). In mucus samples, nine bacterial genera represented a similar percentage of average total relative abundance, with *Arcobacter*, *Cetobacterium*, *Mycoplasma*, *Oceanivirga* and *Gangjinia* ranking among the top five.

Multiple genera that include known pathogenic species were detected in breath and mucus samples, and there was considerable overlap in genera for both sample types (Table 3). In breath samples, the most abundant of potentially pathogenic genera was *Aeromonas* (average relative abundance, 2100), while the remaining genera had relatively low abundance (<1000). In mucus samples, *Mycoplasma*

Table 3: Genera encompassing pathogenic species detected in breath and mucus samples that had an average relative abundance of ≥100; none of these genera occurred at an average relative abundance of ≥100 in seawater samples

Breath samples	Mucus samples
Mycoplasma	*Mycoplasma*
Campylobacter	*Campylobacter*
Staphylococcus	*Staphylococcus*
Porphyromonas	*Porphyromonas*
Helcococcus	*Helcococcus*
Streptococcus	*Serratia*
Hemophilus	*Clostridium*
Aeromonas	*Fusobacter*
	Treponema 2
	Ureaplasma
	Escherichia-Shigella

was a high-abundance genus (average relative abundance, 68 000), while *Porphyromonas*, *Helcococcus*, *Campylobacter*, *Fusobacterium*, *Treponema* and *Clostridium* exhibited moderate abundance (2700–8600; Supplementary Table 2). The remaining potentially pathogenic genera in mucus samples occurred at relatively low abundance (<1000; Supplementary Table 2).

Discussion

Health surveillance of free-ranging marine mammals is an important aspect of their conservation (Hunt *et al.*, 2013), especially for threatened or endangered populations. Non-invasive samples are appealing as they reduce risk of injury or infection, but their value in health evaluation needs assessment. Faecal and exhaled breath samples have been used to measure hormone levels (Ayres *et al.*, 2012; Burgess *et al.*, 2018; Wasser *et al.*, 2017), although breath sampling from active cetaceans can be challenging (Raudino *et al.*, 2019; Robinson and Nuuttila, 2020). In this study, we explored the utility of expelled mucus samples collected over an 11-year period and exhaled breath samples collected over a 4-year period for microbiome assessment, anticipating that these samples would be representative of the upper respiratory tract. The results showed that these types of samples have good potential for microbiome analysis. The observation that long-term storage at ≤−20°C did not have strong effects on the bacterial communities contained in the samples supports the importance of opportunistic sample collection. We also found that mucus and breath microbiomes are distinctive from each other and that microbiomes from both biological samples are different from seawater microbiomes (Fig. 1, Supplementary Figure 2). These observations provide confidence that the samples are representative of different anatomic regions of the animal and that bacterial contamination from seawater is not a major concern for the sampling methods we used.

Five phyla (Tenericutes, Fusobacteria, Spirochaetes, Epsilonbacteraeota, Patescibacteria) occurred exclusively or nearly exclusively in mucus samples compared to breath and seawater samples (Fig. 2). Tenericutes are comprised of a single class, Mollicutes, which have small genomes consistent with a parasitic lifestyle and includes known pathogens of respiratory tracts of mammals (Razin, 2006). Mollicutes have been detected in low abundance in humpback whale breath (Apprill *et al.*, 2017) and bottlenose dolphin upper respiratory tract (Johnson *et al.*, 2009). In our samples, the relative abundance of Mollicutes averaged 6.3% (median, 3.3%) and in one sample represented up to 46.3% of detected taxa, showing that it is a common community member in SRKW mucus. Our findings contrast with those of Bik *et al.* (2016) where Mollicutes were more abundant in dolphin gastrointestinal samples than in expelled blowhole samples. This contrast may be due to a difference in volitional release of mucus by SRKWs in a natural setting and the 'on-demand' release in the dolphin collection. Physiologically, there is potential interconnection between the upper respiratory and gastric tracts in cetaceans, so mucus samples are likely to contain microbiomes from both systems.

Fusobacteria, which includes only two families (*Fusobateriaceae* and *Leptotrichiaceae*), are abundant in oral, gastric and rectal microbiomes of dolphin, harbour seal and sea lion, but at low abundance in upper respiratory tract microbiomes (Bik *et al.*, 2016; Johnson *et al.*, 2009; Palmer *et al.*, 2020; Robles-Malagamba *et al.*, 2020). Interestingly, *Fusobacterium* was detected in exhaled breath of fasting humpback whales at the end, but not at the beginning, of their long annual migrations, suggesting it and other bacterial species could indicate physiological stress (Vendl *et al.*, 2020). The phylum was common in our mucus samples (mean, 10.7%), and among the three genera representing Fusobacteria (*Cetobacterium*, *Fusobacterium*, *Oceanivirga*), *Oceanivirga* was abundant and present in all mucus samples, consistent with prior analysis that the genus is a commensal of marine mammals (Palmer *et al.*, 2020).

The Spirochaetes we detected were from the family *Spirochaetaceae* and the genus *Treponema*. Although the relative abundance was not high (mean, 0.5%), its occurrence in >90% of the mucus samples indicates a common presence of these bacteria. *Treponema* species are known to be the cause of syphilis, yaws and pinta in humans (Radolf, 1996), but not yet reported as a disease agent in marine mammals. *Treponema* has been reported in baleen whale gut microbiomes (Sanders *et al.*, 2015) and in blowhole and genital swabs from bottlenose dolphins (Robles-Malagamba *et al.*, 2020). The presence of this genus deserves higher resolution assessment for abundance and taxonomic identification to understand its potential as a pathogen.

The Epsilonbacteraeota detected in mucus were comprised of two genera, *Arcobacter* and *Campylobacter*, and these genera together exhibited high relative abundance (mean, 14.0%). These bacteria are the leading cause of enteric diseases in humans and are actually isolated more frequently in illness cases than *Salmonella* or *Escherichia coli* (Facciola *et al*., 2017). *Campylobacter* can be indicators for sewage contamination, and *Arcobacter* is abundant in wastewater treatment effluent, even in spite of contemporary standards of treatment (Kristensen *et al*., 2020).

The majority of Patescibacteria detected belonged to the class Gracilibacteria, which has not been reported as a common component of marine mammal microbiomes. The relative abundance in mucus was low (mean, 1.6%) and absent in 6% of the samples. Gracilibacteria likely play a significant metabolic role in oral biofilms in humans, including potential as a parasite of other bacteria, but their abundance in both healthy and diseased conditions suggest a commensal role (Espinoza *et al*., 2018), and perhaps fulfil a similar function in marine mammals.

In addition to these mucus-associated taxa, individual bacterial families *Cardiobacteriaceae* and *Saccharospirillaceae* (phylum Proteobacteria) were differentially prominent (Table 2), with mean relative abundances of 12.7% and 6.6%, respectively. Both families were prominent in the upper respiratory tract and exhaled breath of dolphins (Johnson *et al*., 2009; Lima *et al*., 2012), and *Cardiobacteriaceae* was part of the core microbiome of humpback whale exhaled breath (Apprill *et al*., 2017).

Only one phylum, WPS-2 (Eremiobacterota), was present in breath samples, but not in mucus or water samples (Fig. 2). Metagenomic analyses of this uncultivated phylum revealed it is capable of anoxygenic phototrophy and chemosynthetic fixation of atmospheric CO_2 (Ji *et al*., 2017). Anoxygenic phototrophs tend to be found in specific environments (i.e. not cosmopolitan), and WPS-2 has been identified in cold, acidic, aerobic conditions such as Arctic and Antarctic deserts and boreal mosses (Ward *et al*., 2019). The blowhole cavity of killer whale (and possibly other cetaceans) may be consistent with these conditions, providing an animal niche for WPS-2 habitation.

In seawater samples, the phyla Cyanobacteria and Verrucomicrobia were highly abundant (Fig. 2), and there were eight families found exclusively in seawater (Table 1). The most abundant bacterial families included Oxyphotobacteria chloroplasts, *Rhodobacteraceae* and *Flavobacteriaceae*. The Oxyphotobacteria chloroplast sequences are derived from highly abundant photosynthetic cyanobacteria (e.g. *Synechococcus* and *Prochlorococcus*), from organelles of photosyntheic eukaryotes such as algae, or more likely, from both sources. *Rhodobacteraceae* are another highly abundant marine bacteria in pelagic zones (Ghai *et al*., 2012), and the family exhibits flexibility in nutrient utilization and metabolism (Pujalte *et al*., 2014). The high abundance of *Rhodobacteraceae* and Oxyphotobacteria chloroplast sequences in seawater sample and low abundance in mucus and breath samples suggest that these families can be valuable indicators of seawater in a sample.

This study represents an initial examination of mucus and breath microbiomes from North Pacific killer whales. Although a number of potentially pathogenic taxa were detected (Table 3), it is premature to assign health implications to their presence. The ability of pathogenic bacteria to cause disease can be modulated by the presence of other bacteria and microorganisms as well as the overall health status of the host. A current objective is determination of genetic identification of the samples, allowing assignment to specific individuals. This will permit a higher resolution assessment of the microbiomes with additional information that may be available, such as age, gender, reproductive history, burdens of persistent organic pollutants in blubber and documented body condition.

An earlier effort to examine SRKW breath microbiomes used culture-dependent methods to isolate and identify bacteria and fungi (Raverty *et al*., 2017). The methods of the previous and current studies differ in detection bias, with culture-based methods favouring known pathogenic species, while the current 16S microbial metabarcoding approach can detect many as-yet uncultured taxa. In spite of these differences, there was concordance in detection for *Mycoplasma* (class Mollicutes), *Staphylococcus* (class Bacilli), *Pseudomonas* (class Gammaproteobacteria), *Pychrobacter* (class Gammaproteobacteria), *Microbacterium* (class Actinobacteria), *Bacillus* (class Bacilli), *Kocuria* (class Actinobacteria) and *Arthrobacter* (class Actinobacteria). All but one of these overlapping genera exhibited moderate to high relative abundances with the 16S metabarcoding approach (Supplementary Table 4), suggesting that the culture-dependent approach may have greater sensitivity for certain very low abundance bacteria in breath samples. This comparison highlights the respective value of culture-based and genetic methods in characterizing microbiomes.

Opportunities to examine and assess the health of SRKW have become increasingly limited due to their low population numbers and shifts in their geographic occupancy. Furthermore, conservation scientists wishing to reduce stress or disturbance are investigating methods that minimize contact or close interaction. Utilizing samples that are expelled, shed or excreted has potential that needs to be explored. In diving cetaceans, the regular stress of alveolar collapse due to high barometric pressure and the hypoxia and hyperoxia associated rapid and large tidal volume exchange places heavy demands on the respiratory system (Fahlman *et al*., 2017). SRKWs display wide variation in diving depths, and the majority of dives have a maximum depth of <4 m (Holt *et al*., 2019). However, dives for their preferred prey, Chinook salmon, often exceed 100 m (Tennessen *et al*., 2019), reinforcing the reliance for successful hunting on a robust respiratory system. Low abundance of SRKWs' preferred

prey has been and continues to be a major risk to their population (National Marine Fisheries Service, 2008), and these animals may be compelled to shift dietary choices, geographic ranges or possibly both strategies to obtain sufficient nutrients (Hanson *et al*., 2021). A fully functional respiratory system is essential to SRKWs' ability to respond to this risk, and microbiomes may provide a further evaluation for that system.

Microbiomes are increasingly applied in human health to identify indicators of susceptibility to illnesses such as acute respiratory infections and potentially for diagnostics (e.g. Henares *et al*., 2021; Herivaux *et al*., 2022). Furthermore, microbiomes are evolving for application in prognosis or prediction (Herivaux *et al*., 2022). Human health uses of microbiomes can be guidance for similar application to marine mammal conservation—especially in the data-poor environment of relatively inaccessible species such as SRKWs. Maximizing information from any biological sample is crucial in building our understanding of their physiological condition and health status. However, translating microbiome information into diagnostic or predictive tools requires sufficient sample numbers and adequate contextual metadata.

In the near term, there is good potential for microbiome analysis to become part of a diagnostic evaluation for specific animals of concern, complementing conventional clinical techniques. Samples can be processed and analysed in time scales similar to clinical microbiology testing, an important feature for veterinary decisions. In the case of J50, a young SRKW that had failed to thrive from birth and had visibly poor condition, preliminary molecular analysis of breath samples did not detect a known respiratory bacterial pathogen (Gaydos *et al*., 2019). That conclusion relied upon the assumption that a respiratory infection would be dominated by a single bacterium, but a polymicrobial infection could not be ruled out. A concurrent microbiome assessment could have provided better assurance that J50 was not subject to a more complex respiratory bacterial infection, which was supported by in this report. By utilizing previously undervalued samples such as mucus and breath samples for microbiome analysis, we add capacity for understanding SRKW physiological status and provide information for adaptive management of these endangered marine mammals.

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Credit author statement

L.D.R.: conceptualization, laboratory methodology and analyses, data management and curation, manuscript writing (draft, review, editing), funding acquisition and project administration.

C.K.E.: field collection operations, data management and curation and manuscript writing (review, editing).

G.S.W.: laboratory methodology and analyses and manuscript writing (review, editing).

A.H.W.: laboratory methodology and analyses and manuscript writing (review, editing).

M.B.H.: conceptualization, field collection operations, manuscript (review, editing) and funding acquisition.

Data availability

The data underlying this article are publicly available in the Sequence Read Archive repository of the National Center for Biotechnology Information, under BioProject ID PRJNA752106.

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Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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