Core respiratory microbiome of the blue whale, *Balaenoptera musculus*

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Summary

The number of strandings and unusual mortality events that involve marine mammals may have increased, and potential pathogens of the respiratory tract have been found during examination of individuals in many of these events. Given that the core microbiome is key to understand host-bacteria relationships and to identify their relevance for host health, we characterized the core respiratory microbiome of the Eastern North Pacific blue whale, *Balaenoptera musculus*, using blow samples collected by a small quadracopter drone. 16S rRNA gene high-throughput sequencing revealed 2,732 amplicon sequence variants (ASVs) of which 18 were shared by more than 50% of all blue whales and were considered as the core respiratory microbiome. Sixteen bacterial classes with a relative abundance higher than 0.02% were identified in the blow samples, and eight of these were also found in the seawater samples. Nonetheless, blow samples harboured classes not commonly found in seawater, such as *Acidiomicrobia, Actinobacteria, Campylobacteria, Erysipelotrichia, Leptospirae, Mollicutes*, and *Oxyphotobacteria*. Only one whale presented a potential pathogen, *Mycoplasma*, associated with pulmonary pathology in mammals. Ours is the first study to characterize the respiratory microbiome of apparently healthy blue whales. The core microbiome identified here could be used as a baseline for future long-term studies on blue whale health.

Key words: microbiome, blue whale, *Balaenoptera musculus*, respiratory tract, bacteria, 16S rRNA gene

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Introduction

The advent of modern technologies that allow identification of all bacteria present in environmental or clinical samples (Haegeman *et al.,* 2013; Salter *et al.,* 2014; Rhodes *et al.,* 2022) has led to a myriad of studies on the abundance, diversity and structure of the microbiome of different species (Nelson *et al.,* 2015; Watkins *et al.,* 2022). One of the reasons why it is paramount to increase our knowledge about the microbiome of a given species is because the microbial communities associated with a particular organ can impact the host's physiology (Foster *et al.,* 2017), and even play an important role in its health status (Zaura *et al.,* 2009; Huse *et al.,* 2012; Chaban *et al.,* 2013; Huang *et al.,* 2016; Bierlich *et al.,* 2018; Watkins *et al.,* 2022). For example, respiratory infections can occur when opportunistic microorganisms, which are normally part of the microbiome of a healthy respiratory tract, preferentially flourish under certain conditions (Hilty *et al.,* 2010; Dickson *et al.,* 2016; Rhodes *et al.,* 2022), and *de novo* infections may occur if individuals are exposed to pathogens. In turn, infections can trigger changes in the diversity and composition of the original microbial communities, an event known as dysbiosis (Gagliardi *et al.,* 2018; Infante-Villamil *et al.,* 2020; Sehnal *et al.,* 2021). Therefore, the composition of the microbiome may even be a better predictive marker of progression of a disease, than the simple presence of the specific pathogen commonly associated with the disease. This is why having knowledge about the microbiome composition and how it varies between healthy and sick animals could become an important tool with which to assess the health status of an individual (Shreiner *et al.,* 2015).

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When attempting to use the microbiome to help assess health status, one must distinguish between commensal, opportunistic and transient bacteria (Huang *et al.,* 2016; Infante-Villamil *et al.,* 2020). To do this, it is necessary to identify the bacterial taxa that predominate in the community and that are shared by healthy individuals (Huse *et al.,* 2012; The Human Microbiome Project Consortium; 2012; Willis *et al.,* 2020), a concept known as *the core microbiome*, which plays an important role in maintaining the functional stability and homeostasis of a specific habitat (e.g., skin, gut, lungs) of the host (Shade and Handelsman, 2012; Hernandez-Agreda *et al.,* 2017; Thomas *et al.,* 2017; Björk *et al.,* 2018; Ross *et al.,* 2019). The definition of the core microbiome varies across authors, although they tend to overlap in many of the components of the microbial community (Risely, 2020). Different approaches to define the core microbiome have included the temporal stability (i.e. *dynamic core*, which refers to those bacterial taxa that are present across different stages of the host; Shade and Handelsman, 2012: Ozkan *et al.,* 2017), functional level (i.e. *functional core*: which refers to the set of bacterial genes that are important for host metabolic processes; Dinsdale *et al.,* 2008), ecological influence (i.e. *ecological core*: which refers to bacterial taxa that are important for shaping the structure of their communities; Coyte and Rakoff-Nahoum, 2019; Coyte *et al.,* 2019), host fitness (i.e. *host-adapted core*: which refers to those taxa whose presence increases host fitness; Shapira, 2016), and bacterial occupancy frequency (i.e. *common core*: which refers to the most widespread bacterial taxa that are shared by a considerable proportion of hosts; Huse *et al.,* 2012; Nishida and Ochman, 2017; Bierlich *et al.,* 2018; Ingala *et al.,* 2018; Risely, 2020).

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Regardless of the approach chosen, identifying the common core bacteriome requires setting the detection threshold (relative abundance) and the occurrence percentage (prevalence) of bacterial taxa (Astudillo-García *et al.,* 2017), criteria which have varied widely among published studies (Risely, 2020), with common core annotations ranging from as low as 30% (e.g., Ainsworth *et al.,* 2015) to 100% prevalences (e.g. Huse *et al.,* 2012; Apprill *et al.,* 2017; Hernandez-Agreda *et al.,* 2017; Antwis *et al.,* 2018), and detection thresholds varying from 0.001% to 0.1% (Astudillo-García *et al.,* 2017; Antwis *et al.,* 2018). Given that biological justifications for such prevalence and thresholds values are rare (Risely, 2020), it is important to recognize the arbitrary aspect of the common core definition and to be cautious when interpreting the results. However, the microbiome common core tends to be robust despite varying definitions, particularly when samples from closely related individuals are analysed (Astudillo-García *et al.,* 2017; Risely, 2020).

Bacteria of the mammalian microbiome are found in composite communities (Lee and Mazmanian, 2010; Rhodes *et al.,* 2022) whose diversity and abundance is determined by multiple interactions between species (Shade and Handelsman, 2012; Stubbendieck *et al.,* 2016). The cetacean microbiome has recently begun to be studied and important initial assessments of microbial diversity have been made for a few species (Venn-Watson *et al.,* 2008; Johnson *et al.,* 2009; Lima *et al.,* 2012; Bik *et al.,* 2016; Soverini *et al.,* 2016; Raverty *et al.,* 2017; Rhodes *et al.,* 2022). Among cetaceans, baleen whales play important roles in the marine ecosystem, as they are long-lived, contribute to the movement and storage of carbon (Pershing *et al.,* 2010) and are considered sentinels of ocean health (Moore *et al.,*

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2019; Palmer *et al.,* 2022). However, to date, little is known about the respiratory microbiome of baleen whales, and while some opportunistic pathogens of the respiratory tract have been described for live free-ranging individuals of a few baleen whale species (Acevedo-Whitehouse *et al.,* 2010), to the best of our knowledge, there is only one published study on the core respiratory microbiome of a baleen whale, the humpback whale, *Megaptera novaeangliae* (Apprill *et al.,* 2017).

In this study, we characterized the common core respiratory microbiome of the Eastern North Pacific blue whale, using next generation sequencing on 17 blow samples collected from adult blue whales by a non-invasive drone-based technique (Domínguez-Sánchez *et al.,* 2018) during the boreal winter months in the Gulf of California.

Results

A total of 20 samples were collected and analysed in this study. These samples included 17 photo-identified blue whales and three technical controls (seawater, human sneeze, and PCR blank). Exhaled breath samples were collected from animals in the Gulf of California using a small drone. No adverse behaviour was detected before, during or after sampling (see Domínguez-Sánchez *et al.,* 2018). We identified 379,813 sequences (of which 23,585 were unique sequences) corresponding to the sum of readings identified in the blow samples and technical controls, which ranged from 12,146 to 32,148, and from 12,646 to 20,471 sequences, respectively. These corresponded to 2,732 amplicon sequence variants (ASVs). The sample coverage (i.e., the proportion of the total number of individuals in a community

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that belong to the species represented in the sample; Chao and Chiu, 2016) exceeded 98% in all cases. Alpha diversity measures of all samples (blow and technical controls) (Fig. 1) revealed that species richness (S) ranged from 165 to 924 (mean = 453.35); Simpson's index of diversity (D) ranged from 0.55 to 0.99 (mean = 0.95). Blow samples (S mean = 486.5, D mean = 0.94) were different from the controls (S mean = 265.6, D mean = 0.98) in those metrics ($p = 0.0052$, and $p = 0.017$; respectively). Beta diversity differed significantly between the blow samples and the technical control samples (PERMANOVA, $F= 17.677$, $P>0.001$).

The phylogenetically diverse sequence assemblage of all samples (whale blows and technical controls) reached sixteen identified bacterial classes (Fig. 2). Some of these classes were shared between seawater and blow samples, including *Bacilli, Gammaproteobacteria* (the most abundant class in seawater, representing 20.9% of relative abundance)*, Clostridia, Negativicutes*, and *Verrucomicrobiae.* However, blow samples harboured classes that are not commonly found in seawater, such as, *Actinobacteria, Alphaproteobacteria, Campylobacteria, Erysipelotrichia, Leptospirae,* and *Mollicutes (Fig. 2). Acidiomicrobia* and *Oxyphotobacteria* was identified only in two whales (Bm051 and Bm056) and two technical controls (ControlCADS and ControlWater). Only one whale blow (Bm057) had a high abundance of *Mycoplasma* spp. (34.4% of relative abundance).

Eighteen ASVs were present in more than 50% of the blow samples (Fig. 3) and were considered as the common core microbiome of the respiratory tract of the blue whales in the

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Gulf of California (Table 1). These common core members spanned ten bacterial families (Campylobacteraceae, *Cardiobacteriaceae, Erysipelotrichiaceae, Flavobacteriaceae, Lachnospiraceae, Leptotrichiaceae, Moraxellaceae, Porphyromonadaceae, Prevotellaceae* and *Propionibacteriacea*). Additionally, analysing changes in the pattern of the common central microbiome, based on a range of prevalences and detection threshold values, it was possible to identify that the common central microbiome of blue whale blow can vary from 1166 ASV (5% prevalence and 0.001 detection threshold) to 1 ASV (80% prevalence and 0.02 detection threshold), in all cases revealing *Cutibacterium* spp. as the genus with the highest prevalence in blue whale blow samples. This bacterium was also identified in two technical controls (ControlCADS and ControlLab), with a relative abundance of 14.8%, and 3.6%, respectively. The most abundant genus identified in seawater was *Herbaspirillum* spp. This genus was detected in only three samples (Bm023, Bm043, and Bm059) with relative abundances of 6.1%, 8.3% and 8.7%, respectively, compared to 14.5% in the seawater sample.

Discussion

Recent studies of the human respiratory microbiome have shown that bacterial communities of the respiratory tract are key to maintaining respiratory health (Glendinning *et al.,* 2017; Olufunmilola *et al.,* 2020; Santacroce *et al.,* 2020), not only in terms of their metabolic contribution (Van Treuren and Dodd, 2019), but also because they prevent the colonization of the epithelium by environmental pathogens (Man *et al.,* 2017; Yamamoto *et el.,* 2021).

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Identifying the composition and abundance of the bacterial communities that constitute the microbiome of healthy individuals is an important step to establishing a baseline that will help identify bacteria associated with respiratory diseases (Lemon *et al.,* 2010; Lima *et al.,* 2012), assess chronic states of suboptimal health (Mackenzie *et al.,* 2017) and predict community changes due to perturbation (Shade and Handelsman, 2012; Yamamoto *et al.,* 2021).

We aimed to characterize the respiratory microbiome of the blue whale, the planet's largest extant animal. The results of our study demonstrated that the blow of this baleen whale species supports a diverse and rich community of bacteria. We identified 2,732 ASVs with high sample coverage and with varying levels of richness and relative abundance among samples, which could be an indicator of temporary fluctuations in the composition of the microbiome (Eloe-fadrosh and Rasko, 2013). Richness and relative abundance of microbiome varies among healthy animals (The Human Microbiome Project Consortium, 2012) and this is determined mainly by bacterial immigration from the environment to the lungs during inhalation, bacterial elimination via mucociliary clearance, and a relatively small contribution of growth rate of each bacterial community (Dickson, *et al.,* 2014; Dickson *et al.,* 2016; Huffnagle *et al.,* 2017). Evidently, we cannot rule out that the observed variation was also related to differences in the volume of blow collected, which due to the nature of our collection technique, could not be standardized. On one hand, whales are likely to differ in the amount of exhaled breath condensate exhaled, depending on the whale's size, and the depth and duration of the dive. Furthermore, although drones are safe, minimally

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invasive, and seem to not affect the whales during blow collection (Domínguez-Sánchez *et al.,* 2018), inherent limitations such as flight height and different wind conditions, could potentially result in different volumes of blow being collected (Apprill *et al.,* 2017).

There are various indexes that are used to estimate the diversity of the microbial communities. In our study we used Simpson's diversity index because it considers both richness and evenness (Johnson and Burnet, 2016) and was previously identified as one of the most accurate estimators of diversity in an unknown bacterial community (Haegeman *et al.,* 2013; Johnson and Burnet, 2016). In our study, Simpson's diversity index averaged 0.94 (minimum = 0.55, maximum = 0.98), demonstrating high bacterial diversity in the blow of the whales that we sampled. Some studies have shown that the microbiome of a healthy animal tends to have a high diversity, which presumably allows it to tolerate or counteract changes that may occur due to extrinsic challenges (Chan *et al.,* 2013; Gibson *et al.,* 2019; Jiménez *et al.,* 2019). Bacterial diversity was high in nearly all of the blue whale blow samples, and the composition of the microbiome was dominated mainly by members of the phyla Actinobacteria, Firmicutes and Proteobacteria, which have been reported as major components of the healthy respiratory microbiome of other mammals (Chaban *et al.,* 2013; Dickson *et al.,* 2016, Rhodes *et al.,* 2022). Simpson´s diversity index was similar to that reported for humpback whale blow (Apprill *et al.,* 2017) and bottlenose dolphin blowhole (Johnson *et al.,* 2009; Bik *et al.,* 2016). It is likely that these results are evidence that the respiratory microbiome diversity (based on Simpson´s index) remains preserved among cetaceans. However, the blue whale blow had nearly twice the taxonomic richness than

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reported for humpback whales. In our samples, the percentage of "*others*" (sum of bacteria that does not reach the detection threshold of 0.02%) was higher than in humpback whales (see Apprill *et al.,* 2017). Based on this result, we suggest that the microbiome of the blue whale respiratory system might be more complex than that of the humpback whale. At this stage we can only speculate about the reasons that could give rise to such a difference. This may be due to having used DADA2 for resolving ASVs rather than minimum entropy decomposition (MEDs; Eren *et al.,* 2015), which were used in the previous study, given that although similar in what they report, ASVs are better at removing erroneous sequences (Callahan *et al.,* 2016; Ahlgren *et al.,* 2019). As the algorithm DADA2 allows for the independent analysis or grouping of samples, we conducted pooled analyses to increase sensitivity to detect ASVs that could be present at very low frequencies in multiple samples (Callahan *et al.,* 2016). This approach could explain why a higher percentage (mean = 30.91%) of "rare bacterial biosphere" (Pedrós-Alió, 2012) was identified in our study than in the humpback whale study (see Apprill *et al.,* 2017). This "rare bacterial biosphere", formed by bacteria that are present at low relative abundances are particularly important for dealing with dysbiosis, as they could be considered as a seed bank of genetic resources that can lead the restoration of the core microbiome (Pedrós-Alió, 2012; Skopina *et al.,* 2016; Jousset *et al.,* 2017). A more complex respiratory microbial community is likely to be beneficial to the whales, given that microbiomes with higher richness of species have more synergistic interactions between bacterial taxa, that improve the functioning of the ecosystem (Bell *et al.,* 2005). It is worth mentioning that most studies published to date do not consider the bacterial taxa found in low abundances to be relevant; however, these small populations are

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now thought to play an important role for the functioning of the ecosystem (Willis *et al.,* 2017) and host health (Guss *et al.,* 2011; Jouseet *et al.,* 2017). It has been demonstrated that high diversity of low-abundance bacteria is correlated with less severe bacterial infections in human lungs (Van der Gast *et al.,* 2011).

Eight bacterial phyla were identified in the blow samples, and some of these were also found in the seawater samples. This is unsurprising, as there is likely to be some seawater carried over when the whales exhale. However, the blow samples harboured ASVs belonging to bacterial classes that were not found in the seawater technical control. These bacteria included genera such as *Actinobacteria, Alphaproteobacteria, Campylobacteria, Erysipelotrichia, Leptospirae,* and *Mollicutes.* This shows that despite potential carry over of sea water to the blow during exhalation, bacterial communities of the blue whale respiratory tract are different than those of seawater. Having found *Acidiomicrobia* and *Oxyphotobacteria* in two whales (Bm051 and Bm056) and two technical controls (ControlCADS and ControlWater), may be suggestive of contamination. Nevertheless, is interesting that those bacterial classes were not found in seawater (ControlWater) as they are bacteria reported in marine algae and corals (Hernadez-Agreda *et al.,* 2017; Pearman *et al.,* 2019; Garcia-Pichel *et al.,* 2020).

Eighteen ASVs belonging to the families Campylobacteraceae, *Cardiobacteriaceae, Erysipelotrichiaceae, Flavobacteriaceae, Lachnospiraceae, Leptotrichiaceae, Moraxellaceae, Porphyromonadaceae, Prevotellaceae* and *Propionibacteriacea*; were

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shared in more than a half of the blue whale's samples and we considered them to be the common core respiratory microbiome. It appears that interindividual variability of the blue whales' respiratory microbiome is higher compared to that of the humpback whale, as 25 distinct bacteria were found to be shared among all the animals sampled (Apprill *et al.,* 2017). One bacterial genus (*Porphyromonas*) detected here was previously found in humpback whale skin (Apprill *et al.,* 2014) and humpback whale blow (Apprill *et al.,* 2017). *Porphyromonas* and *Fusobacterium* have been described as bacteria of the core pulmonary microbiome in humans (Erb-Downward *et al.,* 2011; Charlson *et al.,* 2012; Huang *et al.,* 2013; Morris *et al.,* 2013; Cui *et al.,* 2014). These bacteria have also been reported sporadically and in low abundance in the respiratory tract of sheep (Glendinning *et al.,* 2016). We also identified *Moraxella* spp. in the blue whale blow. This bacterial genus is present in the humpback whale blow (Apprill *et al.* 2017) and is commonly found in the lungs of healthy dogs (Tress *et al.,* 2017), although it has also been reported in humans (Yi *et al.,* 2014) and cattle with respiratory diseases (Lima *et al.,* 2016). The bacteria identified in the blue whale respiratory tract are similar to those reported in other mammals, and some of them are known to cause disease. At this stage we are unable to unequivocally establish that the health of the whales sampled was not compromised; however, given that they were present in most of the whales, we can assume that they are part of their respiratory microbiome, and that they are likely to reflect a healthy respiratory tract.

Interestingly, three bacterial genera (*Staphylococcus, Propinebacterium, Corinebacterium*) that were identified in the blue whale blow are associated with the skin of humans and other

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terrestrial mammals (Grice and Segre, 2011; Byrd *et al.,* 2018; Worthing *et al.,* 2018) and were recently identified as part of the skin microbiota of captive bottlenose dolphins (*Tursiops truncatus*), killer whales, and free-ranging humpback whales (Apprill *et al.,* 2014; Chiarello *et al.,* 2017; Hooper *et al.,* 2018; Rhodes *et al.,* 2022). It is likely that their presence in the blow samples indicates that they colonize the blowhole epithelial lining of blue whales and be expelled forcefully during exhalation (Apprill *et al.,* 2017), leading to their presence in the blow samples.

An unexpected finding was *Herbaspirillum* spp., a bacterial genus that tends to be found in soil and freshwater environments (Dobritsa *et al.,* 2010), and that has also been identified as a contaminant in 16S rRNA gene sequencing, most often during sample preparation, as it has been isolated from deionized water (Grahn *et al.,* 2003; Mohammadi *et al.,* 2005; Bohus *et al.,* 2011; Kéki *et al.,* 2013). Nonetheless, this was the most abundant genus identified in seawater and while it was detected in three blow samples (Bm023, Bm043, and Bm059), the relative abundance of this bacterial genus was very low. Using SourceTracker it was possible to verify that there was no contamination of *Herbaspirillum* spp. proceeding from the reagents in the laboratory during the 16S rRNA sequencing. Also, this genus is unlikely to have been detected due to procedural contamination because in the other blow samples *Herbaspirillum* spp. was not detected. Thus, the presence of this genera in the three samples is likely to reflect contamination with seawater.

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In our study, we were able to detect *Mycoplasma* spp. (34.4% relative abundance) in a single blue whale (sample Bm057). This genus, along with 22 other potentially pathogenic bacteria, has been identified in killer whales (Raverty *et al.,* 2017; Rhodes *et al.,* 2022). Having detected only one potentially pathogenic bacterial genus in this study could mean that blue whales are not commonly in contact with coastal areas where spillover of pathogens from humans or domestic animals could occur, unlike killer whales that live in areas where there is a large number of environmental stressors of human origin (Raverty *et al.,* 2017). However, a previous study reported *Entamoeba* spp., *Giardia* spp., and *Balantidium* spp., most likely from sewage discharge, in faeces of blue whales from this region (Pacheco-Armenta 2019), so it is plausible that rather than limited exposure, the presence of *Mycoplasma* in one individual reflects a suboptimal immune status or an underlying upper or lower respiratory condition, which could allow respiratory colonization of this pathogen. The presence of *Mycoplasma* spp. could be indicative of a transient bloom of this bacteria within the respiratory tract, or of an active respiratory infection, because these bacteria are typically present in the respiratory tract at a low abundance, but during active pathological processes, such as pneumonia and other respiratory conditions, their relative abundance increases (Dai *et al.,* 2018). Indeed, this opportunistic bacterial genera has been implicated in respiratory diseases of humans (Chandra *et al.,* 2015; Prince *et al.,* 2018; Qu *et al.,* 2018; Li *et al.,* 2019) and other mammals (Cai *et al.,* 2019; Choi *et al.,* 2019; Tao *et al.,* 2019). In marine mammals, *Mycoplasma* has been related to signs of respiratory disease and has been detected in the lungs of stranded harbour porpoises (*Phocoena phocoena*), Sowerby's beaked whale (*Mesoplodon bidens*) (Foster *et al.,* 2011) and California sea lions (*Zalophus californianus*)

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(Haulena *et al.,* 2013) during unusual mortality events. However, the role of *Mycoplasma* during episodes of disease in cetaceans, their host specificity, their diversity, and their association to cetacean stranding events, remains poorly understood (Foster *et al.,* 2011; Rhodes *et al.,* 2022). It is certainly possible that the whale from which sample Bm057 was collected, was experiencing a respiratory infection that involved *Mycoplasma*. Gaining clinical information that would allow us to establish this beyond any doubt is not feasible, but we propose that future studies consider using the presence of *Mycoplasma* in the blow as an indicator of suboptimal respiratory health.

It is important to note that the respiratory microbiome of the blue whales analysed in our study harboured bacteria that are commonly found in the oropharynx, nasopharynx and the mouth of different terrestrial mammals (German and Palmer, 2006; Guglielmetti *et al.,* 2010) in which those anatomical structures are interconnected in the upper respiratory tract. In contrast, cetaceans have no anatomical connection between the nasopharynx and the mouth (Apprill *et al.,* 2017; Smith *et al.,* 2017). This finding constitutes strong evidence that the core microbiome that we have described belongs to the respiratory system of blue whales and does not include their oral bacteria.

Given the current state of our oceans, which face habitat degradation, pollution, and other anthropogenic stressors (Melcón *et al.,* 2012; Mouton and Botha, 2012; Palmer *et al.,* 2022), suboptimal immune responses can occur in top-predator marine animals (Acevedo-Whitehouse and Duffus, 2009; Van Bressem *et al.,* 2009; Hall *et al.,* 2018), in turn increasing

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the risk of diseases in their populations (e.g. Sós *et al.,* 2013; Van Bressem *et al.,* 2014; Reisfeld *et al.,* 2019). In this sense, it is important to strengthen and expand efforts for health assessment of their populations (Gulland and Hall, 2007), efforts which to date include the determination of body condition by photogrammetry and measures of blubber thickness (e.g. Pettis *et al.,* 2004; Konishi *et al.,* 2008; Bradford *et al.,* 2012; Durban *et al.,* 2016), evaluation of skin integrity (Van Bressem *et al.,* 2015), and quantitation of specific gene transcripts in the skin (Simond *et al.,* 2019). In order to use the respiratory microbiome as a tool to help assess the health of large whales (Apprill *et al.,* 2017; Rhodes *et al.,* 2022), it is imperative to first increase our understanding of the core microbiome of the respiratory tract of different species, which is what our study has done for the Eastern North Pacific blue whale.

Conclusion

Ours is the first study to characterize the microbiome of the respiratory tract in blue whales. We found that the blue whales sampled in the Gulf of California harboured a similar respiratory bacterial composition among individuals. Additionally, our richness and relative abundance results are comparable with those reported in the microbiome of healthy animals and humans, so we propose that the core respiratory microbiome identified here could be used as a baseline for future long-term studies aimed at identifying shifts in the composition and co-occurrence patterns of the respiratory microbiome and identify ASVs related to changes in body condition, as a proxy for poor health condition.

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Materials and Methods

Sample collection. Using a small Phantom 3® quadracopter drone (DJI Innovations, China) with floaters and sterile Petri dishes (Domínguez-Sánchez *et al.,* 2018), we collected 17 blow samples from 17 individual blue whales sampled between February and March of 2016 and 2017 in Loreto Bay National Park (25°51'51''N, 111°07'18''O) within the Gulf of California, Mexico. The number of sampled whales represents 17% of the estimated 100 blue whales that reside during winter/spring in the southwestern Gulf of California (mark-recapture data from 1994-2006; Ugalde de la Cruz, 2006; SEMARNAT, 2018).

Each whale was photo-identified prior to collecting the samples (Gendron and Ugalde de la Cruz, 2012). The approach to the whale with the drone was made from the caudal fin heading towards the head to minimize disturbance and sampling was conducted at a height between 3 to 4 m above the blowhole (Domínguez-Sánchez *et al.,* 2018).

For each sample, the blow droplets were swabbed directly from the Petri dish using sterile cotton-tipped swabs. These were then transferred to a sterile 1.5 mL cryogenic microtube containing 500 µL of 96% molecular grade ethanol and kept frozen in a liquid nitrogen container until processing.

In addition to the blow samples, we collected environmental and technical controls. For this, we collected 1 ml of seawater at a depth of 0.10 m in the same location where we sampled the whales' blow. Two additional types of controls were collected but only one was used. Namely, we flew the drone with a sterile Petri dish attached and maintained the same altitude over the water and the same distance to the boat as we did when collecting the blow samples, but this was done in absence of any whale (this control was not included in this study because

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no bacterial DNA was detected in the sample). The second technical control was a human sneeze (ControlCADS), sampled from the person who collected and processed the samples.

DNA extraction, PCR amplification and sequencing. DNA was isolated from the swabs, seawater and environmental samples using the QIAamp ® DNA Mini Kit (QIAGEN, Germany). The primers used for sequencing the 16SrRNA V3 and V4 regions were 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[CCTACGGGNGGCWGCAG]) and 785R (5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[GACTACHVGGGTATCTAATCC]), which amplified a single product of approximately 460 bp (Thijs *et al.,* 2017). The Illumina overhang adapter sequences for the forward and reverse primers are the first 33 and 34 bp, respectively. The PCR program used an initial denaturation step at 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30s; and a final extension step at 72°C for 5 min. Each 25 µL-reaction contained 12.5 ng of extracted DNA, 5 µM of barcoded primers and 2x KAPA HiFi HotStart Ready Mix (KAPABIOSYSTEM, Cape Town, South Africa). We included one internal control (PCR blank) named ControlLab, as technical control. 1 µl of each sample was run on a 2100 Bioanalyzer (Agilent Technologies, CA, USA) with an Agilent DNA 1000 chip (Agilent Technologies, CA, USA) to verify amplicon size. AMPure XP beads (New England BioLabs, USA) were used to remove unused primers and primer dimer species. The PCR products were pooled into two libraries of equal concentrations. Amplicons were sequenced over 2- by 250-bp MiSeq at the Unit of Sequencing and Identification of Polymorphisms of the

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16S rRNA Sequence data processing. Quality control of the 379,813 raw sequences obtained was performed using the FASTQC pipeline available at the Galaxy web platform (www.usegalaxy.com) according to the creators' instructions (Afgan *et al.,* 2018). This allowed us to obtain a quick impression of the data and avoid downstream problems. We used the Divisive Amplicon Denoising Algorithm 2 (*dada2*, v.1.9.1) (Callahan *et al.,* 2016), to infer exact amplicon sequence variants (ASVs) instead of the rough and less precise 16S rRNA OTU clustering approach (Callahan *et al.,* 2017; Dahan *et al.,* 2018) that groups the sequences with a 97% identity (Edgar, 2013; Edgar, 2017). Firstly, we filtered and trimmed the raw sequences (the quality score "Q" threshold to filter sequences was set at 25). Next, we combined all identical reads into unique sequences, determining the abundance that corresponded to the number of reads of each unique sequence. The forward and reverse reads for each sample were combined into a single merged *contig* sequence. After building the ASV table and removing chimeras (detected using self-referencing), sequences were classified and identified with *DECIPHER* (v.2.0) (Wright, 2016), using the SILVA rRNA sequence database (v.132) as the taxa reference (Quast *et al.,* 2013). We estimated the sampling coverage in blow samples and technical controls using Good´s coverage estimator (Zhauan 2017) with the QsRutils package (v.0.1.4). Finally, we classified and used *phyloseq* (v.1.25.0) (McMurdie and Holmes, 2013) to remove any sequence belonging to archaea, chloroplasts, and eukarya, as well as unknown sequences, and mitochondrial sequences.

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Respiratory microbiome analysis. To identify the common core of the respiratory microbiome, we used the ASVs, and taxonomy table generated with the *dada2* pipeline. Using *phyloseq* (v.1.25.0) (McMurdie and Holmes, 2013), we identified the distribution of reads counts from all the samples, as well as sampling coverage, plot rarefaction curves and the stacked barplot of Phyla to get a sense of the community composition in the samples. To achieve this, we pruned out low abundance taxa and only included those Phyla that contributed more than 0.02% of the relative abundance of each sample. Using *microbiome* (v.1.3.1) (Lahti *et al.,* 2017) we identified the common core microbiome (threshold detection = 0.2/100, prevalence = 50/100). We selected those values because we wanted a more conservative approach and did not want to take into account "rare bacteria" in the analysis. In addition, we analysed how the pattern of the common central microbiome changed based on a sliding prevalence range (5% - 100%) and four detection threshold values (0.001, 0.002, 0.01, and 0.02). With these criteria, a linear model could be built to determine the number of ASVs detected given a detection threshold value for a specific prevalence. We calculated alpha diversity: richness (*S*) and Simpson´s diversity index (*D*) with *vegan* (v.2.5.4) (Oksanen *et al.,* 2019). Alpha diversity measures were tested for deviations from normality with a Shapiro-Wilk test. To examine differences in alpha diversity metrics between the blow samples and controls we performed a Wilcoxon rank sum test. We also used *vegan* (v.2.5.4) to run the permutational multivariate ANOVA (PERMANOVA) on unweighted Unifrac distances to test for differences between the microbiome composition of blue whale blow samples and technical controls. We used SourceTracker (Knights *et al.,* 2013) to estimate the

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proportion of the bacterial community in the blue whales' blows samples that comes from the set of technical controls. All graphs were rendered with *ggplot2* (Wickham, 2016).

Ethics approval and consent to participate. This study complied with the recommendations and methods for approaching blue whales provided by Mexican legislation (NOM-059-SEMARNAT-2010). All procedures were approved by the Bioethics committee of the Universidad Autónoma de Queretaro (Mexico) and sampling was conducted under permits SGPA/DGVS/00255/16 and SGPA/DGVS/01832/17 issued by the Dirección General de Vida Silvestre to D. Gendron.

Competing interests

The authors declare that they don't have competing interests.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author upon reasonable request.

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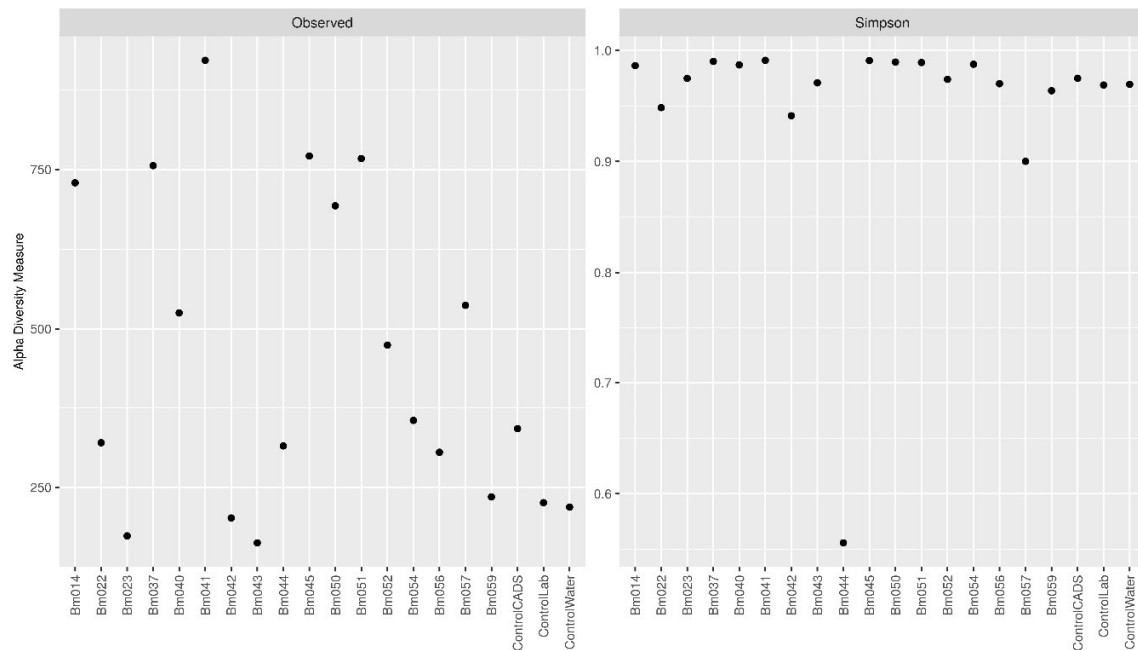**Core respiratory microbiome of the blue whale, *Balaenoptera musculus***

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Table 1. Taxonomic classification of the eighteen ASVs that make up the core microbiome of the respiratory tract of the blue whale.

Domain	Phylum	Class	Order	Family	Genus	Species
Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	*Cutibacterium*	unclassified
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*Oceanivirga*	unclassified
Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	*Dielma*	unclassified
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*Oceanivirga*	unclassified
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Moraxella*	unclassified
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Moraxella*	unclassified
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Moraxella*	unclassified
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Porphyromonas*	unclassified
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Alloprevotella*	unclassified
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*unclassified*	unclassified
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*Oceanivirga*	unclassified
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*	unclassified
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Porphyromonas*	unclassified
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Natranaerovirga*	unclassified
Bacteria	Proteobacteria	Gammaproteobacteria	Cardiobacteriales	Cardiobacteriaceae	*Suttonella*	unclassified
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Porphyromonas*	unclassified
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Natranaerovirga*	unclassified
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Tenacibaculum*	unclassified

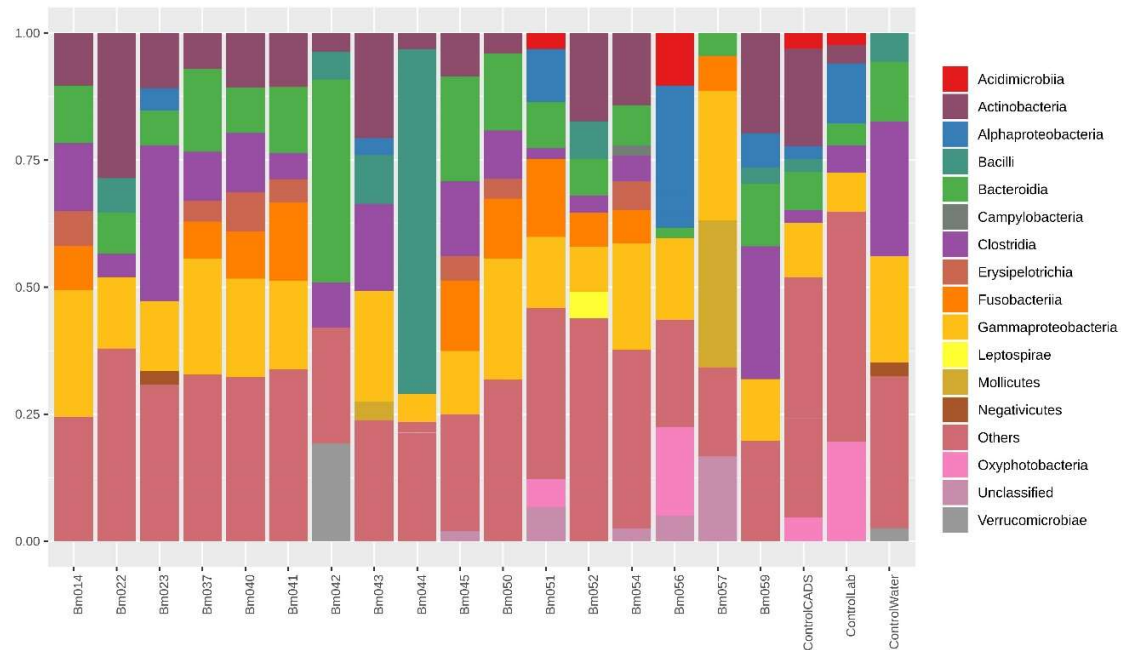# Core respiratory microbiome of the blue whale, *Balaenoptera musculus*

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Figure 1. Bacterial alpha diversity measures in blue whale blow samples and technical controls.

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Figure 2. The phylogenetically diverse assemblage of all samples (whale blows and technical controls). Plot shows the sixteen identified bacterial classes, unclassified, and "others" (sum of bacteria that did not reach the detection threshold of 0.02%).

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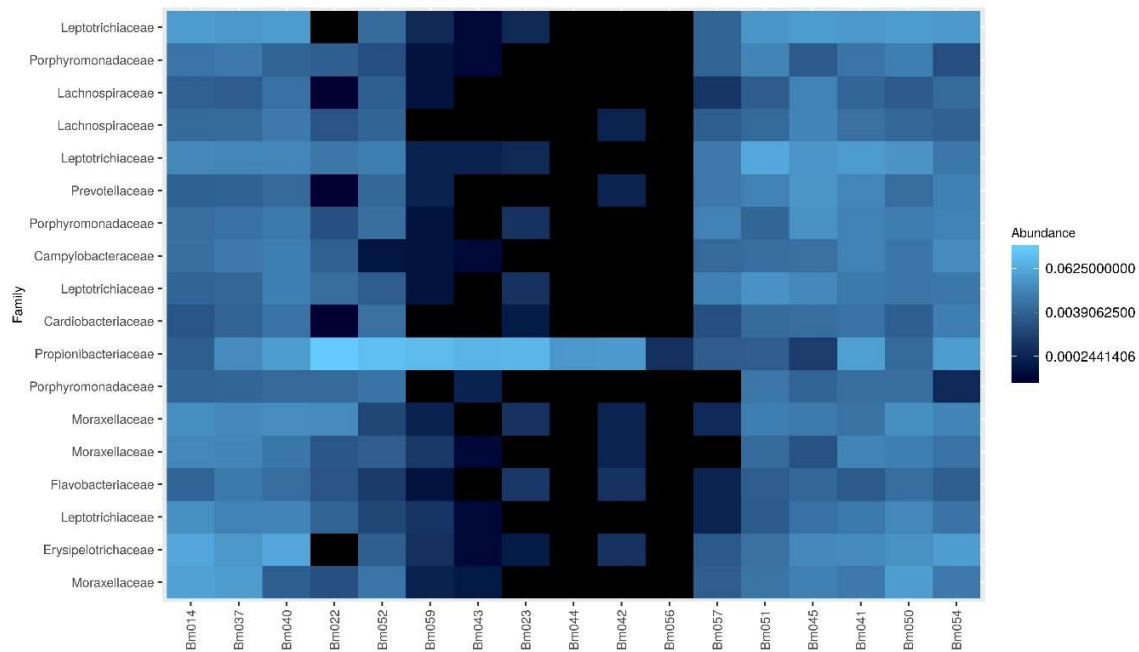
Figure 3. Relative abundance of bacterial classes that form the core respiratory microbiome of the blue whale (Eighteen ASVs present in more than 50% of the blow samples).