Highly abundant core taxa in the blow within and across captive bottlenose dolphins provide evidence for a temporally stable airway microbiota

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Abstract

**Background:** The analysis of blow microbiota has been proposed as a biomarker for respiratory health analysis in cetaceans. Yet, we lack crucial knowledge on the long-term stability of the blow microbiota and its potential changes during disease. Research in humans and mice have provided evidence that respiratory disease is accompanied by a shift in microbial communities of the airways. We investigate here the stability of the community composition of the blow microbiota for 13 captive bottlenose dolphins over eight months including both sick and healthy individuals. We used barcoded tag sequencing of the bacterial 16S rRNA gene. Four of the dolphins experienced distinct medical conditions and received systemic antimicrobial treatment during the study.

**Results:** We show that each dolphin harboured a unique community of zero-radius operational taxonomic units (zOTUs) that was present throughout the entire sampling period (‘intra-core’). Although for most dolphins there was significant variation over time, overall the intra-core accounted for an average of 65% of relative abundance of the blow microbiota. In addition, the dolphins shared between 4 and 52 zOTUs on any of the sampling occasions (‘inter-core’), accounting for a relative abundance between 7 and 33% of any dolphin’s airway microbiota. The majority of the intra-core and all of the inter-core zOTUs in this study are commonly found in captive and free-ranging dolphins and have previously been reported from several different body sites. The blow microbiota of sick dolphins differed significantly from that of healthy dolphins, although we did not find evidence for a common impact of antimicrobial treatment.

**Conclusions:** The airways of dolphins were colonized by an individual intra-core ‘signature’ that varied in abundance relative to more temporary bacteria. We speculate that the intra-core bacteria interact with the immune response of the respiratory tract and support its function. This study provides the first evidence of individual-specific airway microbiota in cetaceans that is stable over eight months.

**Background**
Dolphins harbour rich and diverse bacterial communities in the exhaled breath condensate (blow) that they forcefully expel from their airways through their blowhole when at the sea surface (1–6).
airway microbiota of dolphins are distinct to those of their other body sites, their surrounding seawater (1, 6), air, their prey (fish and squid) and the hand and nose of their human carers in the case of captive dolphins (2). The bacterial communities in the airways are unique to each individual dolphin. The changes the microbiota of an individual dolphin undergo over relatively short periods of time (two months) are minor compared to the differences between individuals (4).

The analysis of blow microbiota has been considered a promising tool for the health assessment of cetaceans (4–8). However, to establish blow microbiota as a viable biomarker, crucial knowledge is still lacking: Is the healthy airway microbiota stable over a period longer than two months? Are certain highly prevalent ‘core’ taxa present that represent a healthy and stable microbiota? Does the airway microbiota of cetaceans reflect the physiological state and the health of its host?

Research on humans and mice has indicated a correlation between airway microbiota and the physical state of the host. Germ-free mice are more susceptible to respiratory infections than conspecifics that carry microbial communities in their airways (9) highlighting the role of the microbiota in disease protection and prevention. Additionally, de Steenhuijsen Piters et al. (10) and Esposito and Principi (11) suggested the human respiratory microbiota to contribute to health regulation. Nevertheless, the temporal stability of the human airway microbiota is under debate (12).

Dickson and Huffnagle (13) and Venkataraman et al. (14) postulated that the bacterial communities in airways of healthy humans are relatively dynamic and transient with few site-specific taxa. In addition, Charlson et al. (15) did not find any shared (core) bacterial sequences in human airways. The same was observed in mice, as Dickson et al. (16) did not find any bacteria common to all mice in their study despite the mice being genetically identical.

Factors, like microbial immigration, elimination and growth rates, as well as the concentration of bacteria in the air determine the community composition of respiratory microbiota in humans (13). The majority of bacteria in the human airways derives from the oral cavity and reaches the airways via microaspiration, as digestive and respiratory tract intersect (14, 17–19). The separation of airways and digestive tract in cetaceans (20–22) makes an overlap of microbiota from these two body regions unlikely. And indeed, Bik et al. (1) showed that the blow microbiota in dolphins are very different from
those in the oral cavity. The blow microbiota might therefore be a better bacterial representation of the airways in cetaceans than the airway microbiota in humans. On the other hand, due to their marine lifestyle cetaceans developed a more forceful breathing mechanism than humans, as they exhale up to 90% of their lung volume in one breath (23–25) compared to only 50% in a physically active human (26, 27). This might cause a higher turnover rate of bacteria in cetacean airways, potentially making them even more transient than in humans.

A state of disease or even a slight change of immunological capacity of the host (16, 19) can change the diversity and overall microbial composition in the airways (28, 29). Yet, very little is known about the specific functions airway bacteria may play and how they interact with their host (30). Furthermore, systemic antibiotic treatment alters (31–33) and suppresses airway microbiota (16) and potentially disturbs the relationship between microbiota and host (34). Shade and Handelsman (35), Hernandez et al. (36) and Apprill et al. (7) hypothesized that highly prevalent and abundant bacterial species, defined as the core, represent a stable and healthy microbial community as these bacteria interact and benefit the immune system of the host.

Here, to test the temporal stability of dolphin airway microbiota, we characterise the blow microbiota of 13 captive bottlenose dolphins sampled at monthly to bi-monthly intervals over an eight-month period. We examine the core bacteria within and across dolphins, as well as the influence of environmental factors, the impact of disease and consequential systemic antimicrobial treatment. We hypothesize that the airways of each dolphin harbour a unique bacterial community throughout the sampling period that maintains a certain level of temporal stability as expressed in a certain number and relative abundance of core bacteria. Furthermore, we predict a significant influence of the antimicrobial treatment and potentially of the compromised health on the microbial blow communities of the dolphins. We seek to contribute new knowledge about the blow microbiota in dolphins to facilitate non-invasive respiratory health assessment in cetaceans in the future.

Results
Sample collection
The dolphin trainers of Sea World Marine Park Gold Coast, Australia, collected samples of exhaled
breath condensate (‘blow’) of 13 captive bottlenose dolphins (Tursiops truncatus) over a period of eight months from April to December 2017. Table 1 presents the sampling schedule listing a total of 87 blow samples, while Table 2 provides additional information on the dolphins, including sex, age and the pool system the animals were kept in. The date of birth was unknown for three dolphins, as they were rescued from the wild. Nine out of the 13 dolphins were in good health conditions throughout the sampling period. Four dolphins (‘Gemma’, ‘Howie’, ‘Nudgee’, ‘Stella’) suffered from a medical condition that required antimicrobial treatment at least once while sampling was performed (Table 2). Table 3 includes their medical condition as well as type and date of antimicrobial treatment.

For the nine healthy dolphins, six blow samples each, collected in weeks 2, 6, 11, 19, 28 and 37, were included into the data analysis (Table 1). For those dolphins that received antimicrobial treatment, we applied a more frequent sampling schedule to be able to closely monitor potential changes of their blow microbiota in response to the treatment (Table 1). Gaps in the extended sampling schedule of the sick dolphins were due to logistical issues at Sea World. Each sampling event was accompanied by one water sample per pool system. Sea World had three large dolphin-holding pool systems (‘Dolphin Bay’, ‘Dolphin Beach’, ‘Endeavour’) and a separate quarantine pool (‘QVC’) (Fig. 1).
Table 1
Sample collection dates. ‘X’ indicates that a blow samples was collected and included in the data analysis. ‘-’ indicates that a blow sample was collected, but was discarded due to its number of reads being below 6,000. A vertical bold line between the columns of ‘Starbuck’ and ‘Stella’ indicates the separation between healthy/untreated dolphins to the left from the treated dolphins to the right. The time period of antimicrobial treatment in ‘Stella’, ‘Gemma’, ‘Howie’ and ‘Nudgee’ is indicated by the bold horizontal cell borders and the shaded cells in the associated columns.

| Date         | Week | Coen | Evie | Kiama | Moki | RB  | Scooter | Sirius | Squeak | Starbuck | Stella | Gemma | Howie | Nudgee |
|--------------|------|------|------|-------|------|-----|---------|--------|--------|----------|--------|-------|-------|--------|
| 05/04/2017  | 2    | X    | X    | -     | X    | X   | X       | X      | X      | X        | X      | X     | X     | X      |
| 12/04/2017  | 3    |      |      |       |      |     |         |        |        |          |        |       |       |        |
| 03/05/2017  | 6    | X    | X    | X     | X    | X   | X       | X      | X      | X        | X      | X     | X     | X      |
| 24/05/2017  | 9    |      |      |       |      |     |         |        |        |          |        |       |       |        |
| 07/06/2017  | 11   | X    | X    | X     | X    | X   | X       | X      | X      | X        | X      |       |       |        |
| 14/06/2017  | 12   |      |      |       |      |     |         |        |        |          |        | X     | X     | X      |
| 21/06/2017  | 13   |      |      |       |      |     |         |        |        |          |        |       |       |        |
| 05/07/2017  | 15   |      |      |       |      |     |         |        |        |          |        | X     | X     | X      |
| 12/07/2017  | 16   |      |      |       |      |     |         |        |        |          |        |       |       |        |
| 19/07/2017  | 17   |      |      |       |      |     |         |        |        |          |        |       |       |        |
| 02/08/2017  | 19   | X    | X    | X     | X    | X   | X       | X      | X      | X        | X      | X     | X     | X      |
| 09/08/2017  | 20   |      |      |       |      |     |         |        |        |          |        | X     | X     | X      |
| 16/08/2017  | 21   |      |      |       |      |     |         |        |        |          |        |       |       |        |
| 06/09/2017  | 24   |      |      |       |      |     |         |        |        |          |        | X     | X     | -      |
| 13/09/2017  | 25   |      |      |       |      |     |         |        |        |          |        |       |       | X      |
| 04/10/2017  | 28   | X    | X    | X     | X    | X   | X       | X      | -      |          |        | X     | X     | X      |
| 06/12/2017  | 37   | -    | X    | X     | X    | X   | -       | -      |        |          |        | X     | X     | X      |
| Total number of samples | 5    | 6    | 5    | 6     | 6    | 5   | 6       | 5      | 6      | 9        | 5      | 9     | 8     |        |
Table 2
Sex, age, pool system the dolphins were kept in and antimicrobial (AB) treatment (Y = Yes, N = No)

| Dolphin ID | Sex | Year of birth | Age level | Pool system | AB treatment |
|------------|-----|---------------|-----------|-------------|--------------|
| Coen       | M   | 1995          | 2         | Dolphin Beach/ Dolphin Bay | N            |
| Evie       | F   | 2008          | 1         | Dolphin Beach | N            |
| Gemma      | F   | 1988          | 3         | Dolphin Beach | Y            |
| Howie      | M   | unknown       | unknown   | Endeavour   | Y            |
| Kiama      | M   | 2003          | 2         | Dolphin Beach | N            |
| Moki       | F   | unknown       | unknown   | Dolphin Beach | N            |
| Nudgee     | M   | unknown       | unknown   | Endeavour/VQC | Y            |
| RB         | M   | 1990          | 3         | Dolphin Bay | N            |
| Scooter    | F   | 1983          | 4         | Dolphin Bay | N            |
| Sirius     | M   | 1979          | 4         | Dolphin Beach | N            |
| Squeak     | F   | 1979          | 4         | Dolphin Beach | N            |
| Starbuck   | M   | 1998          | 2         | Dolphin Beach/ Dolphin Bay | N            |
| Stella     | F   | 2013          | 1         | Dolphin Beach | Y            |

Ten dolphins stayed in the same pool for the entire sampling period. Three dolphins were moved between two pools at least once. We assigned four different age levels: 1: 0–10 years, 2: 11–20 years, 3: 21–30 years, 4: 31–40 years.

Dataset Overview
In 87 dolphin blow and 26 pool water samples we detected a total of 4,190,154 raw 16S rRNA gene sequences, which were clustered into 2,175 zero-distance operational taxonomic units (zOTUs). We removed one zOTU as it had an overall relative abundance of less than 0.0001% and deleted 289 zOTUs from the dataset, which were present and highly abundant in most technical controls. We deleted five dolphin blow samples from the dataset, as these samples did not meet the set cut-off
score of a minimum of 6,000 reads (Table 1). The resulting dataset of 81 dolphin blow and 33 pool water samples contained 1,886 zOTUs with a mean of 15,685 reads per sample (sd = 8,120). The rarefaction curves of dolphin blow and pool water samples (Supplements Fig. 1 and Fig. 2) and Good’s coverage (Table 4) after filtering showed that the majority of samples was sequenced to near-saturation.

### Table 3

| Dolphin ID | Cause of AB treatment                  | AB medication | AB dosage                                      | Period of treatment          |
|------------|----------------------------------------|---------------|-----------------------------------------------|------------------------------|
| Gemma      | Aborted calf, prevention of infection  | Amoxycillin   | Oral administration, 2500 mg, twice a day      | 02/05 to 13/06/2017 (6 weeks) |
| Howie      | Ocular infection                       | Doxycycline   | Oral administration, 250 mg, twice a day       | 30/05 to 07/06/2017 (7 days)  |
| Nudgee     | Septic arthritis in shoulder joint     | Amoxycillin   | Oral administration, 3000 mg, twice a day      | 22/06 to 27/07/2017 (5 weeks) |
|            |                                        | Gentomicin    | Oral administration, 500 mg, once a week       |                              |
| Stella     | Inappetence                            | Amoxycillin   | Oral administration, 4000 mg                  | 05/07/2017 (only once)        |

Alpha diversity parameters differ across individuals but stay stable over time

We calculated four different parameters of alpha diversity (richness, Shannon-Wiener diversity index, Chao1 (37, 38) and ACE (39, 40) species estimators) for each dolphin after rarefying the reads of each sample to the lowest number of reads (6,169) to account for the difference in sampling depth. The alpha diversity parameters averaged to 240 (richness, sd = 88), 4 (Shannon-Wiener diversity, sd = 0.64), 286 (Chao1, sd = 100), 257 (ACE, sd = 93) across all dolphin samples. These parameters differed significantly between dolphins (richness: \( p = 2e-04 \), diversity: \( p = 0.0023 \), Chao1: \( p = 5e-04 \), ACE: \( p = 3e-04 \)) and there was no statistical support for changes over the sampling period within the individual dolphins (richness: \( p = 0.1651 \), diversity: \( p = 0.234 \), Chao1: \( p = 0.0912 \), ACE: \( p = 0.1266 \)) (Table 4).

Beta diversity is unique to individual dolphins, but changes with time

We visualized the beta diversity of microbial communities by creating non-metric multidimensional scaling (nMDS) plots, based on Bray-Curtis dissimilarity and unrarefied data, that showed distinct clustering of dolphin and pool water samples (Fig. 2). To determine if the composition of microbial communities between dolphin blow and pool water differed, we fitted log-link negative binomial
models to each zOTU, with ‘dolphin’ or ‘pool water’ as an explanatory factor (41) with the log of total sequence counts per sample included as an offset to account for differences in sampling depth. Using mvabund, the sum of likelihood ratio statistics and statistical significance was evaluated with anova.manyglm using pit-trap resampling (42), compared to an intercept-only model. These negative binomial models fitted to each zOTU in the dolphin blow and the pool water showed a significant difference between the two groups (sum-of-LR = 22,669, p = 0.001). Although the microbial communities in the blow changed over the eight months of sample collection (sum-of-LR = 2,320, p = 0.001), they remained unique to each individual dolphin (sum-of-LR = 34,482, p = 0.001). The heatmap of Fig. 3 shows the distribution and relative abundance of 63 zOTUs that significantly contributed to the differences across the individual dolphins. Although the microbiota of the water did not differ between the pool systems themselves (sum-of-LR = 6,948, p = 0.056), there was an effect on the blow microbiota depending on which pool system the dolphins were kept in (sum-of-LR = 1,980, p = 0.008). The dolphins’ sex (sum-of-LR = 4.987, p = 0.524) and age (sum-of-LR = 7.011, p = 0.4) did not impact their blow microbiota.

### Table 4

| Dolphin ID | Good’s coverage, mean (sd) | Richness, mean (sd) | Diversity, mean (sd) | Chao1, mean (sd) | ACE, mean (sd) | No of intra-core zOTUs |
|------------|-----------------------------|---------------------|----------------------|-----------------|---------------|-----------------------|
| Coen       | 99.70 (0.10)                | 267 (16)            | 4.19 (0.10)          | 306 (15)        | 283 (9)       | 178                   |
| Evie       | 99.67 (0.09)                | 292 (52)            | 4.24 (0.43)          | 352 (57)        | 314 (54)      | 138                   |
| Gemma      | 99.70 (0.30)                | 173 (106)           | 3.60 (0.70)          | 212 (129)       | 187 (112)     | NA                    |
| Howie      | 99.73 (0.10)                | 325 (67)            | 4.38 (0.50)          | 389 (78)        | 353 (75)      | 268                   |
| Kiama      | 99.71 (0.08)                | 268 (35)            | 4.15 (0.36)          | 335 (55)        | 292 (39)      | 157                   |
| Moki       | 99.58 (0.29)                | 237 (107)           | 3.98 (0.98)          | 285 (113)       | 255 (111)     | 30                    |
| Nudgee     | 99.78 (010)                 | 171 (31)            | 3.66 (0.33)          | 219 (65)        | 185 (38)      | 99                    |
| RB         | 99.74 (0.08)                | 253 (36)            | 4.18 (0.31)          | 293 (43)        | 265 (39)      | 147                   |
| Scooter    | 99.83 (0.11)                | 211 (113)           | 4.22 (0.56)          | 254 (118)       | 228 (117)     | 35                    |
| Sirius     | 99.90 (0.03)                | 183 (73)            | 3.89 (0.47)          | 203 (82)        | 190 (76)      | 32                    |
| Squeak     | 99.79 (0.12)                | 221 (131)           | 4.26 (0.60)          | 254 (148)       | 233 (139)     | 67                    |
| Starbuck   | 99.71 (0.05)                | 325 (24)            | 4.52 (0.23)          | 369 (26)        | 339 (27)      | 260                   |
| Stella     | 99.72 (0.15)                | 189 (80)            | 3.23 (0.85)          | 230 (82)        | 205 (79)      | NA                    |

The table shows the average of Good’s coverage as well as richness, diversity, Chao1 and ACE species estimators of blow samples per dolphin and number of intra-core zOTUs over a period of five months (weeks 6, 11, 19, 28). Gemma and Stella were not included in the calculation of intra-core zOTUs, as they lacked the according samples (No of core zOTUs: NA).

Diverse and abundant intra-core in dolphins present over five months

We determined those zOTUs that persisted in each individual dolphin over time and labelled them as ‘intra-core’. Each dolphin harboured an average of 128 intra-core zOTUs in their blow on each of the four sampling occasions over five months (Table 4). In total, we found 446 intra-core zOTUs. Most
intra-core zOTUs were only present in one or two dolphins. Only two intra-core zOTUs were shared by all 11 dolphins (FIG S3). The relative abundance of the individual intra-core varied over time and among dolphins (Fig. 4), averaging to 65% relative read abundance across all dolphins and time points. Whereas the relative abundance in some dolphins like ‘Starbuck’ and ‘Kiama’ remained fairly stable over time, others showed massive variations. ‘Starbuck’ harboured the highest relative abundance (91%) of intra-core zOTUs, averaged over the four time points measured. In contrast, ‘Moki’s’ intra-core only accounted for an average of 24% of relative read abundance. Most of the intra-core zOTUs (399 of the 446) had been reported previously in the blowhole, mouth or forestomach of bottlenose dolphins as described by Bik et al. (1) (Genbank accession number: e.g., KC260893.1) or Johnson et al. (3) (e.g., FJ959551.1) (Table 5). Those intra-core zOTUs previously found in dolphins accounted for an average of 25% of each dolphin’s total relative read abundance. The taxa with the largest number of intra-core zOTUs present were Gammaproteobacteria, Flavobacteriaceae, Bacteroidia and Helcococcus (Table 5). Those dolphin-associated intra-core zOTUs with a relative abundance above 1% were zOTU12 (2.10%, Gammaproteobacteria, JQ209784.1), zOTU16 (1.65%, Flavobacteriaceae, FJ960162.1), zOTU28 (1.33%, Arcobacter, JQ194090.1), zOTU1768 (1.07%, Bacteroidia, JQ194579.1) and zOTU120 (1.07%, Bacteria, JQ214918.1). The remaining 47 intra-core zOTUs that were not previously detected in dolphins have mostly been reported as present in seawater (e.g., JN489973.1), other environmental sources (e.g., soil MH671536.1), wastewater (e.g., JX515418.1) or on the skin, the gut or mouth of terrestrial mammals (e.g., EU681994.1) (Table 6). The majority of intra-core bacteria were novel and could therefore not be classified to genus level. Actinobacillus, Arcobacter, Helcococcus and Tenacibaculum were some of the few intra-core taxa that were classified to genus level and that were highly abundant within the core (Table 5, 6). In addition, we compared the 446 intra-core zOTUs with those zOTUs present in the pool water controls and 380 of the intra-core zOTUs were exclusively present in dolphin blow.
Table 5
Taxonomic affiliations of 399 intra-core zOTUs that were previously found in the mouth, forestomach or blowhole of bottlenose dolphins and their Genbank accession number, present across weeks 6, 11, 19 and 28 in 11 dolphins. The majority of listed taxa included more than one intra-core zOTU.

| No. of core zOTU with the same taxonomic affiliation | Taxonomic affiliation | Genbank accession (e.g.) |
|----------------------------------------------------|----------------------|--------------------------|
| 111                                                 | Gammaproteobacteria  | JQ209784.1               |
| 105                                                 | Flavobacteriaceae    | FJ959551.1               |
| 41                                                  | Bacteria             | JQ215599.1               |
| 20                                                  | Helcococcus          | FJ959658.1               |
| 10                                                  | Bacteroidia          | FJ959660.1               |
| 8                                                   | Actinobacillus       | KC257787.1               |
| 7                                                   | Clostridiales        | Q214550.1                |
| 7                                                   | SR1_genera_incertae_sedis | JQ216558.1         |
| 7                                                   | Tenacibaculum        | KC260454.1               |
| 5                                                   | Arcobacter           | JQ194090.1               |
| 5                                                   | Bacteroidales        | FJ959660.1               |
| 5                                                   | Bacteroidetes        | JQ215352.1               |
| 4                                                   | Alcaligenaceae       | FJ960346.1               |
| 4                                                   | Campylobacteriales   | FJ959586.1               |
| 4                                                   | Flavobacteriales     | QJ94338.1                |
| 4                                                   | Mycoplasma           | JQ194022.1               |
| 3                                                   | Desulfobulbaceae     | Q214362.1                |
| 3                                                   | Marinicella          | FJ959830.1               |
| 3                                                   | Microbacteriaceae    | JQ194103.1               |
| 3                                                   | Oceanospirillales    | JQ213884.1               |
| 3                                                   | Phoconobacter        | KC260904.1               |
| 3                                                   | Porphyromonadaceae   | JQ209212.1               |
| 2                                                   | Actinomycetales      | JQ208827.1               |
| 2                                                   | Cardiobacteriales    | FJ960414.1               |
| 2                                                   | Guggenheimella       | FJ959658.1               |
| 2                                                   | Lachnospiraceae      | Q214724.1                |
| 2                                                   | Pseudomonadales      | JQ194701.1               |
| 2                                                   | Sphingobacteriа      | FJ959720.1               |
| 1                                                   | Alicycllobacillus    | KC259493.1               |
| 1                                                   | Anaerovorax          | FJ959595.1               |
| 1                                                   | Betaproteobacteria   | FJ959731.1               |
| 1                                                   | Burkholderiales      | FJ959744.1               |
| 1                                                   | Cryomorphaceae       | QJ94623.1                |
| 1                                                   | Deltaproteobacteria  | Q214362.1                |
| 1                                                   | Epsilonproteobacteria| FJ959609.1               |
| 1                                                   | Firmicutes           | QJ94061.1                |
| 1                                                   | Naumannella          | KC259559.1               |
| 1                                                   | Parcubacteria_genera_incertae_sedis | JQ209049.1         |
| 1                                                   | Peptoniphilaceae     | JQ209515.1               |
| 1                                                   | Proteobacteria       | KM823720.1               |
| 1                                                   | Pseudomonas          | CP041013.1               |
| 1                                                   | Salinivibrio         | QJ94108.1                |
| 1                                                   | Spirochaetaceae      | KC259401.1               |
| 1                                                   | Spirochaetes         | KC259034.1               |
| 1                                                   | Spirochaetia         | KC260523.1               |
| 1                                                   | Sulfurospirillum     | JQ215956.1               |
| 1                                                   | Treponema            | KC260218.1               |
Intra-core zOTUs that were not previously found in bottlenose dolphins, their taxonomic affiliation, source, average relative abundance across dolphins and Genbank accession number, present across weeks 6, 11, 19 and 28 in 11 dolphins.

| zOTU no. | Taxonomic affiliation | Environment of most similar sequences | Genbank accession |
|----------|-----------------------|----------------------------------------|-------------------|
| Zotu0033 | Corynebacteriaceae     | Human skin                             | KU689893.1        |
| Zotu0065 | Staphylococcus aureus  | Human blood                            | HG795797.1        |
| Zotu0072 | Staphylococcus         | Frog skin                              | HM330254.1        |
| Zotu0079 | Bacteroidetes          | California sea lion stomach             | KF067368.1        |
| Zotu0138 | Microbacterium esteraromaticum | bioaerosol                        | MG751356.1        |
| Zotu0141 | Betaproteobacterium    | hypersaline lake                       | MG282144.1        |
| Zotu0149 | Microbacteriaceae      | wastewater                             | GQ062150          |
| Zotu0175 | Microbacteriaceae      | soil                                   | GU235593.1        |
| Zotu0180 | Bacteria               | seawater                               | KJ516633.1        |
| Zotu0210 | Salinivibrio costicola | Human skin                             | HN683955.1        |
| Zotu0223 | Bacteriovoracaceae     | seawater                               | JX294354.1        |
| Zotu0237 | Microbiaceae           | cheese rind                            | LT698608.1        |
| Zotu0259 | Saccharibacteria_genera_espertae_sedis | human skin                        | KU689893.1        |
| Zotu0279 | Clostridia             | human skin                             | JQ205124.1        |
| Zotu0300 | Leucobacter sp.        | human skin                             | FN823848.1        |
| Zotu0313 | Okibacterium sp.       | soil                                   | GU235593.1        |
| Zotu0335 | Comamonadaceae         | soil                                   | EU681994.1        |
| Zotu0355 | Marine bacteria        | Human skin                             | MG099642.1        |
| Zotu0368 | Vibrio sp.             | seawater                               | MG705679.1        |
| Zotu0377 | Bacteriovoracaceae     | marine sediment                        | GU235593.1        |
| Zotu0385 | Gracillicibacterium    | oral taxon 873                         | GU235593.1        |
| Zotu0417 | Prevotella melaninogenica | human oral cavity                     | MK129353.1        |
| Zotu0420 | Bacteriovoracaceae     | human skin                             | JN713493.1        |
| Zotu0422 | Gammaproteobacteria    | seawater                               | JQ301272.2        |
| Zotu0460 | Vibrio                 | California sea lion mouth              | EU137548.1        |
| Zotu0491 | Marine bacteria        | unknown                                | CP014053.1        |
| Zotu0508 | SR1_genera_espertae_sedis | Seawater                         | HQ122382.1        |
| Zotu0521 | Vibrio sp.             | Dog gut                                | KP571771.1        |
| Zotu0570 | Microbacterium esteraromaticum | coral                            | CP032548.1        |
| Zotu0590 | Marine bacteria        | bioaerosol                             | MK506675.1        |
| Zotu0645 | Marine bacteria        | seawater                               | KU689893.1        |
| Zotu0723 | Vibrio sp.             | seawater                               | HG795797.1        |
| Zotu0763 | Gracillicibacterium    | marine sediment                        | HM330254.1        |
| Zotu0835 | Bacteria               | dog's oral cavity                      | KF067368.1        |
| Zotu0954 | Bacteria               | coral                                  | FO87814.1         |
| Zotu0959 | Bacteriovoracaceae     | prairie dog flea                      | MG751356.1        |
| Zotu1083 | Vibrio alginolyticus   | Seawater                               | KU689893.1        |
| Zotu1157 | Escherichia coli       | Seawater                               | MG282144.1        |
| Zotu1214 | Bacteroidetes          | human feces                            | JF087814.1        |
| Zotu1449 | Marine bacteria        | Seawater                               | GQ062150          |
| Zotu1620 | Methylophaga           | Seawater                               | GU235593.1        |
| Zotu1639 | Marine bacteria        | fresh water fish                      | KP571771.1        |
| Zotu1644 | Tenacibaculum sp. DSM106434 | Seawater                         | GU235593.1        |
| Zotu1840 | Staphylococcus         | fish skin                              | MK129353.1        |
| Zotu2070 | Staphylococcus         | frog skin                              | JN683955.1        |
| Zotu2659 | Corynebacteriaceae     | human skin                             | HM330254.1        |

Furthermore, we determined any ‘inter-core’ zOTUs and their relative abundance. The inter-core were
defined as those zOTUs that were present across ten dolphins, randomly selected from our 13 study dolphins, at a specific time point. We picked weeks 2, 6, 11, 19, 28 and 37 as these points in time. Ten dolphins shared an average of 24 (sd = 17) inter-core zOTUs at any sample collection point in time (weeks 2, 6, 11, 19, 28, 37). These inter-core zOTUs accounted for a mean relative read abundance of 18% (sd = 9) in each dolphin. All 77 inter-core zOTUs detected were also part of the intra-core and were previously collected from the mouth, blowhole or forestomach of bottlenose dolphins (1, 3). More than half of the inter-core zOTUs were only present across all ten dolphins at one sampling point (FIG S4).

**Few Potential Pathogens Among Intra-core Zotus**

Within the intra-core of healthy and health-compromised dolphins, nine zOTUs belonged to genera that comprise pathogenic ‘species’ linked to infectious disease in marine mammals. These genera included Pseudomonas, Staphylococcus and Mycoplasma (Table 5, 6). Venn-Watson (43) and Venn-Watson (44) associated Pseudomonas and Staphylococcus with pneumonia and mortality in bottlenose dolphins, while Mycoplasma is a common causative agent of infectious disease in pinnipeds (45). We are unable to tell, whether the intra-core, consisting of 399 zOTUs, contained any additional potentially pathogenic genera, as most of the core members were novel and could therefore not be classified to genus level.

**Compromised physiological state of four dolphins impacted their blow microbiota**

To test the potential effect of the medical condition and antimicrobial treatment of four dolphins on their microbial communities, we classified the blow samples into four groups: samples from dolphins that were in good physical conditions and therefore didn’t receive any antimicrobial treatment were labelled as ‘None’. Samples of physiologically compromised and treated dolphins that were collected before the animal was treated received the label ‘Before’. Those samples that were collected (within one week) after the animals were treated were called ‘Directly_After’ and those samples collected at least two weeks after treatment were named ‘After’. The blow microbiota of the dolphins with medical conditions (Table 3) differed from those of the healthy dolphins (sum-of-LR = 3017, p = 0.01). The healthy dolphins’ blow bacterial communities were significantly different to those of the sick dolphins,
before (‘None’ vs. ‘Before’: sum-of-LR = 3751.4, p = 0.001), directly after (‘None’ vs. ‘Directly_After’: sum-of-LR = 2140.1, p = 0.024) and after antimicrobial treatment (‘None’ vs. ‘After’: sum-of-LR = 3443.7, p = 0.001), whereas the samples of the sick dolphins did not differ from each other based on their time of collection (‘Before’ vs. ‘After’: sum-of-LR = 1461.0, p = 0.221; ‘Before’ vs. ‘Directly_After’: sum-of-LR = 967.7, p = 0.554; ‘After’ vs. ‘Directly_After’: sum-of-LR = 773.9, p = 0.554).

Discussion

Dolphins harboured individual-specific ‘signature’ microbiota (intra-core) in blow

The dolphins in this study harboured bacterial communities in their blow that changed in their alpha and beta diversity over the sampling period of eight months, but remained unique to each individual dolphin (Fig. 3). Thus, our findings confirm the short-term results of Lima et al. (4) over a substantially longer time scale. Each dolphin showed a unique intra-core ‘signature’ (Fig. 3), as the majority of intra-core zOTUs were present in a single or two dolphins (FIG S3). With an average of 65% of the relative read abundance the intra-core zOTUs dominated the blow microbiota of individual dolphins. This provides evidence that, unlike the healthy human airways (13), the bacterial communities in dolphin blow are not merely transient. In fact, as much as 25% of the blow microbiota in our study animals had been reported previously in bottlenose dolphins. We propose that dolphins harbour a relatively stable individual-specific microbiota that colonizes the airways. Nevertheless, we found that the relative read abundance of intra-core zOTUs varied across time in most dolphins, including the healthy animals (Fig. 4). Segal et al. (19) and Dickson et al. (16) demonstrated in humans and mice that changes in the airway microbiota can occur even without any obvious clinical signs. Slight shifts in immune function were found to impact the respiratory microbial communities and they may have been at play here. The strong variation in intra-core zOTUs within individuals highlights that the lung microbiota of dolphins is dynamic and there are likely internal physiological factors that impact the community on a day-to-day basis.

Dolphins Shared Significant Number Of Inter-core With Conspecifics

In addition, the dolphins did not only maintain a stable intra-core over time, but also harboured a significant number of inter-core zOTUs that they shared with their conspecifics across pools at certain
time points. With a mean of 18% of relative read abundance, the inter-core in our dolphins was only half of that Apprill et al. (7) found in the blow across 26 humpback whales. Yet, Apprill et al. (7) only determined the inter-core at one single time point, whereas we noticed a large variation in relative abundance of inter-core with a range of 7 and 33% in our dolphins across time. However, it is interesting to notice that Apprill et al. (7) found humpback whales to share more than a third of their blow microbiota across two populations (north Atlantic and Pacific ocean), whereas our study dolphins were kept in the same facility and some even shared the same pool system. This provides additional evidence for the airway microbiota of cetaceans to maintain a certain ratio of stable individual-specific core residents that may be relatively independent of environmental influences.

Impact Of Antimicrobial Treatment Was Not Clearly Evident

We did not find evidence that the antimicrobial treatment that the dolphins received in this study had a significant impact on their blow microbiota. Instead, their microbial communities differed from those of their healthy conspecifics throughout the sampling period, whereas the microbiota of sick dolphins before and after the treatment did not differ. The compromised health of the sick dolphins may already have impacted their airway microbiota before the dolphins received treatment. Yet, all four dolphins suffered from distinct conditions (Table 3), none of which being airway-specific. At this point, we are unable to tell if locally focused physiological issues, like ocular infection or septic arthritis, can have an impact on the respiratory immune response and thus the respiratory microbiota, as such study results have been available neither in dolphins nor humans. In contrast to the compositional differences between the blow microbiota of healthy and sick dolphins, the relative read abundance of their intra-core zOTUs and its variation over time did not show any obvious distinction (Fig. 4).

Social Setting Of Dolphins Appeared To Impact Blow Microbiota

Similar to Lima et al. (4) and Bik et al. (1), we did not find any impact of sex and age of the dolphins on their airway microbiota. Yet, the pool system the dolphins were kept in did make a significant difference. As the bacterial communities in the pool water itself did not differ, we followed speculations of Bik et al. (1) and hypothesized that dolphins kept in the same pool system inoculate each other’s airways with their blow microbiota and therefore contribute to their pool mates’ blow
community composition. A similar effect has been shown for children whose airway microbiota changed with the number of their social contacts (46).

Conclusions
We conclude that the airways of dolphins are colonized by resident core bacteria that can differ in abundance in relation to more transient bacteria. Although we did not find a clear correlation between these core bacteria and the health of the dolphins, potentially due to the small sample size of sick dolphins and the variation of their illnesses, we speculate that these individual-specific core bacteria interact with the immune response of the respiratory tract and support its function. To support our assumption, a larger number of dolphins with a consistent respiratory pathophysiology and additional local immune parameters need to be analysed. For now, we confirm the potential of the analysis of blow microbiota as a future biomarker for the physiological state of the airways in cetaceans.

Methods
Sample collection
Sea World Marine Park Gold Coast, Australia, granted us permission to use the blow samples of 13 captive bottlenose dolphins (Tursiops truncatus) for this study. We tested the microbiota in the blow samples for temporal stability over a period of eight months. The dolphins were kept in three separate pool systems and a quarantine pool (Fig. 1). Three of the 13 sampled dolphins were moved between pools at least once (Table 2). The pools the dolphins were kept in were fed by a constant influx of seawater from the adjacent Pacific Ocean. The pool systems (Fig. 1) could be divided into four to six ‘subpools’ by closing the partition gates. Even when gates between ‘subpools’ were closed, water flow was retained between the pools. Therefore, a water sample of any of the ‘subpools’ was representative for the entire pool system.

The dolphins were trained to exhale on command. Sample collection was generally performed on Monday mornings at 9 am, as part of a feeding and enrichment session. After blow sample collected for this study was completed, the dolphins remained in the care of Sea World. For each sampling event the trainer held a sterile ‘yellow cap’ container (Techno Plas Pty Ltd, St Marys, South Australia, Australia) with a maximum volume of 70 ml, with its lid removed, upside down about 10 cm above the dolphin’s blowhole. Once the dolphin had exhaled, the trainer screwed the lid back on and stored the
sample in an Esky on ice. Each sample contained a volume of approximately 200 µl. The water
samples were obtained from the surface layer. A volume of about 200 ml was collected per water
sample using a sterile ‘yellow cap’ container (Techno Plas Pty Ltd, St Marys, South Australia,
Australia). Samples were transferred from the ice-filled Esky to a -20 °C freezer following collection
and then shipped to UNSW on dry ice.

Dna Extraction And 16s Rrna Gene Sequencing
The water samples were filtered through a Sterivex filter unit (0.22 µm, EMD Millipore Corporation,
Billerica, USA). The liquid of the blow samples was transferred from collection containers into the
tubes provided with the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, California, USA).
Nucleic acids from blow and water samples were extracted following the manufacturer’s protocol (MP
Biomedicals, Santa Ana, California, USA). The V1-V3 region of the bacterial 16S rRNA gene was
amplified using barcoded primers 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 519R (5’-
GWATTACCGCGGCKGCTG-3’) (Lane et al., 1985, Lane, 1991). The final PCR reaction volume was 25 µl
consisting of 0.125 µl of each primer (40 µM, Integrated DNA Technologies, Coralville, IA, USA), 5 µl of
5X Green GoTaq Flexi Buffer (Promega, Madison, WI, USA), 0.3125 µl of Go Taq HotStart Version (5
Units per µl, Promega, Madison, WI, USA), 2 µl of dNTP-mix (10 mM, TaKaRa, Bio Inc., Shiga, Japan),
5 µl of MgCl2 (25 mM; Promega, Madison, WI, USA) and 4 µl of DNA. Negative (no DNA template) and
positive controls (Escherichia coli genomic DNA) were included. Reactions were performed using a
CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad Laboratories Inc, Hercules, CA, USA) under
the following program conditions: Initial denaturation at 98 °C for 2 min; then 35 cycles of
denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s and elongation at 72 °C for 60 s; and a final
elongation at 72 °C for 5 min. PCR products were submitted to the Ramaciotti Centre for Genomics
(UNSW Sydney, Australia) for purification, library preparation and paired-end amplicon sequencing (2
× 300 bp) on the Illumina MiSeq platform.

Sequence Data Processing
An initial quality check was performed with FastQC (47). Paired-end reads were processed with
USEARCH version 10.240 (48). The reads were merged and low-quality sequences (maximum number
of expected errors > 2 and more than 1 ambiguous base) and those shorter than 440 bp were removed. Primers were also removed. Processed sequences of all samples were dereplicated and unique sequences were denoised and de novo clustered into zero-radius operational taxonomic units (zOTUs) with 100% similarity using the unoise3 algorithm. The clustering of zOTUs is therefore a similar approach to the formation of amplicon sequence variants (ASV) (49). A de novo chimera removal was included in the unoise step. Afterwards, remaining chimeric sequences were removed using the uchime2 algorithm (50) in high confidence mode with the SILVA database (version 132) as the reference dataset (51). Subsequently, processed sequences were mapped onto zOTU sequences to calculate the relative abundance of each zOTU in every sample using the otutab command with maxrejects and maxaccepts options disabled. Representative zOTU sequences were assigned a taxonomy using the SILVA rRNA sequence database, release 132 (www.arb-silva.de) (51) and the ribosomal database project (rdp), release 11 (52). The taxonomic information derived from the SILVA rRNA sequence database was exclusively used to identify and delete zOTU sequences derived from mitochondria and chloroplasts.

**Data analysis**

zOTUs that were identified as not being bacteria or as being chloroplasts or mitochondria were deleted from the dataset. We identified 289 zOTUs that were present and highly abundant in most technical controls. We considered those zOTUs as technical contaminants and deleted them from the dataset. We used the package phyloseq (v1.24.2) (53) to perform a rarefaction analysis to test, if a complete representation of the blow and seawater microbiota was achieved given the observed sequence sampling depths. We discarded six dolphin samples, as their number of counts were below 6,000 reads (Table 1). We used the R package nlme (v3.1-140) (54) to assess whether the four alpha diversity parameters were associated with the time of sample collection (55). To determine if the composition of microbial communities between dolphin blow and pool water differed, we fitted log-link negative binomial models to each zOTU, with ‘dolphin’ or ‘pool water’ as an explanatory factor (41). For each outcome, we fitted linear mixed effects models with a fixed effect of pool, random intercepts per dolphin and a continuous AR(1) residual structure and applied a likelihood ratio test to compare
models with and without a fixed effect of time. To assess the importance of the random intercepts in this model (impact of individual dolphins), we performed a restricted likelihood ratio test using the RLRsim package (v3.1-3) in R (56). Bacterial beta diversity of pool water and dolphin blow samples were assessed using the package vegan (v2.5-5) for community ecology analysis (57) and mvabund (v4.0.1) (58).

To ensure comparability of intra-core zOTUs across dolphins, we exclusively considered four samples per dolphin (weeks 6, 11, 19, 28) over five months. We excluded ‘Stella’ and ‘Gemma’ from the intra-core analysis, as they did not have all required samples over the above-mentioned weeks. Missing samples of ‘Howie’ and ‘Nudgee’ were replaced by those obtained a week before or after. ‘Howie’ and ‘Nudgee’ were the only two dolphins included in the intra-core analysis that were treated with antibiotics. Statistical analysis of microbial community results was performed using R statistical software (v3.5.1) (http://cran.r-project.org/).

Declarations

**Ethics approval and consent to participate**

The sample collection was performed under University of New South Wales (UNSW Sydney) animal care and ethics committee (ACEC) permit no. 16/81A.

**Consent for publication**

Not applicable

**Availability of data and materials**

Sequence data of the dolphin blow, water and technical control samples are available in the NCBI Sequence Read Archive under BioProject accession no PRJNA562386.

**Competing interests**

The authors declare that they have no competing interests.
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Authors' contributions

T.N., T.T., T.R., B.F. and C.V. contributed to the design of the study. C.V. performed the collection of the data. C.V. performed the analysis of the data. T.N., B.F., T.T., T.R. and C.V. contributed to the interpretation of the results. T.N., T.T., T.R. and C.V. contributed to the writing of the manuscript. All authors reviewed and approved of the final version of the manuscript.

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Figures
Aerial view (59) of the three pool systems. (a) Dolphin Beach, b) Dolphin Bay, c) Endeavour) at Sea World where sampled dolphins were kept in. Even when partition gates between ‘subpools’ were closed, water flow between ‘subpools’ still was still retained.

nMDS of microbiota, based on Bray-Curtis dissimilarity and unrarefied data, found in 81 dolphin blow and 33 pool water samples. A distinct separation between dolphin blow and pool water samples is present.
Figure 3

Heatmap of fourth-root transformed relative abundance of 63 zOTUs identified as significantly different between dolphins. The microbial communities of blow across individual dolphins were shown to be significantly different. The blow samples per dolphin (columns) are listed in chronological order.
Total relative abundance of intra-core zOTUs in the blow of 11 bottlenose dolphins at four data points over five months. Only three dolphins showed a relatively stable abundance of their intra-core (variations within 10\% of relative abundance), whereas others displayed a large variation over time. Howie and Nudgee were marked with an asterisk, as they received antimicrobial treatment. The transparent orange bars indicate the period of antimicrobial treatment of Nudgee over several weeks, whereas the orange arrows indicate treatment of Howie over a single week.

Supplementary Files
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